CHARACTERIZATION, INVESTIGATION AND CLEARANCE MECHANISM OF NEUROTOXIC PROTEINS IN ALZHEIMER'S AND PARKINSON'S DISEASE

THESIS

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BIOTECHNOLOGY

By

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Dedicated

To

The Almighty

DECLARATION

I hereby declare that the thesis entitled **"Characterization, Investigation and Clearance mechanism of Neurotoxic proteins in Alzheimer's and Parkinson's disease"** submitted by me, for the award of the degree of ***Doctor of Philosophy*** to **Delhi Technological University (Formerly Delhi College of Engineering)** is a record of *bona fide* work carried out by me under the guidance of Prof. Pravir Kumar.

I further declare that the work reported in this thesis had not been submitted and will not be submitted, either in part or in full, for the award of any other degree or diploma in this Institute or any other Institute or University.

Date: 16-10-2019 **Dhiraj**

Place: New Delhi (Reg. No. 2K14/Ph.D/BT/01)

CERTIFICATE

This is to certify that the thesis entitled **"Characterization, Investigation and Clearance mechanism of Neurotoxic proteins in Alzheimer's and Parkinson's disease"** submitted by **Mr. Dhiraj** to **Delhi Technological University (Formerly Delhi College of Engineering)**, for the award of the degree of "Doctor of Philosophy" in Biotechnology is a record of *bona fide* work carried out by him. Dhiraj has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to our knowledge has reached requisite standards.

The results contained in this thesis are original and have not been submitted to any other university or institute for the award of any degree or diploma.

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ABSTRACT

The abnormal accumulations of altered proteins are common pathological hallmarks of various neurodegenerative disorders (NDDs) such as Alzheimer's and Parkinson's disease, the leading cause of disability and death worldwide. Originally, AD and PD are famous for being two distinct NDDs with primarily memory and motor dysfunctions respectively. However, their pathologies share some overlapping proteinopathy along with the standard amyloid-beta (Aβ) plaques, tau associated neurofibrillary tangles, and α-synuclein containing lewy bodies. Recent discoveries have identified amyloid-beta precursor protein (AβPP) central to the pathogenesis of both diseases, including Aβ formation in AD and amyloid intracellular domain (AICD) associated mitochondrial regulation and neurotoxicity in PD. Here, ubiquitination plays an eminent role in the regulation of cellular proteomic balance and triggers distinct cellular responses based on the poly-ubiquitination patterns on their substrates. Therefore, the proteasomal regulation of AβPP is crucial for cell to overcome the amyloid burden and consequent AICD associated pathologies to maintain its healthy state. Moreover, the molecular diagnoses of the UPS components (including E1s, E2s and E3s) and their mechanism for abnormal protein clearance is decisive for developing the promising therapeutic modalities for better public health. Herein, we reviewed the dynamics of UPS machinery for the clearance of toxic metabolites in various neurodegenerative disorders and found that target specific ubiquitin E3 ligases, and their precise mechanism of lysine selectivity and ubiquitination are still rudimentary for most of the markers.

Therefore, main goal of this PhD research is to investigate the ubiquitination mechanism of AβPP and explore the potential ubiquitination sites responsible for AβPP processing and degradation to arrest Aβ formation and consequent pathologies. In addition, we identified the crucial factors for lysine selection during AβPP ubiquitination, including the conserved neighboring residues and secondary structural conformations in protein. Further, we studied the impact of lysine residues on AβPP stability, and their plausible role in non-covalent interactions for ubiquitin-positioning and ubiquitin-AβPP conjugation during ubiquitination and other functions. Furthermore, we elucidated the interaction pattern of AβPP with AD and PD-related proteins and reported the commonly interacting factors among Aβ, Tau and α-

synuclein for governing AD and PD pathogenesis. Moreover, we identified the key ubiquitination enzymes and their interactional network to deduce the regulation of Aβ, AβPP, Tau and α-synuclein ubiquitination. Additionally, we have also identified the aggregation prone regions in Aβ, Tau and α-synuclein that revealed the potential lysine residues for stabilizing the aggregates. These findings reveal novel regulatory mechanisms that would help us to device promising therapies to improve the mental, emotional, social and economic lives of the patients.

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LIST OF ABBREVIATION

AD	Alzheimer's disease
AICD	Amyloid intracellular domain
AIM	Atg8/LC3 binding motif
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
Aβ	Amyloid beta peptide
AβPP/APP	Amyloid beta precursor protein
BLOSUM	BLOcks SUbstitution Matrix
C	Cysteine residue
CAPN1	Calpain 1
CD28	Cluster of Differentiation 28
CHIP/STUB1	C-terminal HSP 70 interacting protein
CREM	cAMP-responsive element modulator
D	Aspartic acid residue
DARs	Dopamine receptors
DJ-1	Parkinsonism associated deglycase
DNA	Deoxyribonucleic acid
DUBs	Deubiquitinases
E	Glutamic acid residue
ER	Endoplasmic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
GABA	Gamma-Aminobutyric acid
GlyRs	Glycine receptor
GSK3B	Glycogen synthase kinase 3 beta
H	Histidine residue
HD	Huntington's disease
HECT	Homologous to E6-associated protein C-terminus domain

HSPs	Heat shock proteins
IFN-γ	Interferon gamma
IκB	Inhibitor of kappa B
K	Lysine residue
K-D	Lysine to Aspartic acid mutation
K-E	Lysine to Glutamic acid mutation
K-H	Lysine to Histidine mutation
K-R	Lysine to Arginine mutation
LRRK2	Leucine Rich Repeat Kinase 2
LTD	Long-term depression
LTP	Long-term potentiation
MAMs	Mitochondrial associated membranes
MAPT/Tau	Microtubule-associated protein tau
MDM2	Mouse double minute 2 homolog
mGluR	metabotropic glutamate receptor
MHC	Major histocompatibility complex
mHtt	Mutant huntingtin protein
MS	Multiple sclerosis
MVB	Multivesicular bodies
nAChRs	Nicotinic acetylcholine receptors
NDDs	Neurodegenerative disorders
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8 protein
NF-κB	Nuclear factor-κB
NMDA	N-methyl-D-aspartate
NUB1	Negative regulator of ubiquitin like proteins 1
PARK2	Parkin
PD	Parkinson’s disease
PHD	Plant homeodomain
PINK1	PTEN-induced kinase 1
PKC	Protein kinase C
PLCB2	Phospholipase C Beta 2

PolyUb	Polyubiquitin chain
PROTAC	Proteolysis targeting chimera
PSMC	26S protease regulatory subunit
R	Arginine residue
RBR	RING between RING finger domain
RING	Really interesting new gene domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SNCA	α-synuclein protein
SNCA	Alpha-synuclein
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase
SUMO	Small Ubiquitin-like Modifier
SUMO1	Small ubiquitin-like modifier 1
TDP-43	TAR DNA-binding protein 43
TNF	Tumor necrosis factor
Ub	Ubiquitin
UbE1s/E1s	Ubiquitin E1-activating enzymes
UbE2s/E2s	Ubiquitin E2-conjugating enzymes
UbE3s/E3s	Ubiquitin E3-ligases
UBL	Ubiquitin like domain
UCH	Ubiquitin C-terminal hydrolase
UPS	Ubiquitin-proteasome system
USP	Ubiquitin specific protease

Chapter I

Introduction and Review of Literature

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 INTRODUCTION

Neurodegeneration is a progressive damage to the neurons because of impaired or misfolded proteins and their aggregates. The underlying cause behind protein aggregation is the disturbed protein homeostasis governed by complex mechanisms of protein folding, protein trafficking, cellular localization, and protein degradation in a cell. Here, heat-stress response (HSR) declines with age that used to induce the expression of chaperones in healthy cells to assist proper folding of misfolded or newly translated proteins. However, beyond a threshold, the chaperones failed to provide a correct folding conformation to the proteins and therefore, UPS comes into action to destruct the misfolded or unwanted toxic proteins/metabolites from the cellular milieu via proteasomal or lysosomal proteolysis. Moreover, with increasing age post mitotic cells lose their potential to regulate proteostatic equilibrium because of down-regulated chaperones, and anomalous translations and defects in protein degradation machinery. Such demise in proteostasis is the key hallmark of a variety of neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD). Therefore, the removal of such misfolded proteins and their toxic aggregates are required to reverse the anomalous conditions in neurons to ensure optimal cell survival. Here, we provided the comprehensive overview of UPS machinery and their mechanisms for the clearance of toxic protein metabolites in various neurodegenerative disorders with their potential therapeutic modalities.

1.2 UBIQUITINATION: A SELECTIVE MARKER FOR PROTEIN DEGRADATION AND OTHER CELLULAR FUNCTIONS

Like every cell, neuron has efficient systems to maintain cellular integrity, repair and mechanisms to remove toxins with the help of a well-coordinated ubiquitin proteasome system. The UPS is playing a cardinal role in clearing the abnormally folded, undesired or overburdened proteins via the ubiquitination process, which otherwise disrupts the cellular homeostasis and aids in the progression of neurodegenerative diseases. Ubiquitination is a post-translational modification that regulates the stability, longevity, functionality and localization of a modified protein

in a cell. The fate of ubiquitin tagged proteins lies on their site of ubiquitination, and the conformations adopted by their site-specific poly-ubiquitin chains. The differently linked poly-ubiquitin chains (K6, K11, K27, K29, K33, K48, K63) are responsible for governing diverse biological and pathological phenomenon, including apoptosis, cell division, DNA transcription and repair, immune and inflammatory responses, organelle biogenesis, antigen processing, receptor modulation, ribosome biosynthesis, stress response pathway and viral infections (Diehl *et al.,* 2010). Additionally, poly-ubiquitination in a neuronal cell regulates DNA damage responses, endocytosis, neuronal development, neural network morphogenesis, synapse remodeling, ribosome biogenesis, cell adaptation and signal transduction pathways (Suresh *et al.,* 2016). There is growing evidence that the biological functions triggered by poly-ubiquitin chains not only regulated by stimulating proteasomal degradation but also by assisting in protein-protein interactions, altering sub-cellular localization and modulating enzyme activities of the ubiquitinated substrates (O'Neill 2009). However, investigations on the model substrates revealed Lys48 as a predominant signal for proteasomal degradation, while Lys6, Lys11, Lys29, Lys33 and Lys63 known to direct protein-protein interactions to form multi-protein complex and modulate enzymatic activities of the substrates. Additionally, the proteasomal targeting of protein is not entirely dependent upon Lys^{48} poly-ubiquitination, but mutually assisted by poly-ubiquitination at multiple sites (Adhikari and Chen 2009). Interestingly, the scientific reports depicting the role of poly-ubiquitination and ubiquitin like molecules are exponentially increasing day-by-day revealing the new realms of cellular functional regulation in neurobiology. For instance, Shcherbik and Pestov discussed the role of ubiquitin and ubiquitin-like molecules in the ribosome biogenesis through p53 regulation by MDM2 ligase activity modulation (Shcherbik and Pestov 2010). Similarly, many proteins are ubiquitinated and destructed under DNA damage responses to imbibe cellular adaptability, as discussed by Price and D'Andrea about the contribution of protein ubiquitination towards chromatin organization around DNA breaks to take cell fate decisions (Price and D'Andrea 2013). In addition, ubiquitination also found to regulate innate immune signaling triggered by Toll like receptors (O'Neill 2008). Likewise, endocytosis, trafficking and turnover of many synaptic proteins, including membrane receptors (AMPA, NMDA,

Acetylcholine, and Dopamine) are regulated by the ubiquitination machinery (Schwarz and Patrick 2012; Goo *et al.,* 2015). Interestingly, the ubiquitination enzymes are themselves regulated by the ubiquitination process to govern the physiology of cellular systems (Weissman *et al.,* 2011). In this manner, ubiquitination controls diverse vital functions within a cell via regulating the levels of different marker proteins in distinct processes.

Interestingly, ubiquitination is a crossroad between cell survival and death using its clearing property of malfunctioned or cytotoxic proteins. It enables the cell to overcome the burden of proteotoxic stress via efficient degradation of abnormal, misfolded or aggregated proteins. It has observed that the cell adopts any of the two mechanisms, including proteasomes and autophagy for selective targeting and degradation of such proteins. Earlier studies have proposed that the state and site of ubiquitination, i.e. polyubiquitin chain structures are the determining factor for the choice of protein degradation and protein targeting for other cellular processes (Nguyen *et al.,* 2014) summarized in **Table 1.1.** For instance, Lys-48 linked polyubiquitin chain form a closed conformation where hydrophobic patches of ubiquitin molecules sequestered at the ubiquitin-ubiquitin interfaces, disallowing them to interact with other target receptors. While Lys-63 liked polyubiquitin chain form an extended structure exposing their hydrophobic patches thereby allowing their interaction with the receptor proteins. In this way, Lys-48 and Lys-63 polyubiquitin chains are predominant markers for the proteasomal and autophagosomal protein clearance (Fushman and Wilkinson 2011). Moreover, in-depth studies have revealed that the choice of degradative pathways depends on the physical properties of the receptors, which recognize their substrates via ubiquitin binding domains (Feng and Klionsky 2017). For instance, in yeast model soluble proteins have shown higher affinity towards proteasomal ubiquitin binding receptor Dsk2 for proteasomal degradation due to its monomeric state, i.e. lack of self-interaction. While the large insoluble protein aggregates are engulfed by phagophore for vacuolar/lysosomal degradation with help of Cue5 ubiquitin binding receptor due to its oligomeric state imparting higher avidity due to bundling of several ubiquitin-binding domains. Although, both the mechanisms recruit ubiquitin binding receptors

but their targeting take place by different binding moieties, such as the ubiquitin like domains for proteasomal degradation while Atg8/LC3 binding motif (AIM) for autophagic degradation (Lu *et al.*, 2017). Moreover, same set of ubiquitinating enzymes, including E2 conjugating Ubc4/Ubc5 and the E3 ligase Rsp5 observed in both the cases, which confirmed the targeting of misfolded proteins to their appropriate degradative pathway based on solubility and selective receptor binding. Therefore, recent researches affirmed the ubiquitination as a shared signal for protein quality control through their targeting for different cellular functions or targeting for degradation via proteasomal or autophagosomal/lysosomal pathway **(Figure 1.1).**

Table 1.1: Ubiquitination architecture and their role in cellular functions

Type of Ubiquitination	Ub-Bonding Architecture	Cellular Functions	References
Mono-ubiquitination	MQIFV**Ub**$_{RLRGG\rightarrow K(n)}$**Substrate**	Receptor transport, Viral budding, DNA repair, Gene expression, Endocytosis, Protein localization	Hicke 2001; Haglund *et al.*, 2003a
Multi-mono-ubiquitination	MQIFV**Ub1**$_{RLRGG\rightarrow K(n1)}$**Substrate** MQIFV**Ub2**$_{RLRGG\rightarrow K(n2)}$**Substrate** MQIFV**Ub3**$_{RLRGG\rightarrow K(n3)}$**Substrate**	Endocytosis, DNA repair and Protein localization	Haglund *et al.*, 2003b
M1-linked Polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow M(1)QIFV}$**Ub3**$_{RLRGG\rightarrow M(1)QIFV}$**Ub2**$_{RLRGG\rightarrow M(1)QIFV}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	Signal transduction	Iwai 2012
K(6) Polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow K(6)TLTG}$**Ub3**$_{RLRGG\rightarrow K(6)TLTG}$**Ub2**$_{RLRGG\rightarrow K(6)TLTG}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	DNA repair	Morris and Solomon 2004
K(11) Polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow K(11)TITL}$**Ub3**$_{RLRGG\rightarrow K(11)TITL}$**Ub2**$_{RLRGG\rightarrow K(11)TITL}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	Cell Cycle control, Endoplasmic reticulum associated degradation (ERAD), Proteasomal degradation	Jin *et al.*, 2008; Matsumoto *et al.*, 2010
K(27) Polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow K(27)AKIQ}$**Ub3**$_{RLRGG\rightarrow K(27)AKIQ}$**Ub2**$_{RLRGG\rightarrow K(27)AKIQ}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	Lysosomal Localization, Proteasomal degradation	Ikeda and Kerppola 2008
K(29) Polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow K(29)IQDK}$**Ub3**$_{RLRGG\rightarrow K(29)IQDK}$**Ub2**$_{RLRGG\rightarrow K(29)IQDK}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	Proteolytic pathway, Regulation of AMP-activated protein kinase (AMPK)-related kinases, Proteasomal degradation	Al-Hakim *et al.*, 2008
K(33) Polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow K(33)EGIP}$**Ub3**$_{RLRGG\rightarrow K(33)EGIP}$**Ub2**$_{RLRGG\rightarrow K(33)EGIP}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	Regulation of AMPK-related kinases, Kinase modification, Immune response i.e. T-cell activation, Stress response	Al-Hakim *et al.*, 2008; Huang *et al.*, 2010
K(48) polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow K(48)QLED}$**Ub3**$_{RLRGG\rightarrow K(48)QLED}$**Ub2**$_{RLRGG\rightarrow K(48)QLED}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	Proteasomal degradation	Mallette 2012
K(63) polyubiquitination	MQIFV**Ub4**$_{RLRGG\rightarrow K(63)ESTL}$**Ub3**$_{RLRGG\rightarrow K(63)ESTL}$**Ub2**$_{RLRGG\rightarrow K(63)ESTL}$**Ub1**$_{RLRGG\rightarrow K(n)}$**Substrate**	Protein Kinase activation, Signal transduction,DNA repair, Autophagy, Endocytosis	Hayden and Ghosh 2008

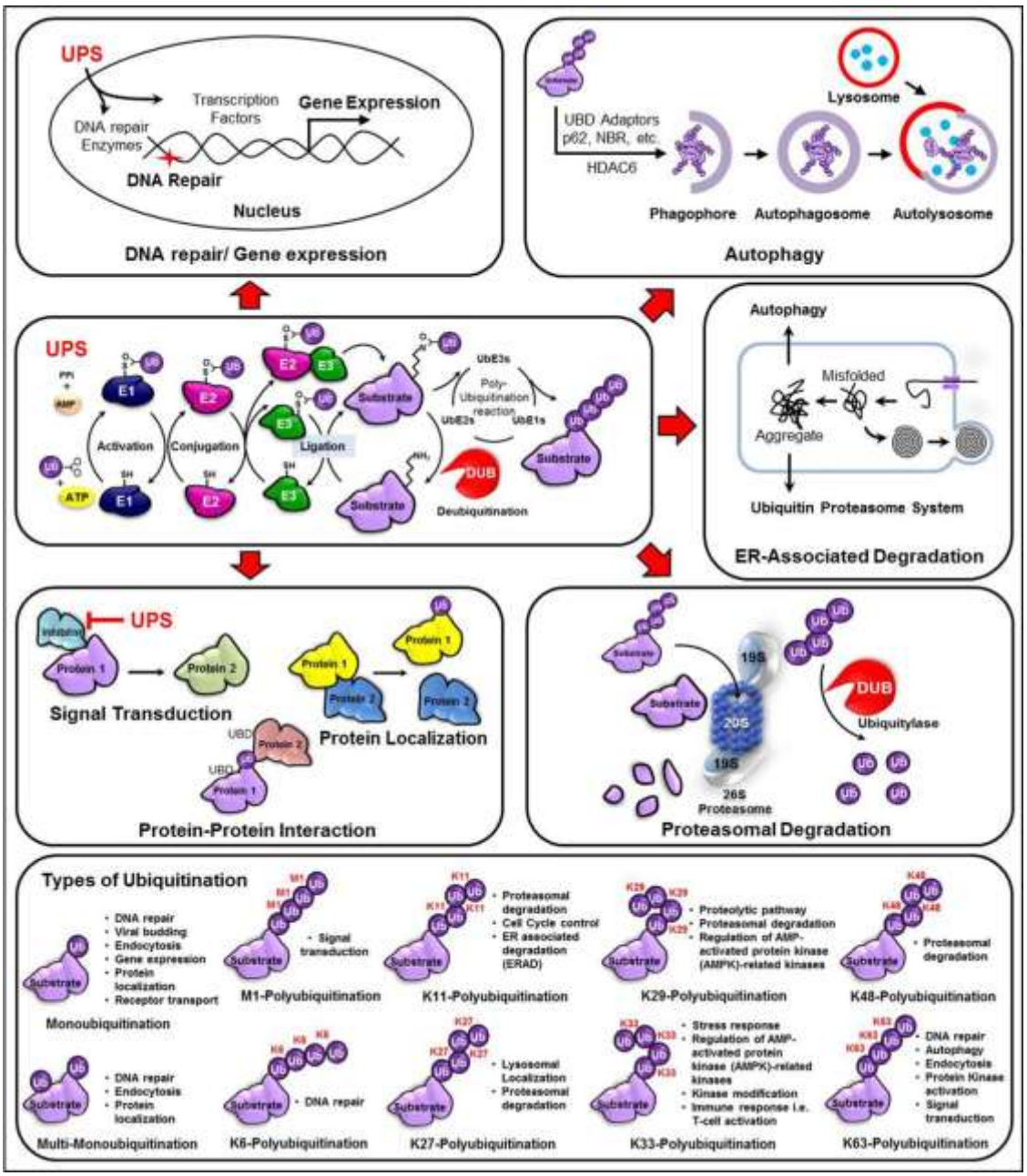


Figure 1.1: The ubiquitination process and their cellular responses based on the ubiquitin chain architecture: The ubiquitination involves the complex interplay of ubiquitin activating enzymes (E1s), ubiquitin conjugating enzymes (E2s), ubiquitin ligases (E3s) and deubiquitinases (DUBs) to form a variety of ubiquitin tags, including mono-ubiquitin, multi-mono-ubiquitin and poly-ubiquitin chains (M1, K6, K11, K27, K29, K33, K48, K63) that perform a variety of functions in a cell. **UPS-** Ubiquitin proteasome system; **Ub-** Ubiquitin, **UBD**- Ubiquitin binding domain; **p62**- Nucleoprotein 62, **NBR**- Neighbor of BRCA1 gene; **HDAC6**- Histone deacetylase 6.

1.3 UPS IN NEURONAL SIGNALING AND BRAIN HOMEOSTASIS

Over hundred billion interconnected wire-frame networks of neurons transmit well-coordinated electrochemical signals to regulate complex physiological functions in our brain. In general these functions include Pre- and Post synaptic regulation, neurotransmitter's vesicle transport and release, synaptic density and plasticity, and neuronal receptor modulations (DiAntonio *et al.*, 2001; Lin and Man, 2013). Moreover, ubiquitination determines learning and memory functions via regulating the abundance of selected proteins involved in the synaptic plasticity (Caroni *et al.*, 2012). Interestingly, ubiquitinaton is also associated with drug addiction mediated behavioral changes and neuronal adaptations via targeting drug induced proteins for proteasomal degradation (Massaly et al., 2015). Furthermore, it also controls transcription process in neurons by regulating the degradation of transcriptional repressors like IκB and CREM (cAMP-responsive element modulator) (Liu and Chen, 2011). Alike neurons, ubiquitination also acts in glial cells like astrocytes and microglia to maintain protein homeostasis, cytokine balance and healthy environment in the brain. Therefore, it is necessary to elucidate the ubiquitin system's biology in brain for proper understanding of complex biophysiological functions to regulate brain homeostasis.

1.3.1 Redox regulated UPS activity governs lipid metabolism, inflammatory responses and cellular immunity in the brain

The brain is a chief metabolizer of oxygen, but with relatively delicate defensive antioxidant mechanisms. Here, mitochondrial machinery metabolizes oxygen to produce ATP that yields several reactive oxygen species (ROS) including oxygen ions, free radicals, and peroxides in neurons. In fact, low level of ROS ensures the activation of various signaling pathways through transcriptional activation, while its abundance causes oxidative or nitro-oxidative stress in neurons. Moreover, it triggers severe inflammatory responses against the degenerated neurons, oxidized proteins, and lipid peroxidation, or glycated products in the brain (Popa-Wagner *et al.*, 2013). For instance, ROS activated IκB kinase (IKK) phosphorylates NF-κB inhibitor and proteasomally degrade IκB and release of NF-κB that migrate into nucleus and promote transcription of pro-inflammatory mediators along with c-Fos/Activator protein 1 (Hayden and Ghosh, 2012). Another regulator of redox signaling; NRF2

remains bound to its adaptor KEAP1 under basal conditions and are maintained at a low level in the cytoplasm through ubiquitination. Though in the diseased state it migrates into the nucleus and binds to the ARE sequence and consequently, trigger transcriptions of multiple genes involved in the expression of antioxidants, efflux transporters, glutathione, ATP synthesis, and several proteasomal subunits, etc. (Sivandzade *et al.,* 2019). Furthermore, oxidative stress initiates the release of pleiotropic cytokines like IFN-γ that induces the proteasomal activator PA28 (11S multimeric complex) to trigger over expression of immunoproteasomes and consequent MHC class- I mediated antigenic peptide's presentation in astrocytes and microglial cells (Jansen *et al.,* 2014; Launay *et al.*, 2013). Besides, oxidative stress as well linked with the PI3K/AKT pathway mediated over expression of the FOXO transcription factors that guides neuronal survival and stress responses in nervous system (Lefaki *et al.,* 2017). In addition, UPS also protects against lipid peroxidation and consequent toxin production, which may otherwise cause neural apoptosis (Popa-Wagner *et al.,* 2013). Likewise, a UPS component, Parkin monoubiquitinates CD36 and stabilize it at plasma membrane to facilitate the uptake of fatty acids stored in lipid droplets or oxidized by mitochondria. Moreover, under cellular stress, CD36 triggers Ca^{2+}- and ERK1/2-mediated activation of cPLA2 to release arachidonic acid from ER and nuclear membrane phospholipids that aids in synaptic transmission and its conversion into bioactive eicosanoids by microglia to have pleiotropic effects on inflammation (Abumrad and Moore, 2011). UPS also regulates both T-cell receptor (TCR) and co-stimulatory CD28 signaling via Cbl family ubiquitin ligases and USP9X deubiquitinase mediated regulation of pro-inflammatory cytokines and their receptor's expression to direct effective immunity and self-tolerance (Naik *et al.,* 2014). These scientific advancements have improved our understanding about the ubiquitin system mediated redox biology, lipid metabolism, inflammation and immune system regulation in brain cells by checking the levels of ROS, oxidized proteins, transcriptional factors, and pro-inflammatory agents within limits to maintain cellular homeostasis.

1.3.2 UPS regulates the shuttling of neuronal receptors for proper synaptic transmission

UPS is known to control the trafficking or internalization of various receptors, including Glutamate (mGluRs, AMPARs, NMDARs, KARs, and TrkB) and non-glutamate receptors (GABA, nAChRs, GlyRs, DARs) for maintaining proper synaptic transmissions. For instance, metabotropic glutamate receptors (mGluRs) reported to be ubiquitinated by E3 ligase, seven in absentia homolog (Siah1A) and marked for degradation (Moriyoshi *et al.,* 2004). Moreover, a mGluR-interacting protein, Homer-3 found to act as an adaptor to shuttle the ubiquitinated mGluRs to the proteasome via interacting with its S8 ATPase regulatory subunit (Rezvani *et al.,* 2012b). In another study, kainate receptor's subunit GluR6/GluK2 is ubiquitinated by Cullin-3 (an E3 ligase) with the help of adaptor protein actinofilin and directed for proteasomal degradation (Salinas *et al.,* 2006). Further, in case of NMDA receptors, its glycosylated subunits GluN1 found to be ubiquitinated by E3 ligase SCFF-box protein (Fbx2) and degraded by ERAD pathway, while Fbx2 carried out ubiquitination of GluN2A subunit in association with CHIP (Mabb and Ehlers, 2010). Additionally, it's GluN2B subunit's ubiquitination is reported by a different E3 ligase called Mindbomb-2 (Mib2) (Goo *et al.,* 2015). In addition, studies on AMPA receptors have revealed implication of many E3s, including APC (anaphase promoting complex), RPM1 (Regulator of Presynaptic Morphology 1) and KEL-8/Cul3 in C. elegans (Schaefer and Rongo, 2006; Tulgren *et al.,* 2011; Wang *et al.,* 2016b). Likewise, Nedd4 (neural precursor cell expressed developmentally downregulated-4), RNF167 (Ring Finger Protein-167) along with APC in mammals for ubiquitination and degradation of GluA1 subunits (Lin *et al.,* 2011; Lussier *et al.,* 2012). Interestingly, brain derived neurotrophic factor (BDNF) and Neurotrophin-4 reported to trigger ubiquitination and consequent differential endocytic sorting, degradation of TrkB receptors ensuing diverse biological functions (Proenca *et al.,* 2016). On the other hand, non-glutamate receptors, GABARs are shown to be ubiquitinated and proteasomal degraded in an activity dependent manner in ER of the neurons with help of ubiquitin like protein Plic-1 (Saliba *et al.,* 2007, 2008). Similarly, D4 subunit of dopamine receptors is targeted for ubiquitination by BTB protein KLHL12 with the help of Cul3-based E3 ligase (Rondou *et al.,* 2008).

However, ubiquitination of other receptors such as acetylcholine (nAChR) and glycine (GlyR) have been reported to control the synaptic transmissions, but their molecular ligases still have not been identified (Rezvani *et al.,* 2010; de Juan-Sanz *et al.,* 2013). Moreover, how neuronal activity coupled to the processes of activation, translocation and deactivation of regulatory proteins, and proteasomal units need to be elucidated.

1.3.3 UPS mediated synaptic plasticity: A gateway for learning and memory functions in the brain

The substantial research in several model systems has forced us to believe that synapses are the principal site for information storage in the brain. However, memory consolidation relies on the strength of the synapses in different parts of the brain, including their initial storage in the hippocampus followed by their transfer to the cortex region (Abraham *et al.,* 2019). Interestingly, UPS plays a vital role in controlling these synaptic connections during memory and learning processes of synaptic plasticity via regulating protein synthesis and degradation (Bingol and Sheng, 2011). Both short-term and long term synaptic plasticity's regulation included the turnover of diverse protein kinases- cAMP-dependent protein kinase A (PKA), calcium-calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK). For instance, NMDA receptor's stimulation triggered CaMKII autophosphorylation and consequent redistribution of proteasomes from dendritic shaft to the spine and its proteasomal activity in hippocampal neurons (Hell 2014). Similarly, serotonin stimulated sensory neurons exhibited proteasomal degradation of inhibitory R subunit of PKA and subsequent PKA and PKC mediated degradation of CREB repressor- CREB1b to regulate long-term facilitation in *Aplysia* model (Hegde 2017). Likewise, PKA mediated phosphorylation of AMPA receptor subunits- GluR1 and GluR4 triggered their synaptic incorporations in neuronal membrane during long-term potentiation (Woolfrey and Dell'Acqua, 2015). On the other hand, MAPK family proteins such as ERK1/2 is responsible for gene transcription, protein translation and posttranslational modification during information processing and memory formation while others, including ERK5, p38 or JNKs have a distinct role in learning and memory (Medina and Viola, 2018). Besides, proteasomal degradation of NF-kB regulators- IjB and IkB

kinases regulated gene transcription and its associated formation and stability of fear memory in the amygdala (Jarome *et al.,* 2011). Moreover, UPS also regulates both pre- and post-synaptic protein densities and receptor shuttling to govern synaptic transmission activity during memory and learning process. For instance, NMDAR activity dependent proteasomal degradation of MOV10 resulted in translational activation of alpha-CaMKII, Limk1, and the depalmitoylating enzyme lysophospholipase1 (Lypla1) during synaptic plasticity (Banerjee *et al.*, 2009). Other synaptic proteins like Dunc-13 (pre-synaptic vesicle priming protein), Syntaxin 1 (synaptic vesicle exocytosis protein), and RIM1α (pre-synaptic scaffolding protein) are proteasomally degraded by different E3 ligases namely Fbxo45, STARING (Syntaxin 1-interacting RING finger protein) and SCRAPPER respectively (Hegde 2010). Similarly, UPS also modulates structural proteins, neurotransmitter receptors and regulatory proteins in postsynaptic terminal. For instance, proteasome inhibitor MG132 prevented internalization of membrane receptors, including ionotropic GLR-1 glutamate receptors and NMDA-induced AMPA receptors (Goo *et al.,* 2015). In addition, postsynaptic scaffold protein PSD-95 stabilized the surface expression of AMPA receptors, while its proteasomal degradation by Mdm2 (E3 ligase) triggered AMPA receptor internalization. Similarly, NMDA receptors also retrotranslocated and degraded by UPS with the help of SCF-type ligase and contribute towards short-term synaptic plasticity regulation (Hegde 2010). Likewise, the glycine and GABA receptors also internalized and degraded upon ubiquitination in the neurons (Lin and Man, 2013). Furthermore, other postsynaptic scaffolding proteins such as GRIP1, GKAP, Shank, SPAR, and Arc have a role in synaptic strength regulation by their own proteasomal degradation to control the surface expression of diverse receptors, including AMPA (GluR2) and NMDA by different E3 ligases- TRIM3, SCFβ-TRCP, and Triad3A (Ferrara *et al.,* 2019; Lin and Man, 2013; Mabb *et al.,* 2014). There is growing list of E3 ligases regulating the synaptic proteins and receptors to modulate learning and memory process in the brain **(Table 1.2).** In summary, kinase action contributes towards memory formation by two ways, including the regulation of ion channel density/conductivity, i.e. regulation of membrane receptor trafficking and regulation of gene transcription/local translation that governs synaptic transmission and synaptogenesis respectively.

Table 1.2: Key role of E3 ligases in synaptic plasticity mediated memory and learning processes within the brain

E3 ligases	Role in Memory and Learning	Target Protein	Experimental Model	References
APC^{Cdh1}	Interacts with EphA4 receptor to mediate ephrin-induced proteasomal degradation of AMPARs	AMPAR	EphA4 knockout mice	Fu *et al.*, 2011
CBX4	Contextual fear conditioning memory Formation	HDAC7	C57BL6J mice	Jing *et al.*, 2017
CRL4	Target voltage- and calcium-activated BK channel for ER retention	Cereblon, BK channel	CRBN mutant mice	Song *et al.*, 2018
Fbx2	Controls synaptic NR1and contribute to homeostatic regulation of NMDA receptor	NR1	Hippocampal neurons	Kato *et al.*, 2005
MDM2	Glutamate receptor trafficking	PSD-95	Rat forebrain crude synaptosomes	Colledge *et al.*, 2003
MGRN1	Potentiates the transcriptional cellular response to proteotoxic stress	-	C57BL/6J mice and Brain lysate	Benvegnù *et al.*, 2017
Nedd4	Role in trafficking of GluA1-containing AMPAR	GluA1, AMPAR	Nedd4(+/-) mice	Camera *et al.*, 2016
Parkin	Role in short term memory through regulation of hippocampal snaptic plasticity	α-synuclein, synaptotagmin XI, Pael receptor, tubulin, mitofusin	Parkin-deficeint mice	Rial *et al.*, 2014
RNF167	Regulates GluA2 associated synaptic AMPAR currents	GluA2, AMPAR	Sprague–Dawley rats	Lussier *et al.*, 2012
$SCF^{\beta\text{-}TRCP}$	Regulate postsynaptic density of spine-associated Rap GTPase activating protein (SPAR) in association with Polo-like Kinase 2	SPAR	E19 Long Evans rat hippocampi	Ang *et al.*, 2008
SCRAPPER	Role in RIM1 (Rab3-interacting molecule-1) mediated synaptic vesicle release and neurotransmission	RIM1	SCR (+/−) mice	Yao *et al.*, 2011
TRIM3A	Role in γ-actin turnover at hippocampal synapses regulating spine densities, long-term potentiation, and short-term contextual fear memory consolidation	γ-actin	Trim3−/− mice	Schreiber *et al.*, 2015
TRIM67	Role in appropriate brain development and behavior	Netrin receptor DCC	Trim67−/− mice	Boyer *et al.*, 2018
TRIM9	Role in neuronal morphogenesis and spatial learning and memory	SNAP25, Netrin-1	Trim9−/− mice	Winkle *et al.*, 2016
UBE3A	Regulate postsynaptic SK2 levels for NMDA receptor activation. Also regulate glutamate receptor trafficking and CaMKII abundance	SK2, Arc	UBE3A-deficient mice	Mabb and Ehlers, 2010; Sun *et al.*, 2015

1.3.4 UPS in drug addiction mediated behavioral changes and neuronal adaptations

Drug addiction is a compulsive pattern of drug seeking and enduring propensity to relapse it, in effect, of the neuro-adaptations in specific brain regions analogous to long-term memory consolidation. Latest research has defined drug addiction mechanism to be associated with alterations in synaptic transmission in mesocorticolimbic and corticostriatal pathways and the transcriptional changes in neurons for their durable changes in plasticity and behavior. In fact, UPS is evident to control the trafficking or internalization of various receptors, including Glutamate (mGluRs, AMPARs, NMDARs) and non-glutamate receptors (GABA, nAChRs) for maintaining proper synaptic transmissions. For instance, cocaine addiction mediated synaptic plasticity changes has been observed in the nucleus accumbens (NAcc) (Massaly *et al.,* 2013). Similarly, proteasomal degradation of N-ethylmaleimide-sensitive fusion (NSF) protein (a post synaptic density protein), modulate AMPA glutamate receptor's expression and involved in cocaine addiction mediated memory reconsolidation (Ren *et al.,* 2013). In addition, repeated exposure to both amphetamine and cocaine altered glutamatergic neurotransmission in NAcc and potentiated AMPAR synaptic transmission, while synaptic de-potentiation was only associated with cocaine-induced protein synthesis and metabotropic glutamate receptor-5 (mGluR5) activation in NAcc medium spiny neurons (Jedynak *et al.,* 2016). Likewise, proteasomal degradation of GKAP and Shank destabilized PSD-95 and consequently, down regulated NR2B subunit of glutamate N-methyl-Daspartate receptors (NMDARs) to trigger behavioral sensitization in amphetamine addictions in NAcc (D'Souza 2015). Another instance where protein ubiquitination is involved in the neuroplastic changes in nAChR's associated synaptic transmission underlying alcohol and nicotine addictions (Feduccia *et al.,* 2012). Furthermore, morphine, cocaine, and nicotine evoked synaptic plasticity was reported with nitric oxide and BDNF induced ubiquitination and reduction in GABA and GABARs in ventral tegmental area and prefrontal cortex of the brain (Lüscher and Malenka, 2011). These studies have highlighted the importance of investigating diverse ubiquitination enzymes involved in the regulation of drug-induced neuroplastic changes to mimic behavioral pathology in human addicts. This way, ubiquitination system regulates

protein turnover in neurons and glial cells to facilitate multifaceted processes of neuronal signaling, synaptic regulation and neuronal adaptations to maintain brain homeostasis **(Figure 1.2).**

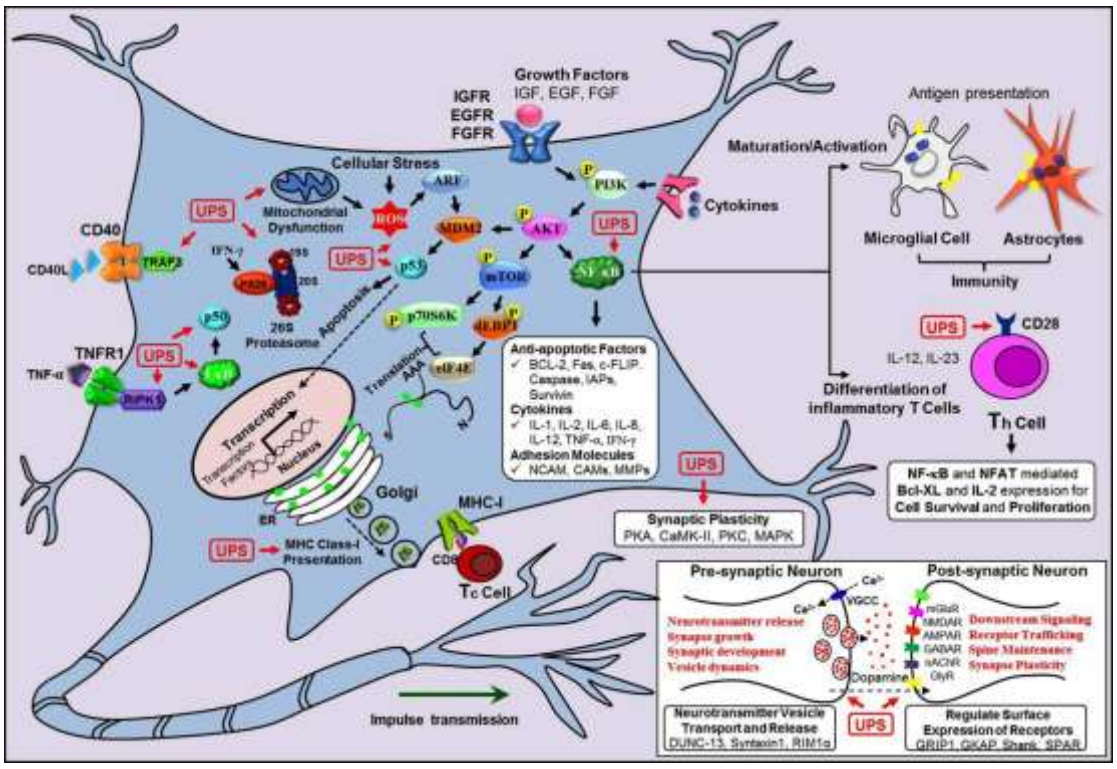


Figure 1.2: Role of Ubiquitin Proteasome System in Brain signaling, Synaptic regulation and Cellular homeostasis: UPS is involved in multifaceted functions, including MHC Class-I antigen presentation, Cellular immunity, T-cell receptor and CD28-signaling, transcription and translation process, and synaptic transmission within the brain. Moreover, it governs mitochondrial trafficking; ROS mediated signaling, and p53 linked apoptotic regulation; NF-kB mediated inflammatory responses (release of anti-apoptotic factors, cytokines and adhesion molecules), diverse receptor's expressions (CD40, TNFR1, Growth factor receptors) to maintain cellular-homeostasis. It also activates microglia and astrocytes to trigger immunoproteasome linked antigen presentation and subsequent T-cells response to generate various cytokines and associated immune defense under cellular stress. Additionally, UPS governs the synaptic transmission by regulating neurotransmitter release, Synapse growth, Synaptic development, Vesicle dynamics in the Pre-synaptic neurons while receptor trafficking, Downstream signaling spine maintenance, and Synaptic plasticity in Post-synaptic neurons. **IGF**-Insulin growth factor; **EGF**-Epidermal growth factor; **FGF**-Fibroblast growth factor; **IGFR**-Insulin growth factor receptor; **EGFR**-Epidermal growth factor receptor; **FGFR**-Fibroblast growth factor receptor; **CD8**-Cluster of differentiation 8; **CD40**-Cluster of differentiation 40; **TNFα**-Tumor necrosis factor alpha; **TNFR1**-Tumor necrosis factor receptor 1; **MHC**-Major histocompatibility factor; **ER**-Endoplasmic reticulum; **TRAF3**-TNF Receptor Associated Factor 3; **RIPK1**-Receptor-interacting serine/threonine-protein kinase 1; **I-κB**-Inhibitor of kappa B; **NF-κB**-Nuclear Factor kappa beta; **IFNγ**-Interferon gamma;

PA28-Proteasome activator 28; **ARF**-ADP ribosylation factors; **MDM2**-Mouse double minute 2 homolog; **PI3K**-Phosphatidylinositol-4,5-bisphosphate 3-kinase; **AKT**-Protein kinase B; **mTOR**-mammalian target of rapamycin; **eIF4**E-Eukaryotic translation initiation factor 4E; **p70S6**K-Ribosomal protein S6 kinase beta-1; **IAPs**-Inhibitor of apoptosis; **IL**-Interleukins; **NCAM**-Neural cell adhesion molecule; **CAMs**-Cell adhesion molecules; **MMPs**-Matrix metalloproteinases; **NFAT**-Nuclear factor of activated T-cells; **PKA/PKC/CaMK-II**-Protein kinases; **VGCC**-Voltage gated calcium channel; **DUNC-13**-Drosophila homolog UNC-13; **RIM1α**-Regulating synaptic membrane exocytosis 1 alpha; **GRIP1**-Glutamate Receptor Interacting Protein 1; **GKAP**-Guanylate kinase-associated protein; **SPAR**-Spine-associated Rap-Gap; **mGluR**-Metabotropic glutamate receptor; **NMDAR**-N-methyl-D-aspartate receptor; **AMPAR**-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; **GABAR**-Gamma-Aminobutyric acid; **nAChR**-Nicotinic acetylcholine receptor; **GlyR**-Glycine receptor.

1.4 INTRICACIES OF UPS IN THE CELLULAR PHYSIOLOGY OF NEURODEGENERATIVE DISORDERS

The functional UPS machinery comprised of multiple components, including ubiquitin, ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), ubiquitin-ligating enzyme (E3s), deubiquitinating enzymes (DUBs), and the proteasome. Among them, E2s and E3s are the key components that determine the substrate specificity for the clearance of toxic metabolites from a cell. Humans have ∼40 E2s that centrally govern the attachment and transfer of ubiquitin or ubiquitin like protein (Ubl) to the target proteins or to the E3 ligases. The peculiarities of E2s are their ability to interact with hundreds of E3 ligases; for instance, UBE2R1 interacts with SCF E3 ligase complex; UBE2C and UBE2S interact with APC E3 ligase complex, UBE2N and UBE2V1 interact with TRAF6 E3 ligase. The proposed mechanism of ubiquitin conjugation by E2 enzymes during poly-ubiquitination is the orientation of acceptor ubiquitin in a manner to expose the selective lysine residue at their active site charged with donor ubiquitin to form lysine site-specific ubiquitin-linked chains (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) (Ye and Rape 2009). Moreover, there is increasing evidence for the role of E2 enzymes in toxic protein clearance in various neurodegenerative disorders. For instance, UBE2A reported to coordinate the clearance of Aβ, through proteolysis in Alzheimer's disease (Zhao *et al.,* 2016) and to regulate the clearance of dysfunctional mitochondria in Parkinson's disease (Martin *et al.,* 2011). In another study, UBE2K found to regulate Aβ neurotoxicity by regulating the function of different kinases, including apoptosis signal-regulating kinase 1 and c-Jun N-terminal kinase (JNK) (Hong *et al.,* 2014).

Further, a spectrum of E2 enzymes, including UBE2C, UBE2D, UBE2E1, UBE2K, UBE2L3, UBE2L6 and UBE2N reported to govern Parkin's activation and its translocation to mitochondria for triggering mitophagy (Fiesel et al 2014). Similarly, UBE2A, UBE2D2, UBE2K, UBE2V1, and UBE2W are found to ubiquitinate mHtt protein and regulate their solubility in Huntington's disease (Howard *et al.,* 2007; de Pril *et al.,* 2007; Wang *et al.,* 2018). However, UBE2E3 is identified to induce TDP-43 ubiquitination and its proteasomal clearance in Amyotrophic lateral sclerosis (Lee *et al.,* 2010; Hans *et al.,* 2014). Most of the E2s have exhibited their intrinsic reactivity towards free lysine (aminolysis) and cysteine (transthiolation) for conjugating ubiquitin to E3 ligase or direct transfer of ubiquitin to the target protein, while few have shown some unexpected features (Stewart *et al.,* 2016). For instance, UBE2L3 (UbcH7), an E2 is found to be solitary responsive to cysteine while unreactives towards lysine thereby reactive against only HECT-type and RING/HECT hybrid type E3s. They usually catalyze ubiquitination through an obligate thioester-linkage with a conserved cysteine residue in RING2 of RING-in-between-RING (RBRs) family of RING/HECT hybrid type E3s (Wenzel *et al.,* 2011). Other E2s like UBE2W is capable of catalyzing ubiquitination reaction with N-terminal α-amino group of proteins as well as with α-amino group of small lysine-less peptides but not with free lysine, whereas UBE2D3 can facilitate the ubiquitination at lysine but not α-amino group (Vittal *et al.,* 2015). Interestingly another E2, UBE2J2 reported to ubiquitinate major histocompatibility complex via hydroxyl groups (serine/threonine) with the help of murine K3, a viral RING E3 ligase (Wang *et al.,* 2009a). Likewise, another E2, ATG3 reported to conjugate Ubl proteins to the phosphatidylethanolamine (a non-protein primary amine) in the outer phagosomal membrane (Slobodkin and Elazar, 2013). Another interesting finding about E2s is their increase in aminolysis-activity upon interaction with RING type E3s (Wenzel *et al.,* 2011). For instance, UBE2D families of E2 proteins have shown rapid reactivity with lysine in presence of an E3 RING domain while slowly in its absence.

Apart from E2s, the ubiquitin E3 ligases (~800) govern UPS machinery with a high degree of substrate specificity. Post translation modification such as hydroxylation or phosphorylation is often required prior to its specific selection by different E3 ligases.

They facilitate ER associated degradation (ERAD) of misfolded or unfolded proteins with the help of their diverse domains, including HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene), U-box (a modified RING motif without the full complement of Zn^{2+}-binding ligands), Ring between Ring finger domain and PHD finger domain (Kostova *et al.,* 2007). There is a majority of RING-E3 ligases, including CBL, RNF4, MDM2, CHIP, APC/C; Cullin RING ligases, etc. along with HECT E3 ligases SMURF1/2, ITCH, E6AP, HERC family ligases, etc. Some of the RBR E3 ligases include Parkin, Parc, RNF144, HOIP, HHARI, etc. and some U-box E3 ligases include UFD2a, UFD2b, CHIP, KIAA0860, CYC4, PRP19; and little PHD finger E3 ligases are UHRF1, UHRF2, TIF1A, TRI33, and SHPRH (Morreale and Walden, 2016; Hatakeyama *et al.,* 2001). Here, HECT E3 ligases directly catalyze ubiquitination reaction with the substrate, while RING and U-box E3 ligases serve as an adaptor-like molecule, which brings E2, and substrate in near proximity for ubiquitination reaction. Moreover, sequence conservation analysis of ubiquitin ligating domains revealed the conservation of cysteine residues in all the domains along with other critical residues important for ubiquitination, including leucine, isoleucine, valine, histidine, proline, etc. shown in **Figure 1.3** (Hall, 1999). Furthermore, HECT type E3 ligases have a variety of conserved amino acid residues, while PHD finger E3 ligases have a unique conserved histidine apart from cysteine, isoleucine, leucine and valine. Interestingly, U-Box type E3 ligases have shown the conservation of only proline. Conservation of histidine was significant for Zn binding in PHD domains while proline conservation in U-Box domain signified their conjugation activity by creating a folded architecture. Further, the conservation of key amino acid residues in different domains of E3 ligases prominent for ubiquitination has summarized in **Table 1.3.** Additionally, with the help of diverse E3 families, they target a wide array of substrates that perform critical roles in cellular development, cell signal maintenance, and cellular homeostasis (Metzger *et al.,* 2014). For instance, SCF complex found to degrade BACE1 enzyme and their associated Aβ toxicity that improved synaptic functions in AD (Gong *et al.,* 2010). Likewise, another E3 ligase HRD1 found to control the ubiquitination and degradation of misfolded and unfolded proteins from endoplasmic reticulum through ERAD to regulate ER stress in neurodegenerative disorders (Kaneko, 2016). Similarly, Gigaxonin reported to

regulate intermediate filaments in neurons via controlling the level of microtubule-associated protein-1B (Allen *et al.,* 2005). In this manner, there are enormous E3 ligases that govern diverse biological functions in the neuronal cells like synaptic regulation, signal transmission, mitophagy, autophagy, protein quality control, inflammatory responses, etc. There is another class of enzymes called deubiquitinases (DUBs ~100 in humans), which controls the ubiquitination levels in a cell. They are classified into distinct families (majority are cysteine proteases) based on the architecture of their catalytic domains, which includes the ubiquitin C-terminal hydrolases (UCHs), ubiquitin specific proteases (USPs), Machado-Joseph disease proteases (MJDs), ovarian tumor proteases (OTUs), MIU containing novel DUB family proteases (MINDY) and JAB1/MPN/Mov34 (JAMM) domain proteases (Leznicki and Kulathu, 2017; Wilkinson, 2009). These deubiquitinases possess distinct specificity towards distinct ubiquitin-chain topologies and perform four different catalytic activities such as i) ubiquitin recycling during proteasomal processing, ii) ubiquitin chain editing, iii) reversal of ubiquitin conjugation, and iv) processing of ubiquitin-precursors. Altogether, these activities regulate diverse cellular functions, including apoptosis, chromosome segregation, cell-cycle progression, DNA repair, activation of kinases and proteasome or lysosome mediated protein degradation (Wilkinson, 1999; Clague *et al.,* 2012; Suresh *et al.,* 2015). Additionally, studies have identified their roles in the pathologies of various neurodegenerative disorders. For instance, UCH-L1 is evidenced to improve memory and synaptic functions in the suffering patients (Gong *et al.,* 2016). Likewise, USP30 is involved in the clearance of dysfunctional mitochondria in association with parkin and PINK1 in PD patients (Gersch *et al.,* 2017). Another deubiquitinase, USP14 found to reduce cellular aggregates and associated degeneration by overcoming unfolded protein response triggered by IRE1 activation under ER stress (Hyrskyluoto *et al.,* 2014). In summary, DUBs support the UPS machinery by recycling the free ubiquitin for their functional regulation in a cell. In this way, the multifaceted properties of E1s, E2s, E3s and their interactions with DUBs empower them to provide a specific and effective mechanism for protein clearance from the cell and thus prove them as potential targets for therapeutic actions.

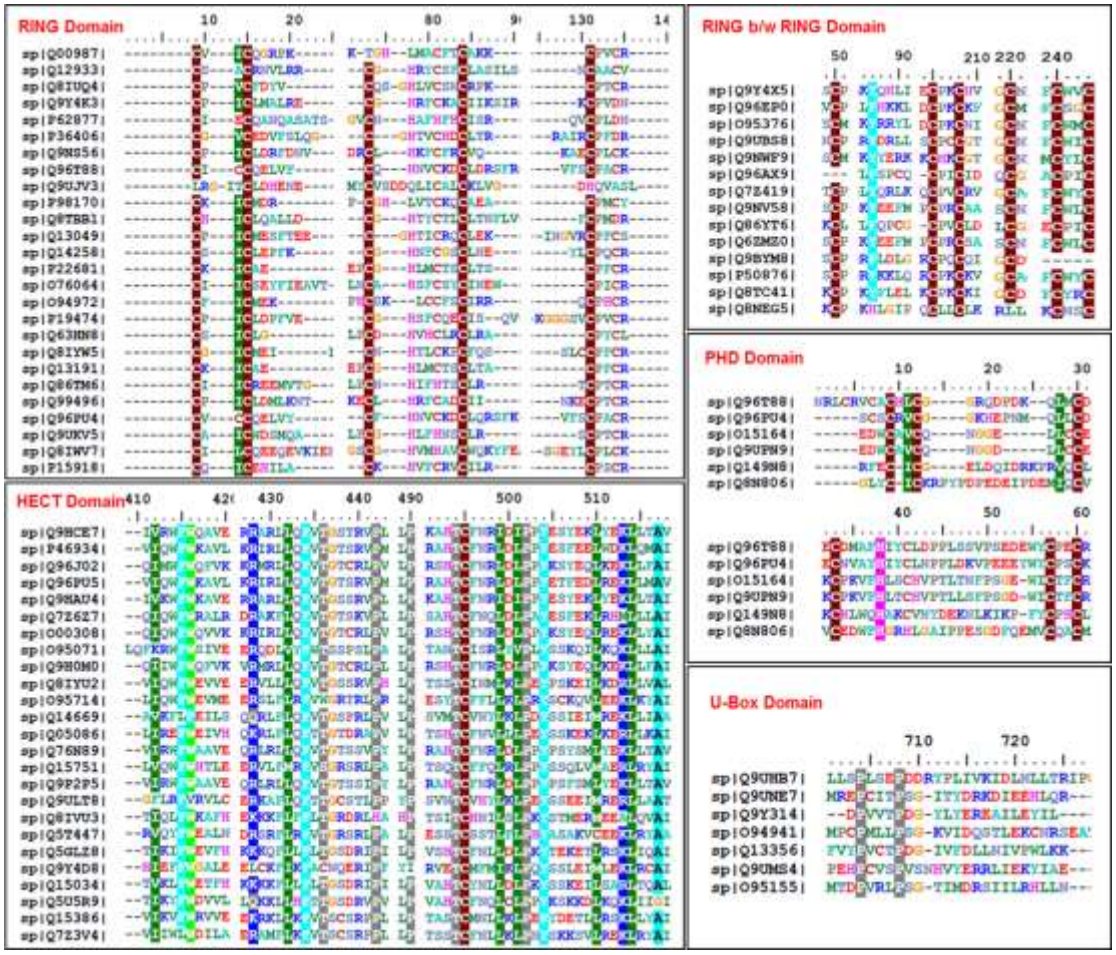


Figure 1.3: Multiple sequence alignments of different ubiquitin ligase domains. (A) RING Domain: Five conserved cysteine (position 9, 15, 73, 84, 131), and one conserved leucine/isoleucine/valine (position 14). **(B) HECT Domain:** Six conserved leucine/isoleucine/valine (position 412, 432, 499, 501, 510, 514), three conserved proline (position 442, 490, 502), two conserved phenylalanine (position 415, 434), arginine/lysine (position 428, 513), tyrosine (position 436, 494), and one conserved cysteine (position 495), tryptophan (position 416), tyrosine (position 504), alanine (position 517). **(C) RING b/w RING Domain:** Six conserved cysteine (position 50, 205, 208, 220, 240, 243) and one conserved tyrosine/phenylalanine (position 87). **(D) PHD Domain:** Six conserved cysteine (position 9, 12, 30, 33, 57, 60), two conserved leucine/isoleucine/valine (position 11, 28), and one conserved histidine (position 38). **(E) U-Box Domain:** Two conserved proline (position 704, 708).

Table 1.3: Key amino acids crucial for the ubiquitin-ligation by different ubiquitin ligase domains

E3 ligase Domain	Alignment Position	Conserved Amino acids	Similarity Threshold (%)	Different E3 ligases in Human	Importance in Ubiquitination	References
RING	9	Cysteine	95	AMFR, BRCA1, BRE1A/B, CBL, CBLB/C, CHFR, CIP1, CNOT4, DTX1/2/3/3L/4, DZIP3, FANCL, GOLI, HAKAI, LIN41, LNX1, LRSM1, LTN1, MARH1/2/3/4/5/6/7/8/9/A/B, MDM2, MGRN1, MKRN1/2/3/4, MSL2, MYCB2, MYLIP, NEU1B/L1/L3, NHLC1, PJA1/2, PZRN3, RAD18, RAG1, RBBP6, RBX1, RFWD2/3, RING1/2, RN103/111/114/115/123/125/126/128/133/135/138/139/146/149/152/167/168/169/170/180/181/182/185/187/213/220, RNF4/5/6/8/12/13/25/34/38/41/43, RO52, SH3R1/2, SHPRH, SIAH1/2, SYVN1, TIF1A, TM129, TOPRS, TRAF2/6/7, TRAIP, TRI11/13/17/18/22/23/25/27/31/32/33/36/37/38/39/41/50/56/58/62/63/68/69, TRIM1/4/8/9/L, TTC3, UBR1/2/3/7, UHRF1/2, UNKL, XIAP, ZN645, ZNRF1/2/3/4	Transthiolation to E2 conjugating enzyme followed by E3 ligase directed ubiquitin transfer to ε-amine of substrate lysine	Metzger *et al.*, 2014
	14	Isoleucine Valine Leucine	90			
	15	Cysteine	99			
	73	Cysteine	90			
	84	Cysteine	90			
	131	Cysteine	90			

E3 ligase Domain	Alignment Position	Conserved Amino acids	Similarity Threshold (%)	Different E3 ligases in Human	Importance in Ubiquitination	References
HECT	412	Isoleucine Valine	90	HACE1, HECD1/2/3/4, HECW1/2, HERC1/2/3/4/6, HUWE1, ITCH, NEDD4, NED4L, SMUF1/2, TRIPC, UBE3A/B/C/D, UBR5, WWP1/2	Transthiolation to HECT E3 ligase active site cysteine followed by ubiquitin transfer to ε-amine of substrate lysine	Metzger *et al.*, 2014; French *et al.*, 2017
	415	Phenylalanine	90			
	416	Tryptophan	95			
	428	Arginine Lysine	95			
	432	Leucine Isoleucine Valine Methionine	100			
	434	Phenylalanine	100			
	436	Threonine Serine	90			
	442	Proline	95			
	490	Proline	95			
	494	Threonine	100			
	495	Cysteine	100			
	499	Leucine Isoleucine	100			
	501	Leucine Isoleucine Valine	100			
	502	Proline	100			
	504	Tyrosine	100			
	510	Leucine Methionine	95			
	513	Lysine Arginine	90			
	514	Leucine	100			
	517	Alanine	95			

E3 ligase Domain	Alignment Position	Conserved Amino acids	Similarity Threshold (%)	Different E3 ligases in Human	Importance in Ubiquitination	References
RING b/w RING	50	Cysteine	90	ARI1/2, HOIL1, MIB1/2, PRKN2, R144A/B, RN19A/19B/216/217, RNF14/31, ZSWM2	A RING-HECT-hybrid E3 that utilizes a RING domain for E2-binding and second RING with active site cysteine for ubiquitin transfer to ε-amine of substrate lysine	Dove and Klevit 2017
	87	Tyrosine Phenylalanine	90			
	205	Cysteine	100			
	208	Cysteine	100			
	220	Cysteine	90			
	240	Cysteine	90			
	243	Cysteine	90			
PHD	9	Cysteine	100	SHPRH, TIF1A, TRI33, UHRF1/2	A type of complex Zn finger protein with proposed ubiquitin ligase activity induced upon histone binding	Agricola *et al.*, 2011
	11	Leucine Isoleucine Valine	100			
	12	Cysteine	100			
	28	Leucine Isoleucine Valine	100			
	30	Cysteine	100			
	33	Cysteine	100			
	38	Histidine	100			
	57	Cysteine	100			
	60	Cysteine	100			
U-Box	704	Proline	100	AFF4, CHIP, NOSIP, KIAA0860, PPIL2, PRPF19, UFD2	Identified as a ubiquitin chain assembly factor for E1, E2, E3 to catalyze the formation of ubiquitin chain	Hatakeyama and Nakayama 2003
	708	Proline	100			

1.4.1 Proteasome assembly and the dynamics of protein clearance

There are two different types of proteasomes including 20S (without regulatory unit; smaller) and 26S (with regulatory unit; larger) that functions to degrade unfolded and folded proteins respectively in the eukaryotic cell. Here, the 20S proteasome comprised of only a catalytic barrel shaped core unit that proteolyses unstructured, intrinsically unfolded non-ubiquitinated proteins, while the 26S proteasome comprised of additional two 19S regulatory units at both ends along with the 20S barrel-shaped proteolytic core unit that proteolyses the ubiquitinated substrates. The 19S caps identify the substrate, deubiquitinate and unfold it to further process with hollow core of the 20S catalytic center to produce free amino acids. During the course of toxic metabolite clearance, firstly, ubiquitinated proteins get docked with its ubiquitin chain to the 19S proteasome regulatory particle base subunits, including Rpn10/PSMD4 (26S Proteasome non-ATPase regulatory subunit-4) and Rpn13/ADRM1 (Adhesion Regulating Molecule 1) evident in several studies (Schreiner *et al.,* 2008). Further, the docking between proteasome and ubiquitin tagged protein is followed by the cleavage of the entire ubiquitin chain from the substrate protein by 19S regulatory particle Rpn11/Poh1 (also called PSMD14) thereby recycling free ubiquitin and facilitate substrate's entrance to the catalytic 20S core unit. The ubiquitin chain cleavage is dependent on ATPase activity of Mts2/Rpt2/S4 subunit of 19S regulatory protein, which aids in the translocation of targeted protein to 20S through a conformational change in the Rpn11n, i.e. 19S regulatory particle (Kleiger and Mayor, 2014). Such conformational change triggers the movement of 20S core alpha subunit's N-terminal tails to open the axial pores that enhance proteolytic activity (Miller and Gordon, 2005) and release cleaved peptides. Moreover, the complex interaction between ubiquitin E3 ligases and the proteasome is a precisely controlled process that governs the clearance of toxic metabolites. For instance, Parkin, an E3 ligase found to interact via its N-terminal ubiquitin-like (Ubl) domain with 19S proteasomal ubiquitin receptors Rpn10/S5a and Rpn13/ADRM1 (Safadi and Shaw, 2010). Where, Rpn13/ADRM1 reported to interact with the help of its pleckstrin-like receptor for ubiquitin (PRU) domain. In addition, PRU domain interaction with Parkin attributed to the hydrophobic patch surrounding Ile44 in the parkin Ubl domain (Aguileta *et al.,* 2015). Another instance where E3 component of

two different ubiquitin ligases Ubr1p and Ufd4p are found to interact with 19S particle via Rpt1p, Rpt6p, Rpn2p and Rpt6p proteins respectively in yeast (Xie and Varshavsky, 2000). Moreover, there are proteasome adaptor proteins that enable the binding of proteasome with the ubiquitin-tagged proteins. For instance, Rad23, Dsk2, and Ddi1 found to interact with proteasome via their ubiquitin like (Ubl) domain and to the polyubiquitinated chains by their ubiquitin associated (UBA) domains (Schrader *et al.,* 2009). Apart from E3s, few E2 proteins, including Ubc1, Ubc2, Ubc4, and Ubc5 also found to interact with 26S proteasome, where their interaction gets strongly induced by heat stress (Tongaonkar *et al.,* 2000). However, the potential roles of conjugating E2s are still elusive in direct interaction with proteasome for the clearance of polyubiquitinated proteins.

1.5 MUTATIONAL CONSEQUENCES OF UBIQUITIN PROTEASOME SYSTEM

The UPS governed processes are highly orchestrated with the help of multi-level checkpoints for protein quality control, including ubiquitin tag, substrates undergoing ubiquitination, ubiquitinating enzymes i.e. activating E1s, conjugating E2s and ligating E3s, proteasomal subunits and deubiquitinating enzymes. Therefore, genetic disorders pertaining to the ubiquitination and proteasomal degradation are associated with the mutations in the UBB ubiquitin gene, substrate genes for ubiquitination, ubiquitinating enzymes (E1, E2s and E3s), deubiquitinating enzymes and proteasomal subunits (Jiang and Beaudet, 2004). Many of these factors reported mutated in a variety of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis. For instance, a mutant isoform of ubiquitin, i.e. UBB^{+1} (G76Y mutation followed by 19 additional residues) is evident in Alzheimer's disease to be more pathogenic than its α-synuclein aggregates thereby aggravating its symptoms (Chojnacki *et al.,* 2016). Moreover, UBB^{+1} linked aberrancy has also been reported as hallmarks of disease in other neurodegenerative diseases like Parkinson's disease, Pick's disease and Huntington's disease (Yim *et al.,* 2014). There are some other instances where mutations in the hydrophobic core of ubiquitin impaired their interaction with the proteasome (Haririnia *et al.,* 2008). Similarly, mutations in ubiquitin substrate NF-κB essential

modulator (NEMO) has reported compromised ubiquitination and consequent loss of NF-κB signaling leading to immunodeficiency, ectodermal dysplasia, immune thrombocytopenic purpura and incontinentia pigmenti (Ramirez-Alejo *et al.,* 2015). Likewise, mutation in another ubiquitin substrate epithelial Na^+ channel's (ENaC) PY (Pro-Pro-x-Tyr) motif disrupted its ubiquitination by Nedd4-2 E3 ligase and triggered hypertension linked Liddle syndrome (Rotin, 2008).

Furthermore, there are innumerable instances of mutations in ubiquitinating enzymes that govern a variety of diseases, including neurodegeneration. For instance, two missense mutations in UBE1 gene (c.1617 G→T, p.Met539Ile; c.1639 A→G, p.Ser547Gly) found among the patients with X-linked infantile spinal muscular atrophy (XL-SMA) exhibiting clinical features like areflexia, hypotonia and multiple congenital contractures (Ramser *et al.,* 2008). However, a spectrum of pathologies has been diagnosed with mutant conjugating E2s such as UBE2A in cancer, UBE2A mutant c.382C→T in the novel X-linked mental retardation syndrome (Nascimento *et al.,* 2006) while UBE2N both in cancer (Voutsadakis, 2013) and synaptosomal accumulation of mutant huntingtin in Neurodegeneration (Yin *et al.,* 2015). Likewise, UBE2W knockout exhibited postnatal lethality and defects in male reproductive systems (Wang *et al.,* 2016a). Alike conjugating E2s, there are numerable E3 ligases that are involved in the pathogenesis of diverse disorders in their mutant state. A survey identified 83 E3 ligase gene mutants representing around 70 different neurological diseases, including ~51 rare and ~19 common (George *et al.,* 2018). For instance, mutant E3 ligases such as FBXL7, FBXL13 and TTC3 are evident to aggravate the pathology of Alzheimer's disease (Floudas *et al.,* 2014; Tosto *et al.,* 2015; Kohli *et al.,* 2016). Similarly, many E3 ligases, including FBXO7, LRSAM1 and PARK2 found, mutated and contributing to the pathogenesis of Parkinson's disease (Lohmann *et al.,* 2015; Aerts *et al.,* 2015; Konovalova *et al.,* 2015). Additionally, altered functioning of different E3 ligases like UBE3A and WWP1 reported in Huntington's disease (Maheshwari *et al.,* 2012; Lin *et al.,* 2016). Likewise, a spectrum of CCNF mutations is identified in the patients affected with Amyotrophic lateral sclerosis and Fronto temporal dementia (Pan *et al.,* 2017). Apart from ubiquitinating enzymes, several deubiquitinating enzymes mutations have

diagnosed in a spectrum of diseases, including cancer and NDDs. For instance, UCH-L1 mutants found to contribute in the pathogenesis of AD, PD and HD (Xu *et al.*, 2009; Cartier *et al.,* 2012; Zhang *et al.,* 2014), while ataxin3 mutants, a deubiquitinating enzyme is well identified for the occurrence of polyglutamine disorders like spinocerebellar ataxia (Konno *et al.,* 2014). Similarly, other DUB mutants CYLD, USP6 and TNFAIP3 are found to be involved in the progression of Cylindromatosis, Sarcomas and B-cell/T-cell lymphomas respectively (Hussain *et al.,* 2009).

Another UPS factor that triggers a variety of disorders is mutations in the Proteasomal subunit genes. However, huge reports are available signifying the involvement of proteasomal mutants (PSMA6/7, PSMB8/9) in variety of disorder, including diabetes, heart disease, inflammatory disease, Cancer, etc. (Gomes, 2013), but there is hardly any evidence of mutated proteasomal subunits in neurological disorders. Instead, numerous studies have reported the structural aberrancies in proteolytic or regulatory subunits of UPS in diverse neurodegenerative disorders. For instance, in a study on cell lines HAW, HEK293 and APP23 transgenic mice model, overexpression of AβPP significantly lowered the expression of two 20S proteasomal subunits α-type-5 and β-type-7 (Wu *et al.,* 2015). Another study identified calpain mediated cleavage of S5A (Rpn10) subunit of 19S regulatory units upon mitochondrial dysfunction in neurons (Huang *et al.,* 2013). Likewise, Zouambia *et al.,* identified the immunoreactivity of S6b (Rpt3) subunit of 19S regulatory ATPase at dystrophic neurites and neurofibrillary tangles in Alzheimer's Disease, Down's syndrome, Frontotemporal dementia, Pick's disease, and Progressive supranuclear palsy except Lewy body disease (LBD) and multiple system atrophy (Zouambia *et al.,* 2008). However, Barroso-Chinea *et al.,* reported that impaired proteasomal activity could be due to D1 receptor mediated disassembly of 26S proteasome rather than defects in proteasomal subunits (Barroso-Chinea *et al.,* 2015). Similarly, heterozygous deletion of ATPase subunits of 19S regulatory subunit S4 (Rpt2) in mice derived fibroblasts exhibited reduced proteasomal activity (Rezvani *et al.,* 2012a). Another evidence of S8 (Rpt6), a 19S subunit reduction is observed in the cortex for cognitive impairment in Lewy Body Dementia (Alghamdi *et al.,* 2017). Likewise, Bedford *et al.,* demonstrated Psmc1 knockout mice mediated 26S proteasome depletion in targeted neurons that

resulted in intraneuronal Lewy-like inclusions and extensive neurodegeneration in forebrain and nigrostriatal pathway (Bedford *et al.,* 2008). Moreover, some studies have identified the elevated proteasomal action upon overexpression of 20S core β-5 subunit and non-ATPase 19S regulatory subunit-11 (Rpn6) indirectly supporting the fact that 19S and 20S proteasomal subunits get affected during diseased conditions unable to clear protein burden (Pathare *et al.,* 2012; Vilchez *et al.,* 2012a, b). Consequently, there are growing instances where mutations are reported in the UPS components governing the pathology of neurological disorders summarized in **Table 1.4.**

Table 1.4: Mutational aberrancies in UPS components triggering neurological disorders

Neurological Disorders [OMIM ID]	Gene (UPS-Domains)	Reported Mutations		Disease Phenotypes	References
		SNV	SNP		
Anencephaly [208900]	TRIM36 (E3-RING)	–	rs79290430 (K→R), rs17137481 (N→S), rs2974617 (D→N), rs3749745 (Q→E)	Neural tube defects lead to the absence of brain tissues. Infants born with intact spinal cord, cerebellum, and brainstem, but lack formation of neural structures above this level causing death in utero or perinatally	Singh *et al.*, 2017a
Angelman's syndrome [105830]	UBE3A (E2-HECT)	F750D	rs587784511 (R→H), rs587781241 (T→K), rs587782907 (C→R), rs587782915 (V→G), rs147145506 (A→T), rs587780581 (D→V), rs587780582 (L→H/P), rs587780583 (L→W), rs1059383 (V→G), rs587782908 (N→T), rs141984760 (S→T), rs587781242 (L→P), rs587780584 (P→L), rs587781243 (R→P), rs587782916 (M→I), rs587781233 (G→R), rs587781244 (M→K/R), rs587781235 (E→Q), rs587782918 (Q→E), rs587782919 (Q→P/R), rs587781236 (T→S), rs587782920 (L→R), rs587781237 (F→C), rs587782910 (V→I/L), rs587781239 (P→L)	Neurodevelopmental disorder characterized by severe motor and intellectual retardation, ataxia, flapping of arms and hands, hypotonia, seizures, absence of speech, frequent smiling and episodes of paroxysmal laughter with open-mouthed expression	Ronchi *et al.*, 2014; Sadikovic *et al.*, 2014
Ataxia [608984]	RNF170 (E3-RING)	—	rs397514478 (R→C)	A degenerative disease caused by loss of posterior columns of spinal cord. Affected individuals have a reduced ability to feel pain, temperature and vibration especially in hands and feet	Lu *et al.*, 2011, Valdmanis *et al.*, 2011
Autosomal Recessive Mental Retardation [615979, 615516]	FBXO31 (E3-F-box)	C283Rframeshift→Ter, S278A, S400A	—	A neurodevelopmental disorder characterized by significantly below average general intellectual functioning associated with impairments in adaptive behavior. Disease manifestations include mild to moderate intellectual disability and dysmorphic features	Mir *et al.*, 2014
	HERC2 (E3-HECT/ RCC1 like)	C2708S, C2711S, T4827A	rs397518474 (P→L)		Puffenberger *et al.*, 2012

Neurological Disorders [OMIM ID]	Gene (UPS-Domains)	Reported Mutations		Disease Phenotypes	References
		SNV	SNP		
Charcot-Marie-Tooth disease [607678, 614436, 615490]	EGR2 (E3-Zn finger)	D305V, R381H	rs104894158 (I→N), rs104894161 (R→W), rs281865137 (R→H), rs104894160 (D→Y), rs104894159 (R→W)	A hereditary motor and sensory neuropathies of peripheral nervous system characterized by progressive weakness and atrophy, initially of the peroneal muscles and later of the distal muscles of the arms and loss of touch sensation across various parts of body	Pareyson *et al.*, 2000; Yoshihara *et al.*, 2001
	LRSAM1 (E3-CC/LRR/RING)	Deletion(649-664), C675A, H692A	rs1539567 (N→D), rs759312530 (C→R), rs886041051 (C→Y)		Amit *et al.*, 2004
	TRIM2 (E3- beta-propeller (NHL))	—	rs587777063 (E→V)		Thompson *et al.*, 2011
Congenital Neuropathy, Hypomyelinating or Amyelinating [605253]	EGR2 (E3-Zn finger)	—	rs104894158 (I→N), rs104894161 (R→W), rs281865137 (R→H), rs104894160 (D→Y), rs104894159 (R→W)	Severe degenerating neuropathy results from a congenital impairment in myelin formation. It is clinically characterized by early onset of hypotonia, areflexia, distal muscle weakness, and very slow nerve conduction velocities	Funalot *et al.*, 2012
Dejerine-Sottas syndrome [145900]	EGR2 (E3-Zn finger)	R359W	rs104894158 (I→N), rs104894161 (R→W), rs281865137 (R→H), rs104894160 (D→Y), rs104894159 (R→W)	A severe degenerating neuropathy characterized by motor and sensory neuropathy with very slow nerve conduction velocities, increased cerebrospinal fluid protein concentrations, hypertrophic nerve changes, and delayed age of walking	Timmerman *et al.*, 1999
Gordon Holmes syndrome [212840]	RNF216 (E3-RING)	—	rs387907368 (R→C)	An autosomal recessive adult onset neurodegenerative disorder characterized by cerebellar and brain stem atrophy, cerebellar ataxia, lack of secondary sexual characteristics, and infertility	Margolin *et al.*,2013

Neurological Disorders [OMIM ID]	**Gene (UPS-Domains)**	**Reported Mutations**		**Disease Phenotypes**	**References**
		SNV	**SNP**		
Intellectual Developmental Disorder with Dysmorphic Facies, Seizures, and Distal Limb Anomalies [617452]	OTUD6B (DUB-OTU)	C158S	rs368313959 (R→Ter), rs3210518 (R→Q/L)	An autosomal recessive severe multisystem disorder characterized by poor overall growth, developmental delay, early-onset seizures, intellectual disability, and dysmorphic features	Santiago-Sim *et al.*, 2017
Macrocephaly, Dysmorphic Facies and Psychomotor Retardation [617011]	HERC1 (E3-HECT/RCC1 like)	—	rs1063423 (L→F), rs3764187 (L→F), rs36089909 (G→V), rs7162519 (H→Y), rs16947363 (S→A), rs2255243 (G→A/V), rs2228512 (T→A/P/S), rs2228510 (I→V), rs35122568 (A→T/P), rs2228513 (S→F), rs7182782 (G→R), rs2229749 (E→D/E), rs2228516 (I→V), rs769677823 (G→A)	An autosomal recessive neurodevelopmental disorder characterized by large head and somatic overgrowth, intellectual disability, facial dysmorphism, seizures, hypotonia, and gait ataxia features	Ortega-Recalde *et al.*, 2015
Macrocephaly, Macrosomia, Facial Dysmorphism Syndrome [614192]	RNF135 (E3-RING)	—	rs7225888 (H→Q/H), rs7211440 (S→P) rs111902263 (R→K), rs121918162 (R→H), rs61749868 (W→C/Ter)	An autosomal dominant disorder characterized by the association of macrothrombocytopathy, progressive sensorineural hearing loss without renal dysfunction and variable learning disability	Douglas *et al.*, 2007
Neurodevelopmental Disorder with Hypotonia, Seizures, and Absent Language [617268]	HECW2 E3-C2/WW/HECT)	—	rs878854416 (R→Q), rs878854422 (F→V), rs878854417 (R→W), rs878854424 (E→G)	A neurodevelopmental disorder characterized by severely delayed psychomotor development, absent speech, epilepsy, encephalopathy, hypotonia, dystonia/dyskinesia, and macrocephaly	Berko *et al.*, 2017

Neurological Disorders [OMIM ID]	Gene (UPS-Domains)	Reported Mutations		Disease Phenotypes	References
		SNV	**SNP**		
Parkinson's Disease [260300, 168600, 600116, 606324, 605909]	FBXO7 (E3-F-box)	T22M, V253E	rs11107 (M→I), rs71799110 (R→G/C), rs148272407 (R→C)	A complex neurodegenerative disorder characterized by bradykinesia, resting tremor, postural tremor, muscular rigidity and postural instability, spasticity, mainly in the lower limbs, anxiety, hyperreflexia and psychotic episodes. The pathology involves the loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies (intraneuronal accumulations of aggregated proteins), in surviving neurons in various areas of the brain.	Burchell *et al.*, 2013
	PARK2 (E3-UBL/ RING)	S65E, C332S, C337A, C365S, W403A, C421A, C431S, H433N/A, E444Q/A	rs532703934 (V→M), rs147757966 (R→Q), rs148990138 (P→L), rs368134308 (R→P), rs137853059 (V→E), rs55774500 (A→E), rs566229879 (A→V), rs137853057 (K→N), rs1801474 (S→N), rs9456735 (M→L), rs9456735 (M→V), rs137853060 (K→N), rs137853058 (C→Y), rs137853054 (T→M/R), rs747427602 (C→Y), rs150562946 (R→C), rs34424986 (R→W), rs72480422 (D→N) , rs751037529 (G→R), rs55961220 (C→G), rs199657839 (R→C), rs56092260 (R→W), rs1801582 (V→L), rs1801334 (D→N), rs55830907 (R→C), rs778125254 (T→N), rs191486604 (G→D), rs397514694 (C→F), rs149953814 (P→L), rs778305273 (C→R)		Oliveira *et al.*, 2003; Foroud *et al.*, 2003
	PARK7 (E3)	E18A/D/N/Q, C46A/S, V51A, C53A/S, C106A/D/S, H126A, K130R	rs74315351 (M→I), rs137853051 (A→S/T), rs74315353 (E→D), rs71653619 (R→Q), rs774005786 (A→T/S), rs74315352 (D→A), rs368420490 (G→S), rs74315354 (E→K), rs28938172 (L→P), rs777026628 (A→S)		Bonifati *et al.*, 2003
	PINK1 (E3-Protein kinase)	K219A, D362A, D384A	rs763142730 (L→F), rs575668171 (R→W/G), rs148871409 (Q→L/P), rs45604240 (T→M), rs138050841 (R→H), rs56297806 (L→S), rs768091663 (A→P), rs143204084 (K→N), rs138302371 (P→L), rs35802484 (P→S), rs34677717 (P→L), rs371854396 (P→L/R), rs74315360 (A→D), rs573931674 (E→K), rs370906995 (T→I), rs372280083 (L→V), rs28940284 (H→Q/H), rs548506734 (R→Q), rs74315358 (R→H), rs772510148 (A→T), rs779060308 (P→L), rs7349186 (P→R), rs74315355 (G→D), rs74315359 (T→M), rs200949139 (V→I), rs139226733 (M→L), rs768019187 (P→L), rs55831733 (A→T), rs3738136 (A→T), rs35813094 (M→I), rs28940285 (L→P), rs34203620 (C→F), rs45515602 (A→T), rs1035071310 (G→V), rs119451946 (P→L), rs556540177 (R→Q/P/L), rs45478900 (G→S), rs554114655 (P→S), rs74315361 (Y→H), rs747400197 (N→S/I), rs764328076 (R→H), rs115477764 (E→K), rs34416410 (S→T), rs1043424 (N→T), rs531477772 (D→N), rs771032673 (A→T)		Geisler *et al.*, 2010

Neurological Disorders [OMIM ID]	Gene (UPS-Domains)	Reported Mutations		Disease Phenotypes	References
		SNV	SNP		
Periventricular Nodular Heterotopia [617201]	NEDD4L (E3-C2/WW/HECT)	S448A, C942S	rs767136811 (P→L), rs879255599 (Y→C), rs879255598 (Q→H), rs879255597 (E→K), rs879255596 (R→Q)	A disorder of neuronal migration from ventricular zone to the cortex during development, resulting in the formation of nodular brain tissue lining the ventricles characterized by delayed psychomotor development, intellectual disability, and seizures	Broix *et al.*, 2016
Progressive myoclonus epilepsy [254780]	NHLRC1 (E3-RING)	E280K	rs28940575 (C→S), rs757759398 (F→S/C), rs779507031 (E→Q/K/Ter), rs28940576 (P→A), rs10949483 (P→L/H), rs950907157 (L→P), rs769301934 (D→N), rs200595273 (C→R), rs121917876 (I→N), rs879745047 (L→P), rs757858146 (Q→P), rs137852859 (D→A)	An autosomal recessive and severe form of adolescent-onset progressive epilepsy manifested typically as seizures which increase in frequency, cognitive impairment, ataxia and other neurologic deficits	Couarch *et al.*, 2011
Spinocerebellar Ataxia [109150, 615768]	ATXN3 (DUB-UIM/Poly-Gln)	C14A, S236A, S256A, S335A	rs1048755 (V→M)	A group of hereditary ataxia characterized by progressive incoordination of gait and often poor coordination of hands, speech and eye movements, due to cerebellum degeneration with variable involvement of the brainstem and spinal cord	Mao *et al.*, 2005
	STUB1 (E3-U-box/TPR)	K30A, H260Q, P269A	rs690016544 (N→S), rs587777347 (A→D), rs587777346 (A→T), rs587777344 (L→V), rs587777341 (N→I), rs146251364 (K→Q), rs587777342 (W→Ter), rs587777340 (L→F), rs587777345 (M→T), rs587777343 (T→M)		Depondt *et al.*, 2014; Synofzik *et al.*, 2014
Stankiewicz-Isidor Syndrome [617516]	PSMD12 (PS-PCI)	—	rs2230680 (V→A)	A neurodevelopmental disorder characterized by delayed psychomotor development, intellectual disability, behavioral disorders, ophthalmologic anomalies, feeding difficulties, deafness, and variable congenital malformations of the cardiac and/or urogenital systems	Kury *et al.*, 2017

Neurological Disorders [OMIM ID]	Gene (UPS-Domains)	Reported Mutations		Disease Phenotypes	References
		SNV	SNP		
Wilms Tumor, Spastic Paraplegia and Psychomotor Retardation with or without Seizures [616756]	HACE1 (E3-ANK/HECT)	C876A/S	rs17853353 (R→H), rs17857038 (I→T)	A form of spastic paraplegia, a neurodegenerative disorder characterized by a slow, gradual, progressive weakness and spasticity of the lower limbs	Hollstein *et al.*, 2015
X-linked Mental Retardation [300984, 300919, 300928, 300978]	USP27X (DUB-USP)	—	rs886038211 (Y→H)	A form of mental retardation associated with macrocephaly and variable contractures, characterized by significantly below average general intellectual functioning	Hu *et al.*, 2015
	USP9X (DUB-USP)	—	rs587777317 (L→H), rs587777319 (L→I/L)		Homan *et al.*, 2014
	FBXO25 (E3-F-box)	S244L	rs17665340 (N→D), rs10090550 (R→H/P)		Hagens *et al.*, 2006
	MID2 (E3-RING/CC/ B-box)	—	rs551253128 (N→S), rs587777605 (R→Q), rs12849510 (A→D/G)		Geetha *et al.*, 2014
	RLIM (E3-RING)	—	rs786205133 (Y→C)		Tonne *et al.*, 2015
X-linked Syndromic Martin-Probst Type Mental Retardation [300519]	RAB40AL (E3)	—	rs145606134 (D→G)	A rare neurodevelopmental disorder characterized by mental retardation, sensorineural hearing loss, short stature and craniofacial dysmorphisms	Bedoyan *et al.*, 2012
X-linked Syndromic Mental Retardation [300354]	CUL4B (E3-NEDD)	Deletion(55-58), K55A, K56A, R57A, K58A, K703Ter	rs61759504 (L→P/R), rs763692058 (T→I), rs121434615 (R→C)	A recessive neurodevelopmental disorder characterized by severe intellectual deficit associated with short stature, craniofacial dysmorphism, small testes, muscle wasting in lower legs, small feet, and abnormalities of the toes	Tarpey *et al.*, 2007
X-linked Syndromic Nascimento-Type Mental Retardation [300860]	UBE2A (E2-UBCc)	—	rs387906728 (R→Q)	A neurodevelopmental disorder characterized by dysmorphic features, including large head, almond-shaped and deep-set eyes, large ears, wide mouth, micropenis, and onychodystrophy along with intellectual deficiency	Budny *et al.*, 2010
X-linked Syndromic Turner type Mental Retardation [300705, 300706]	HUWE1 (E3-UBA/UIM/WWE/HECT)	Y4268S, C4341A/D	rs41307640 (N→S), rs121918526 (R→H), rs121918525 (R→W), rs121918527 (R→C)	A neurodevelopmental disorder characterized by the association of mental retardation with macrocephaly and variable contractures, poor intellectual functioning is associated with impairments in adaptive behavior	Zhao *et al.*, 2008

E2-Ubiquitin conjugating enzyme; **E3-**Ubiquitin ligase; **DUB-**Deubiquitinase; **PS-**Proteasomal Subunit; **NEDD-**Neddylation

1.6 THE UPS MEDIATED AMELIORATION OF NEURODEGENERATIVE PROTEINOPATHIES

Neurodegeneration governed by various pathological events associated with proteinopathies such as mitochondrial dysfunction, ER stress, oxidative stress, unfolded protein response and neuro-inflammation. Inside a cell, protein quality control system and protein waste elimination pathway, for instance, UPS plays a decisive role in compensating the adverse effects of proteinopathies and thereby ameliorating the symptoms of neurodegenerative disorders.

1.6.1 Alzheimer's disease: Clearance of Aβ, AβPP and Tau

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the deposition of amyloid-β (Aβ) peptides as senile plaques and tau as neurofibrillary tangles in the brain, where mutant AβPP and ERAD/ER stress are playing a prominent role in mediating Aβ production, secretion, and related neurotoxicity. Various cell line studies have identified the Aβ oligomer's accumulation upon AβPP mutation in ER lumen along with ER stress markers such as eIF2α- phosphorylated eukaryotic initiation factor-2, GRP78- 78kDa glucose-regulated protein, caspase-3 and caspase-4 that triggered apoptosis and autophagy (Pereira, 2013). Likewise, animal studies have also shown similar results. For instance, transgenic mice (E693Δ), expressing mutant amyloid precursor protein (AβPP) lead to Aβ oligomeric deposition in the ER of hippocampal neurons which is attributed to the over expression of GRP78 and HRD1, an ERAD E3 ubiquitin ligase (Umeda *et al.,* 2011). Likewise, Aβ oligomeric-pool reported in ER lumen due to defective kinesin-1-dependent axonal transport due to JNK-mediated phosphorylation and AβPP cleavage through amyloidogenic pathways (Muresan and Muresan, 2012). Here, a clear role of the ubiquitin proteasome system (UPS) has reported in both early and late-stage AD characterized by synaptic dysfunction and neurodegeneration respectively. Moreover, increasing evidence reported the intriguing relationship between Aβ and UPS activity playing a cardinal role in AD pathogenesis. For instance, UPS has a role to play in Aβ clearance, while at the same time Aβ also inactivates proteasomal activity of UPS subsequently leading to impairment in multivesicular bodies (MVB) sorting pathway (Almeida *et al.,* 2006).

Further studies have identified the mutant ubiquitin (Ub) and ubiquitin-like (Ubl) ubiquilin-1 protein linked with Aβ accumulation while up regulated proteasomal degradation of BACE1 and γ-secretase components leads to decrease in Aβ accumulation (Hong *et al.,* 2014). Another E3 ligase FBXO2 (an ERAD family protein) is evidenced to expedite the clearance of AβPP, which otherwise reduced in AD (Atkin and Paulson, 2014). Similarly, another E3 ligase, Parkin over expression curtailed AβPP expression, Aβ burden, and inflammation in the AD mouse model (Sweeney *et al.,* 2017). Another protein C-terminus of HSC70-Interacting Protein (CHIP) is found to curtail the level of Aβ as well as hyper phosphorylated tau through ubiquitination with help of Hsp90 and Hsp70 (Gadhave *et al.,* 2016). Here, binding of Hsc70 and phosphorylation of tau is a prerequisite signal for its ubiquitination by E3 ligase CHIP and E2 conjugating enzyme UbcH5B (Shimura *et al.,* 2004). Further, a proteasome conjugator, Nedd8 ultimate buster 1 (NUB1) is conjugated tau aggregates for proteasomal degradation via disrupting the interaction between GSK-3β and tau (Richet *et al.,* 2012). Likewise, MDM2 (mouse double minute 2 homolog), another E3 ligase also contributed in tau clearance in the brain by inhibiting the interaction between GSK-3β and p53 (Proctor and Gray, 2010). Interestingly, inhibition of proteasomal deubiquitinating enzyme (Usp14) and intrusion of proteasome activator (Blm10) showed facilitated tau degradation *in-vitro* studies (Boselli *et al.,* 2017). Similarly, HRD1 (an ERAD-associated E3 ubiquitin-protein ligase) triggered AβPP ubiquitination and degradation leading to Aβ clearance (Wang and Saunders, 2014). Besides, the proteasomal inhibition by Aβ, is found to act through elevated expression of E2-25K/Hip-2 thereby stabilizing and activating caspase-12 (Song *et al.,* 2008). Moreover, Aβ also found to induce ER stress mediated JNK3 activation and AβPP phosphorylation that triggered its endocytosis and further processing for Aβ production (Yoon *et al.,* 2012). Collectively, the above results depicted the progression of ER stress mediated neurotoxic protein burden, which triggered proteasomal dysfunction and thus caused pathogenic symptoms in Alzheimer's disease.

1.6.2 Parkinson's disease: Clearance of α-synuclein and lewy bodies

Parkinson's disease (PD) is one of the prevalent neurodegenerative movement disorders characterized by progressive loss over muscle coordination and control with α-synuclein deposits as lewy body. However, the disease pathogenesis poorly understood, but several lines of evidence presented an intimate relation between UPS aberrations and PD progression. Typically, failure of UPS machinery under the influence of various stresses governed by noxious protein accumulation is responsible for altered protein homeostasis and consequent toxic depositions detrimental to neuronal health. As an instance, deletion of PSMC1/Rpt2 (ATPase Subunit) and variations in PSMC4/Rpt3 in dopaminergic neurons has exhibited ubiquitin and α-synuclein positive inclusions suggesting the role of proteasome in α-synuclein degradation (Vilchez *et al.,* 2014). Similarly, other cellular and animal models developed the prominent features of PD upon UPS disruption. For instance, aberrations in the RING E3 ubiquitin ligase Parkin and the deubiquitinase enzyme, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) has been reported to cause early-onset familial forms of PD with the characteristic features of loss in midbrain dopaminergic neurons and symptoms of rigidity, tremor, and bradykinesia (Mabb and Ehlers, 2010). Since, mutant parkin failed to clear toxic accumulation of their substrates, including, parkin-associated endothelial-like receptor (Pael-R) and cell division control-related protein (CDCrel-1) thereby contributing in disease pathogenesis. Moreover, the loss of Parkin's stability has altered α-Synuclein clearance due to impaired interaction with Beclin-1, otherwise their elevated interaction in response to increased parkin ubiquitination via tyrosine kinase inhibition triggered α-Synuclein clearance (Lonskaya *et al.,* 2013). Further, Parkin acted as a part of macromolecular E3 ligase complex along with the peptidases DJ-1 and PTEN-induced kinase-1 (PINK1) where, any aberrations reported to cause a hereditary form of PD. These proteins together facilitate proteolytic degradation of Parkin's substrate synphillin-1 that is known to interact with α-synuclein and present in Lewy bodies in PD. Furthermore, A mis-sense mutation (I93M) in deubiquitinase enzyme UCHL1 observed in familial forms of PD, which reduced its deubiquitinating activity and consequently, caused accumulation of toxic aggregates like α-synuclein in neurons. Further, It also evidenced to function as E3 ligase to mediate K63-linked

ubiquitination of alpha-synuclein to promote its clearance. Unfortunately, mutant α-synuclein is found to induce filament formation and to curtail proteolytic activity of UPS via direct interaction with its 20S core preoteolytic unit. Another instance has shown the interaction between ubiquitin E3 ligases seven in absentia homolog-1/2 (SIAH-1/2) with synphilin-1 to promote its proteasomal degradation thereby preventing inclusion formation in association with α-synuclein (Liani *et al.,* 2004). Moreover, Nonaka *et al.,* identified the potential ubiquitination sites at Lys6, Lys10, and Lys12 in filament like α-synuclein while Lys21, Lys23, Lys32, and Lys34 in the soluble form of α-synuclein. Interestingly there in-vivo studies identified the ubiquitination sites to be common to the sites found in filamentous α-synuclein signifying the importance of structural conformation of protein aggregates in determining their ubiquitination process (Nonaka *et al.,* 2005). Several other ubiquitin-linked proteins such as HRD1, MDM2 and CHIP governed an important role in maintaining protein homeostasis to prevent neurotoxicity in case of PD. For instance, HRD1 ubiquitinated Pael-R for proteasomal degradation and reduced endoplasmic reticulum stress mediated neuronal loss in PD (Omura *et al.,* 2006). Likewise, CHIP has prevented α-synuclein aggregation via inhibiting its oligomerization and by triggering its proteasomal degradation (Tetzlaff *et al.,* 2008).

1.6.3 Huntington's disease: Clearance of mHtt and inclusion bodies

Huntington's disease (HD) is a poly-glutamine expansion disorder in huntingtin protein causing progressive neurodegeneration. The pathological hallmark of this disease is the intracellular mutant huntingtin (mHtt) aggregates prominently seen with the components of the ubiquitin proteasome system. Numerous studies have reported the aggregate as well as soluble forms of polyubiquitinated mHtt in HD transgenic R6/2 mice model, HD patient material or post-mortem brains (Chang *et al.,* 2015; Juenemann *et al.,* 2015). However, mHtt ubiquitination has reported but researches revealed that proteasomes are incapable of cleaving poly glutamine sequences leading to its aggregation in neurons. As seen in case of mammalian 26S proteasomes, this found to cleave mHtt within the flanking sequences or after first glutamine of a PolyQ peptide leaving intact PolyQ repeat in the neuron (Venkatraman *et al.,* 2004). Thus, released undigested PolyQ sequences obstructed their passage through proteasome,

caused its impairment and aggregation in neurons, and triggered cytotoxicity. This has verified by FRET experiments, which have shown the interaction between mutant huntingtin (mHtt) and proteasomal catalytic subunit LMP2 (Holmberg *et al.,* 2004), whose impairment affected their chymotrypsin and caspase like activities thereby compromising the long peptide cleavage in HD patients (Schipper-Krom *et al.,* 2012). Further, their interaction is evident to be irreversible with the help of FRAT experiment that signified the entrapment of proteasomes in poly Q aggregates (Schipper-Krom *et al.,* 2012). It has also observed that oligomeric forms or soluble forms of mHtt triggered proteasomal impairment while inclusion bodies failed to do so (Diaz-Hernandez *et al.,* 2006). Scientists have further shown the improvement in mHtt clearance by altering the activities of proteasomes. For instance, proteasome activators such as PA28, PA200, PA700 and 20S catalytic immuno subunits- LMP2 (PSMB9), LMP7 (PSMB8), and Mecl-1 (PSMB10) found to facilitate PolyQ cleavage and its clearance in response to IFN-γ stimulation (Hendil *et al.,* 1998; Heink *et al.,* 2005; Koyuncu *et al.,* 2017). Interestingly, optical pulse chase experiments have shown faster mHtt removal in the cytoplasm of the cell body than in neuronal processes indicating their selective compartmentalized degradation (Zhao *et al.,* 2016). Apart from proteasome alteration, other proteins have also identified to govern the clearance through ubiquitination of mHtt. For example, Iwata et al demonstrated the facilitated degradation of mHtt aggregates with the help of nuclear ubiquitin ligases SAN1P and UHRF-2 (Iwata *et al.,* 2009). Another study revealed K48 mediated ubiquitination of Htt fragments in UBE3A dependent manner (Bhat *et al.,* 2014). Similarly, UBE2W found to regulate the ubiquitination and solubility of mHtt aggregate in HdhQ200 KI mice (Wang *et al.,* 2018). Another protein Bcl-2 found to decrease the affinity of p62/SQSTM1 to polyubiquitinated proteins that reduced mHtt aggregation and allowed their proteasomal degradation (Zhou *et al.,* 2015; Cohen-Kaplan *et al.,* 2016). Interestingly an E3 ligase WWP1 found to inhibit proteasome mediated mHtt degradation via K63 linked ubiquitination (Lin *et al.,* 2016). Similarly, other atypical ubiquitination (K6, K27, K29) of mHtt by tumor-necrosis factor receptor associated factor 6 (TRAF6) evident to form aggregates in HD (Zucchelli *et al.,* 2011), while, another inflammatory kinase like IKK found to promote the degradation of mHtt by proteasomes and lysosomes (Thompson *et al.,* 2009).

Other UPS pathway proteins such as conjugating E2s and E3-ligases affect the ubiquitination, degradation and toxicity of mHtt. For instance, hE2-25k, a conjugating E2 interacted with N-terminus of mHtt and promoted its inclusion body formation thereby reducing cytotoxicity (de Pril *et al.,* 2007). While other E2 conjugating enzyme, named CDC34 found to promote toxicity in response to the decreased inclusion body (Saudou *et al.,* 1998). Likewise, other E3 ligases- HRD1, Parkin and CHIP decreased inclusion bodies with facilitated clearance of mHtt with the ubiquitin proteasome system (Tsai *et al.,* 2003; Jana *et al.*, 2005; Yang *et al.*, 2007).

1.6.4 Amyotrophic lateral sclerosis: Clearance of SOD1, TDP-43 and inclusion bodies

ALS is a rare progressive motor-neurodegenerative disorder affecting brain and spinal cord. The loss of motor activity triggers advanced paralysis with troubles in chewing, talking, walking and consequent death after few years of onset. In fact, studies have reported impaired turnover of several proteins, including SOD1 (Superoxide Dismutase 1), FUS (Fused in Sarcoma), Optineurin (OPTN), Ubiquilin-2 (UBQLN2), TDP-43 (Transactivation Response DNA-binding protein 43), and translational product of intronic hexanucleotide repeats of C9ORF72 gene, as the most susceptible cause for ALS (Simon-Sanchez *et al.,* 2012; Farrawell *et al.,* 2015). Molecular investigations reported the presence of prion like domains in these proteins, including FUS, TDP-43 and other RNA binding proteins (RBPs) enriched with asparagine, glutamine, glycine and tyrosine residues that can adopt aggregation prone conformation (Blokhuis *et al.,* 2013). Various cellular and animal models have shown the role of dysfunctional UPS in protein-inclusion formation and consequent motor neuronal death (Bendotti *et al.,* 2012). For instance, conditional knockout of proteasomal subunit PSMC4/Rpt3 showed aggregates of TDP-43 and FUS proteins with affected locomotor function and motor neuronal loss while not in the case of autophagy related protein Atg7 knockout, signifying the involvement of proteasome in ALS development (Tashiro *et al.,* 2012). Moreover, it has been observed that inducible subunits of proteasome like LMP2, LMP7 and MECL-1 get overexpressed early during disease progression and provide immunity by generating peptides for

major histocompatibility class-I molecules in the SOD1 mice model of ALS (Bendotti *et al.,* 2012). Furthermore, current researchers have identified the role of UPS in the clearance of pathological proteins in ALS. Specifically, the clearance of mutated or excessive SOD1 in ER triggered by an E3 ligase NEDL1 with the help of translocon protein TRAP while the cytosolic SOD1 reported to be clear by E3 ligases E6-AP and Dorfin (Mishra *et al.,* 2013). Interestingly, Dorfin has the potential to ubiquitinate mutant SOD1 without affecting the wild-type SOD1 (Niwa *et al.,* 2002). Another E3 ligase, CHIP along with ubiquitin binding proteins- Bag1 and VCP (p97) have facilitated mutant SOD1 degradation triggering its translocation from ATPase subunit of 19S to the core 20S proteasome particle (Choi and Lee, 2010). Moreover, a mitochondrial E3 ligase MITOL found to ubiquitinate mutant SOD1 and thereby mitigated mSOD1 induced reactive oxygen species (ROS) generation (Yonashiro *et al.,* 2009). Similarly, TDP-43 found to be ubiquitinated by Parkin in association with HDAC6 and facilitate its accumulation in the cytosol. The TDP-43 aggregates are reported to inhibit the proteasome and thus evident to increase the cellular burden (Hebron *et al.,* 2013). Further, a CCNF missense mutation has identified to alter E3 ligase complex (SCFCyclin-F) thereby causing abnormal ubiquitination of ubiquitinated proteins like TDP-43 (Williams *et al.,* 2016). Likewise, C9ORF72 gene associated mutations are responsible for dipeptide repeat proteins that aggregated and have shown ubiquitin and p62 immunoreactivity in the cell (Freibaum and Taylor, 2017). Recently, a p53 family protein p63 has been reported to augment the cellular level of an E3 ligase called TRIM63 (MuRF1) that triggers the degradation of muscular proteins during muscular atrophy in ALS (von Grabowiecki *et al.,* 2016). Similarly, an ubiquitin-binding protein CG5445 has been identified to attenuate the cytotoxicity and aggregation of ALS linked TDP-43 in Drosophila (Uechi *et al.,* 2018).

1.6.5 Multiple sclerosis: Clearance of auto-antigens and inflammatory agents

Multiple sclerosis (MS) is a chronic inflammatory progressive neurodegenerative disorder of the central nervous system triggering demyelination and muscular atrophy with prominent visual impairment. It primarily affects the nerves in the brain and spinal cord resulting in a range of phenotypes, including physical, mental and

psychiatric problems. Earlier diagnosis reported human leukocyte antigen as first genetic factor while later several other causes have been identified such as chronic oxidative injury, mitochondrial damage, and limited axonal transport with affected protein homeostasis (Campbell and Mahad, 2018). However, numerous studies have reported impaired activity and composition of UPS, but exact mechanism of UPS associated MS is remained elusive. For instance, abnormally ubiquitinated axons are reported in normally myelinated white matter in MS brain (Giordana *et al.,* 2002). Further, UPS has also reported to trigger the inflammatory process in MS, which abrogated in response to interferon-beta-1b treatment (Minagar *et al.,* 2012). Another study evidenced the ubiquitination mediated pathological immunity triggered by Th17 cells via targeting its activator RORγt (retinoic acid-related orphan receptor-γt) and coactivator SRC1 (steroid receptor coactivator 1) for proteasomal degradation (He *et al.,* 2016). Another UPS component, Ubiquitin specific protease-18 (USP18), a deubiquitinating enzyme is evident to be deficient under diseased conditions and unresponsive to therapeutic IFNβ treatment (Malhotra *et al.,* 2013). Similarly, another deubiquitinase enzyme A20 has been implicated in the regulation of inflammation and cell death in multiple sclerosis (Wertz *et al.,* 2015). Interestingly, a recent study has also shown ubiquitination independent proteasomal degradation in multiple sclerosis. For instance, Belogurov *et al.,* identified the degradation of an auto antigen of MS, i.e. myelin basic protein (MBP) to be 26S proteasomal degraded independent of the ubiquitination process (Belogurov *et al.,* 2014). Supporting this notion, a pleiotropic cytokine osteopontin found to be process by extracellular proteasomes to form chemotactic fragments thereby triggering inflammatory responses in MS (Dianzani *et al.,* 2017). Further, impaired elimination of harmful autoimmune cells reported in MS involving the affected UPS components. For instance, pro and anti-apoptotic protein BAK and MCL1 depressed in response to altered expressions of its associated E3 ligases HUWE1 and β-TrCP in MS patients (Mandel *et al.,* 2012). Likewise, other E3 ligases CBL-b and ITCH are associated with T-cell activation considerably diminished in MS brains (Sellebjerg *et al.,* 2012). Additionally, UPS has a more complicated role to play in MS pathogenesis since, it is governed by both inflammatory and neurodegenerative pathways. Therefore, further identification of

UPS components is required to reveal their molecular pathogens to devise novel therapies. Moreover, UPS mediated neurodegeneration mechanism in neurodegenerative disorders, including AD, PD, HD, ALS, and MS has elucidated in **Figure 1.4** and the UPS component associated mechanistic roles in the neurodegenerative disease specific clearance of toxic metabolites has formulated in **Table 1.5.**

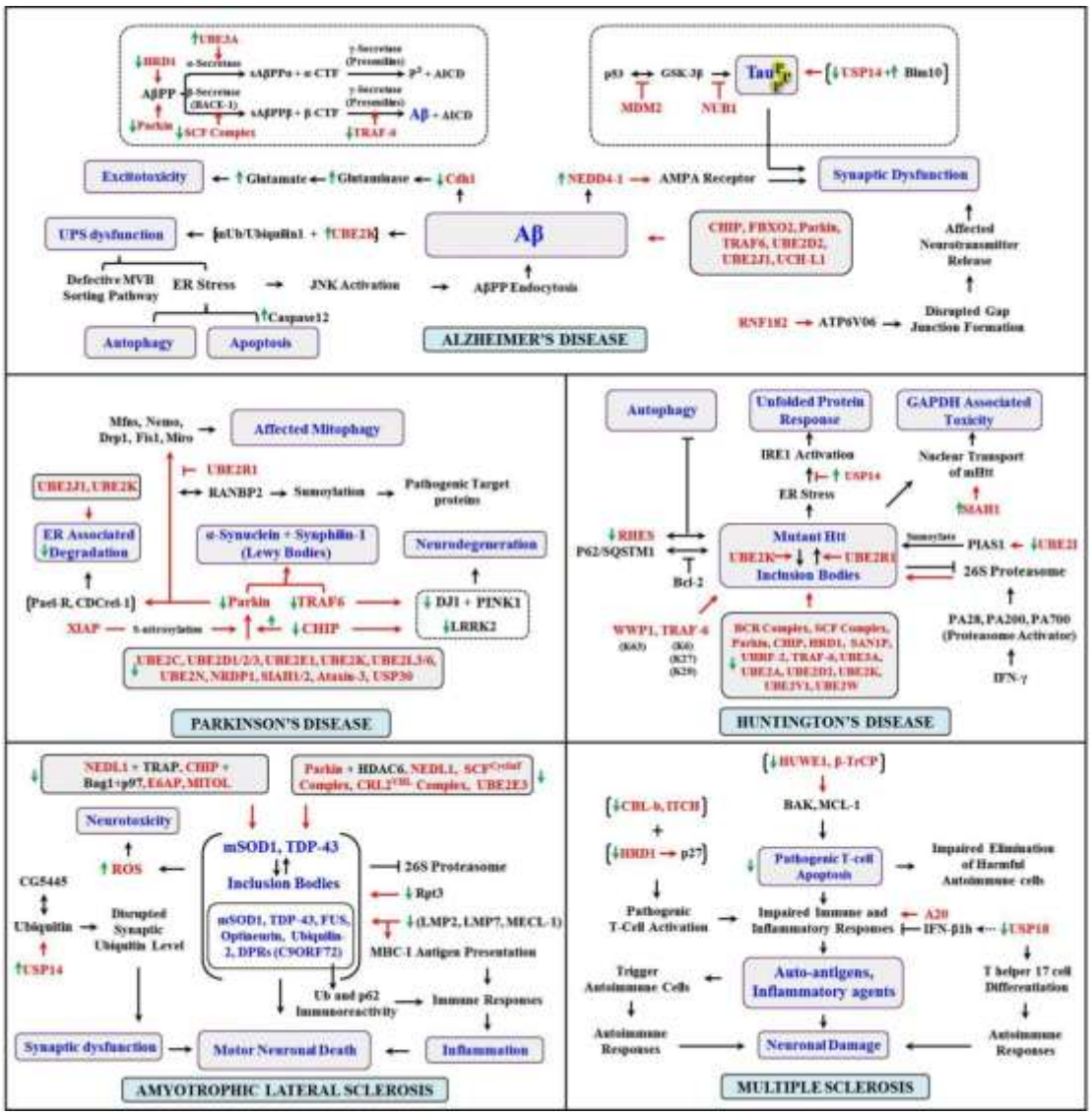


Figure 1.4: Ubiquitination mediated neurodegeneration mechanism in neurodegenerative disorders: (A) Alzheimer's Disease- The AβPP is proteolytically processed to generate non-amyloid (p3) and amyloid (Aβ) peptide with help of α- and β-secretase respectively, followed by γ-secretase. Under pathogenic state, E3 ligases HRD1 and Parkin failed to regulate the level of AβPP and give rise to its amyloidogenic products. Similarly, down-regulated SCF complex, TRAF-6 and up regulated UBE3A triggered the selection of amyloidogenic pathways for Aβ synthesis. Moreover, disrupted Cdh1 activity, lead to glutaminase overload associated excited-toxicity in neurons. Furthermore, Neurotransmitter release and synaptic

functions are compromised due to impaired AMPA signaling, and gap junction formation due to NEDD4-1 and RNF182 mediated proteasomal degradation of AMPA receptors and ATP6V06 (Endosomal proton pump member). Likewise, multi vesicular body-sorting pathway for lysosomal degradation is affected due to UBE2K mediated mutant ubiquitin or ubiquitin like ubiquilin-1 conjugation. In addition, ER-stress trigger JNK activation and induces endocytosis of AβPP to produce Aβ peptides. Consequently, It governs autophagy and caspase-12 mediated apoptosis as a facultative response against Aβ toxicity. Several other UPS components like CHIP, FBXO2, Parkin, TRAF6, UBE2D2, UBE2J1 and UCH-L1 have reported to clear Aβ that get affected in the diseased state. Like Aβ, Tau protein is marked by down regulated USP14 and elevated proteasome activator Blm10 for proteasomal degradation. Moreover, NUB1 prevents hyper-phosphorylation of Tau by Glycogen synthase kinase-3beta (GSK-3β) to alleviate its toxicity, while MDM2 mediates inhibition of interaction between GSK-3β and p53 that enables p53 to trigger transcriptional activation of protective genes to clear off hyper phosphorylated tau. These UPS components get disrupted in diseased state and caused hyper-phosphorylated tau mediated neurotoxicity due to the neuro-fibrillary tangles. **(B) Parkinson's Disease-** The lewy body (α-synuclein and Synphilin-1) pathology is mainly governed by defective E3 ligase Parkin. It regulates mitochondrial membrane proteins (Mfns, Nemo, Drp1, Fis1, and Miro) and is affected in the diseased state that triggers impaired mitophagy. Likewise, defective parkin disrupted endoplasmic reticulum associated degradation (ERAD) by affected Pael-R and CDCrel-1 ubiquitination. It also triggers SUMOylation via interacting with RANBP2 for the clearance of pathogenic target proteins. Similarly, CHIP and TRAF6 are associated with the ubiquitination of LRRK2 and PINK1/DJ1 respectively that get affected in the disease state and cause neurodegeneration. **(C) Huntington's Disease-** The mutant huntingtin forms inclusion bodies- both soluble and insoluble forms. Here, Proteasome activators PA28, PA200 and PA700 are helpful in reversing the inhibition of proteasome by mHtt and inclusion bodies. However, it governs ER stress mediated unfolded protein response and induces GAPDH associated toxicity through its nuclear transport by SIAH1. Many UPS enzymes act differently i.e. positively and negatively to regulate the clearance of mHtt, including WWP1 and TRAF6 that direct K63 and K6, K27, K29 poly-ubiquitination respectively, and retard proteasomal degradation under diseased state. **(D) Amyotrophic Lateral Sclerosis-** Inclusion bodies in ALS is populated with mutant SOD1, TDP-43, FUS, Optineurin, Ubiquilin2 and dipeptide repeat proteins that affect the different cellular process in neurons. Impaired proteasome and MHC-I mediated immunity and inflammation triggers the neuronal death in the diseased state. Moreover, CG5445 and USP14 scavenge the ubiquitin protein and thereby interfere with the synaptic functions in ALS. Furthermore, mitochondrial dysfunction mediated ROS production also triggers inflammation and associated neurotoxicity in ALS. (E) Multiple Sclerosis- Auto-antigens and inflammatory agents are the principal cause for the progression of MS. Where depleted expression of certain E3s, including HUWE1, β-TrCP, CBL-b, ITCH and HRD1 lead to compromised pathogenic T-cell apoptosis and its activation to aggravate the symptoms of MS. However, IFN-β1b treatment enabled the suppression of immune and inflammatory responses by targeting auto-antigens and inflammatory agents who prevented neuronal damage in Multiple sclerosis. **Dashed arrow**- Regulation; **Red arrow**- Ubiquitination and degradation; **Black arrow**- Activation; **Up arrow (Green)** - Elevated expression; **Down arrow (Green)** - Reduced expression

Table 1.5: Neurodegenerative disease specific role of ubiquitinating enzymes in clearance of toxic protein aggregates

NDDs	UPS Components		Molecular Functions	Mechanism of Action	References
Alzheimer's Disease (Aβ, Tau)	Involved E3s	APC/Cdh1	Aβ linked deficiency of Cdh1 lead to increase in glutamate level and consequent excitotoxicity in neurons	Alzheimer's disease characterized by two main protein aggregates including extracellular senile plaques (Aβ component) and intracellular neurofibrillary tangles (pTau component). Ubiquitin E3 ligases such as SCF complex, TRAF6, and HRD1 regulate proteasomal degradation of β-secretase, Presenilin (γ-secretase) and AβPP respectively thereby controlling the level of Aβ in brain. Other E3s Parkin and CHIP directly associated with the clearance of Aβ in brain. While UBE3A downregulated and prevent α-secretase degradation thereby inhibiting Aβ formation. Moreover, Aβ also inhibit few E3s including Cdh1 and NEDD4-1 that affect membrane receptors, synaptic density and neurotransmission. Multiple E2s including UBE2A, UBE2D1, UBE2K, and deubiquitinases UBE2J1 and UCH-L1 are associated with the conjugation and recycling of ubiquitin to regulate protein turnover. Alterations in these E2s, E3s and DUBs under diseased state affect gap junctions, synaptic protein densities, membrane receptors, thus synaptic transmission, and neurological behaviors in AD patients.	Vina *et al.*, 2014
		CHIP	Ubiquitinates phosphorylated Tau and increased level promote Aβ degradation		Petrucelli *et al.*, 2004; Kumar *et al.*, 2007
		HRD1	Ubiquitinate and clear misfolded or unfolded proteins like misfolded MHC-I heavy chains, Pael-R and AβPP through ERAD and reduces Aβ formation		Saito *et al.*, 2014
		NEDD4-1	Triggers Aβ mediated AMPARs ubiquitination and degradation thereby affecting synaptic function, strength and dendritic spine density		Rodrigues *et al.*, 2016
		Parkin	Reduces Aβ levels and improve long term potentiation and behavioral abnormalities		Rosen *et al.*, 2010
		RNF182	Ubiquitinate ATP6V06 protein, disrupt the formation of gap junctions and neurotransmitter release in diseased state of AD brain		Liu *et al.*, 2008
		SCF Complex (SKP1, CUL1, Fbx2)	Attenuate amyloidosis by degrading BACE1 enzyme to improve synaptic function		Gong *et al.*, 2010
		TRAF6	Abrogate Aβ-mediated inhibition of p75 neurotrophin receptor degradation and restore neuronal cell survival, also degrade mutated Presenilin		Geetha *et al.*, 2012
		UBE3A	It's deficiency prevents α-secretase (ADAM10) degradation thereby triggering non-amyloidogenic pathway to reduce Aβ level		Singh *et al.*, 2017b
	Regulatory E2s	UBE2A	Coordinates for the clearance of amyloid peptides through proteolysis and get affected in diseased state		Zhao *et al.*, 2016
		UBE2D1	Genetically associated with the late onset Alzheimer's susceptible gene APOE		Morgan *et al.*, 2007
		UBE2K (E2-25K/Hip2)	Regulate Aβ neurotoxicity by acting as upstream regulator of apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK). Colocalize with mutant ubiquitin (UBB+1) and inhibit proteasome activity to cause Presenilin accumulation		Hong *et al.*, 2014
		UBE2J1,	Increase cellular levels of mono-ubiquitin and therefore is involved in the increase of protein turnover by the UPS that improves contextual memory, restores synaptic function, while its low level is linked with Aβ accumulation in AD		Burr *et al.*, 2011
	Recycler DUBs	UCH-L1			

NDDs	UPS Components		Molecular Functions	Mechanism of Action	References
Parkinson's Disease (Lewy bodies; α-synuclein, Synphilin1)	Involved E3s	CHIP	Enhance parkin's activity and ubiquitinates α-synuclein and LRRK2 to promote their degradation	Parkinson's disease characterized by lewy body i.e. abnormal α-synuclein depositions in the substantia nigral neurons affecting motor functions. Multiple E3s are directly or indirectly ubiquitinate and clear α-synuclein from the cell, which includes Parkin, TRAF6, SIAH1, UBE3A, CHIP and DJ1. Parkin is the key E3 ligase playing a central role in the clearance of alpha synuclein with help of other E3 ligases- NRDP1, XIAP, CHIP and DJ1. NRDP1 and XIAP1 stabilize Parkin, while CHIP reported to increase the activity of Parkin. Parkin also regulates the ubiquitination of other E3 ligase RANBP2 and its SUMOylation target mitochondrial membrane proteins to regulate mitophagy. While gigaxonin ubiquitinates MAP1B and regulate stability of intermediatory filaments. These E3 ligases along with a diverse set of conjugating enzymes including UBE2A, UBE2C, UBE2D, UBE2E, UBE2J, UBE2K, UBE2L, UBE2N, UBE2R and various deubiquitinases like Ataxin3, UCHL1, USP8, USP9X, USP30 regulate membrane trafficking, synaptic regulation and microtubular organization.	Ko *et al.*, 2009; Kalia *et al.*, 2011
		Gigaxonin	Regulate degradation of microtubule associated protein 1B and thus intermediatory filaments to maintain neuronal health		Allen *et al.*, 2005; Johnson-Kerner *et al.*, 2015
		NRDP1	Regulate Parkin's stability and activity via regulating its proteasomal degradation		Zhong *et al.*, 2005
		Parkin	Ubiquitinate and clear α-synuclein aggregates and various mitochondrial membrane proteins and thus regulate mitophagy		Miklya *et al.*, 2014
		RANBP2	SUMOylate its pathogenic target and trigger consequent degradation upon interaction with Parkin.		Dorval and Fraser 2007
		SIAH1	Ubiquitinate and degrade alpha-synuclein and synphilin-1 proteins		Franck *et al.*, 2006
		TRAF6	Promotes ubiquitination of α-synuclein, DJ1 and PINK1		Zucchelli *et al.*, 2010; Murata *et al.*, 2013
		UBE3A	Colocalize with alpha synuclein to clear it in a proteasome-dependent manner		Mulherkar *et al.*, 2009
		XIAP	Trigger S-nitrosylation of Parkin and thereby affect neuronal survival in PD		Tsang *et al.*, 2009
	Regulatory E2s	UBE2A	Associate with parkin and regulate the clearance of dysfunctional mitochondria in mice and human cells		Martin *et al.*, 2011
		UBE2C, UBE2D family members, UBE2E1, UBE2K, UBE2L3, UBE2L6, UBE2N	Contribute towards mitophagy by redundantly charging RING-HECT hybrid ligase Parkin with ubiquitin		Geisler *et al.*, 2014; Fiesel *et al.*, 2014
		UBE2K, UBE2J1	Role in ubiquitination and selective degradation of misfolded membrane proteins from the endoplasmic reticulum (ERAD)		Araki and Nagata 2011
		UBE2R1	Act as a negative regulator of Parkin via reducing parkin translocation to mitochondria		Fiesel *et al.*, 2014
	Recycler DUBs	Ataxin-3	Coupled with parkin and govern ubiquitination and decomposition of polyubiquitin chain		Durcan *et al.*, 2012
		UCH-L1, USP9X	Mutated form lead to impairment in abnormal protein clearance		Cartier *et al.*, 2012; Rott *et al.*, 2011
		USP30	Associated with PINK1 and Parkin for the clearance of damaged mitochondria in PD		Gersch *et al.*, 2017
		USP8	Associated with the clearance of α-synuclein and modify its toxicity in PD		Alexopoulou *et al.*, 2016

NDDs	UPS Components		Molecular Functions	Mechanism of Action	References
Huntington's Disease (PolyQ, mHtt)	Involved E3s	BCR Complex (BTB-CUL3 -RBX1)	Associated with the proteasomal degradation of mHtt in association with NUB1-NEDD8-CUL3 pathway	Huntington's disease is an autosomal dominant inherited disorder characterized by the presence of mutant huntingtin (mHtt) forming a larger form of huntingtin, which is toxic to the nerve cells. Diverse E3s including BCR complex, SCF complex, TRAF6 and CHIP regulate mHtt levels via ubiquitination and proteasomal degradation. Other E3s such as SIAH1 regulate nuclear transport associated toxicity of mHtt, while UBE3A regulate dopamine regulated phosphoprotein (DARPP-32) and directs neurotransmission. Similarly, RHES binds Beclin1 to regulate autophagy process to overcome the cytotoxicity caused by protein aggregates. Collectively these E3s with the assistance of multiple E2s and DUBs - UBE2A, UBE2D2, UBE2K, UBE2V1, UBE2W, UBE2I, and USP14 effectively remove the cellular aggregates.	Lu *et al.*, 2013
		CHIP	Involved in the suppression of aggregation and toxicity of PolyQ huntingtin		Miller and Gordon 2005
		RHES	Binds autophagy regulator Beclin-1 to activate autophagy, while its co-expression with mHtt blocks Rhes-induced autophagy activation		Mealer *et al.*, 2014
		SCF Complex (Skp1-Cul1-F-box)	Deficient Cul1 and Skp1 are associated with increased mHtt levels in brain		Bhutani *et al.*, 2012
		SIAH1	Reported to enhance the nuclear translocation of mHtt and cytotoxicity in association with GAPDH		Bae *et al.*, 2006
		TRAF6	Associated with the ubiquitination and degradation of mHtt and PolyQ proteins		Zucchelli *et al.*, 2011
		UBE3A	Its deficiency trigger protein overload and also decrease dopamine regulated phosphoprotein DARPP-32 crucial role in HD progression		Maheshwari *et al.*, 2014
	Regulatory E2s	UBE2A, UBE2D2, UBE2V1	Ubiquitinates polyglutamine aggregates and controls the size and number of protein aggregates		Howard *et al.*, 2007
		UBE2I	Conjugate SUMO protein to E3 SUMO ligase PIAS1 that regulate the SUMOylation of huntingtin protein		Ochaba *et al.*, 2016
		UBE2K (E2-25/Hip2)	Interacts directly with huntingtin and may mediate ubiquitination of neuronal intranuclear inclusions in Huntington disease		de Pril *et al.*, 2007
		UBE2W	Markedly decreases mHTT aggregate formation and increases the level of soluble monomers		Wang *et al.*, 2018
	Recycler DUBs	USP14	Effectively reduce cellular aggregates and counteract cellular degeneration by counteracting ER stress-mediated IRE1 activation		Hyrskyluoto *et al.*, 2014

NDDs	UPS Components		Molecular Functions	Mechanism of Action	References
Amyotrophic Lateral Sclerosis (TDP43, mSOD1, TRAP, Dvl1 and other insoluble protein aggregates)	Involved E3s	$CRL2^{VHL}$ Complex (Cul2-Rbx1-EloBC-VHL)	Associated with the ubiquitination and proteasomal degradation of misfolded TDP-43	ALS is a neuromuscular disorder with motor dysfunctions typically characterized by protein aggregates like TDP-43, mSOD1, TRAP, Dvl1 and insoluble protein aggregates. Multiple E3 ligases such as $CRL2^{VHL}$ complex, NEDL1 and Gigaxonin trigger ubiquitination and proteasomal degradation of TDP-43, mSOD1 and MAP1B proteins to ameliorate the symptoms of ALS. These E3s along with E2s UBE2E3/UBCM2 and deubiquitinase USP14 regulate ERAD, intermidiatory filaments, neuromuscular junctions and synaptic functions.	Uchida *et al.*, 2016
		Gigaxonin	Regulate intermediatory filament by ubiquitination and degradation of microtubule associated protein 1B (MAP1B) thereby contributing to neuronal health		Allen *et al.*, 2005; Johnson-Kerner *et al.*, 2015
		NEDL1	Associated with the ubiquitination and degradation of mutant SOD1, translocon-associated protein-delta (TRAP), and Dishevelled-1 (Dvl1)		Miyazaki *et al.*, 2004
	Regulatory E2s	UBE2E3/ UBCM2	Enhance the ubiquitination of TDP-43 and controls the solubility and neurodegeneration		Hans *et al.*, 2014
	Recycler DUBs	USP14	Maintain synaptic ubiquitin level for proper synaptic functions and for the development of neuromuscular junctions		Chen *et al.*, 2009
Multiple Sclerosis (Soluble oligomers, auto-antigens and auto-inflammatory agents)	Involved E3s	HRD1	Facilitates T-cell proliferation by the destruction of cyclin-dependent kinase inhibitor $p27^{kip1}$ and thus aids in T-cell mediated immunity	Multiple sclerosis is a neuroinflammatory disorder characterized by soluble oligomers aggravating the inflammation. Multiple E3s regulate cellular functions such as HRD1 ubiquitinate p27 (a CDK inhibitor) and elevate T-cell expression. HUWE1 and FBXW1A regulate the expression pattern of anti- and pro-apoptotic proteins MCL1 and BAK respectively to govern T-cell survival. ITCH and CBLB also regulate T cell antigen surface expression. Collectively these UPS enzymes regulate T-cell expression, inflammation, and autoimmune responses mediated mylein damage.	Xu *et al.*, 2016
		HUWE1	Regulate the levels of anti-apoptotic protein MCL-1 and pro-apoptotic BAK, which get disrupted and thus enhance the survival of pathogenic T-cells		Mandel *et al.*, 2012
		FBXW1A (β-TrCP)			
		ITCH	Maintain the cell surface expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) on $CD4^{+}CD25^{high}$ T cells thereby regulating T cell function in MS		Sellebjerg *et al.*, 2012
		CBLB			
	Recycler DUBs	USP18	Acts as a negative regulator of type-I interferon signaling and thus a therapeutic response to IFN-β in MS pathology. Also necessary for T helper-17 cell differentiation and autoimmune responses		Malhotra *et al.*, 2013; Honke *et al.*, 2016

1.7 THERAPEUTIC APPROACHES FOR UPS MEDIATED TARGETING OF NEURODEGENERATIVE DISORDERS

The great potential of UPS for intervention in numerous pathologies like Cancer, viral infections, immunological and neurodegenerative disorders have served it as a potent pharmaceutical target for drug development. Moreover, their inhibition is an effective tool widely studied in cancer to induce cancerous cell death in comparison with the normal ones. The best example is Bortezomib; a proteasomal inhibitor approved by FDA as a therapeutic agent in multiple myelomas (Buac *et al.,* 2013). However, the scenario is different in case of neurodegenerative disorders, where the stimulation of UPS components along with the inhibition of specific UPS components lethal for cell is the most promising approach to clear the toxic metabolites in neurons. For instance, alteration in the activity of many E3s- Parkin, CHIP, TRAF6, HRD1 along with multiple E2s and DUBs- UBE2D, UBE2J, UBE2K, USP14, USP18 and UCH-L1 have been reported in distinct NDDs to trigger various neurologic pathologies, including synaptic dysfunction, motor impairment, mitochondrial and ER stress induced neurotoxicity. In addition, many active forms of ubiquitination enzymes- E2s, E3s, and DUBs have aggravated the neurological pathologies. Namely, RNF182 and NEDD4-1 are reported to trigger proteasomal degradation of ATP6V06 (gap junction protein) and AMPA receptors to impair gap junction formation and AMPA reception thereby affecting synaptic functions (Rodrigues *et al.,* 2016; Liu *et al.,* 2008). Likewise, UBE3A identified to degrade α-secretase and thus induce amyloidogenic pathway to form toxic Aβ in Alzheimer's disease (Singh *et al.,* 2017b). Similarly, SIAH1 found to enhance nuclear transport of mHtt thereby inducing GAPDH associated toxicity in Huntington's disease (Bae *et al.,* 2006). Additionally, UBE2R1, an E2 enzyme reduced Parkin's translocation to mitochondria that interfered in the process of mitochondrial biogenesis leading to mitochondrial dysfunctions in Parkinson's disease (Fiesel *et al.,* 2014). In the same manner, there are numerous instances where inhibition of UPS components is necessary for the reversal of neurodegenerative phenotypes. Therefore, certain novel therapeutic strategies can effectively alleviate the nuisance caused by the impaired ubiquitin proteasome system as explained in **Figure 1.5.**

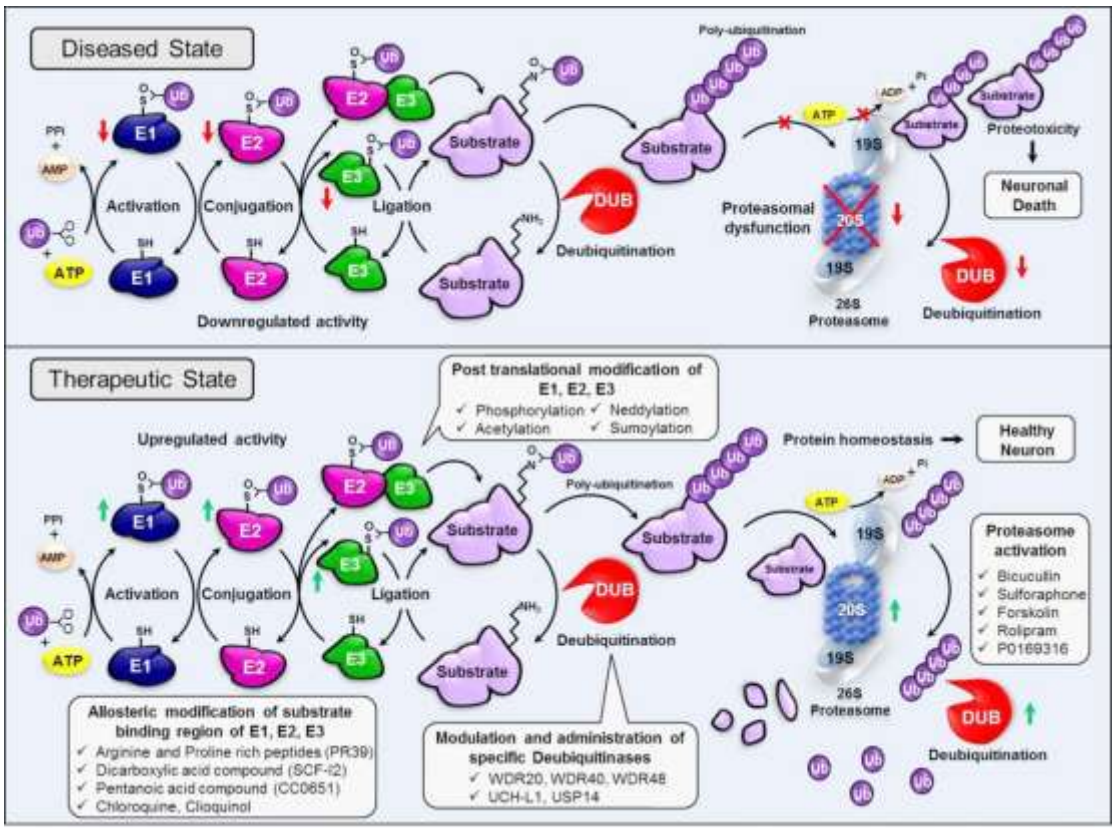


Figure 1.5: Therapeutic approaches to alleviate neurodegenerative pathologies: Under diseased conditions the activity of ubiquitinating enzymes (E1s, E2s, and E3s), deubiquitinase enzymes (DUBs), proteasome assembly and functions are compromised leading to proteotoxic stress in the neurons causing its death. The disease pathologies might be reversed upon adapting the following remedial strategies- i) Allosteric modification of the substrate binding region, which could increase or decrease the activity of E1, E2 and E3 enzymes in order to regulate the clearance of toxic, misfolded or unwanted proteins. ii) Post translational modification of E1, E2, and E3 enzymes lead to the protection of these enzymes from their self-ubiquitination and degradation thereby enabling them to be active during diseased conditions for elevated clearance of harmful proteins. iii) Modulation and administration of specific deubiquitinases could aid in overcoming the burden of native or mutant ubiquitin charged enzymes and substrates, facilitating the UPS machinery for the efficient clearance; iv) Proteasomal activation aids in the mitigation of proteotoxic burden of neurons enabling them to maintain their homeostatic state. **E1**-Ubiquitin E1 activating enzyme; **E2**-Ubiquitin E2 conjugating enzyme; **E3**-Ubiquitin E3 ligase; **DUB**-Deubiquitinase enzyme; **Ub**-Ubiquitin protein; **ATP**-Adenosine triphosphate; **AMP**-Adenosine monophosphate; **PPi**-Pyrophosphate.

1.7.1 Therapeutic strategies to target UPS mediated pathologies

One of the strategies could be *(i) the allosteric modification of the substrate binding region of ubiquitination enzymes* with the help of small molecules, which would enable us to increase or decrease the E1's, E2's, E3's specificity towards substrate and thus controlling the accumulation of ubiquitinated substrates (Paiva *et al.,* 2018). Such allosteric drugs include arginine and proline rich peptides, chloroquine and its derivatives, clioquinol, dicarboxylic acid compound (SCF-I2), and pentanoic acid compound (CC0651) that showed promising therapy against various diseases and can be studied for their efficacies in NDDs (Gaczynska *et al.,* 2003; Wang *et al.,* 2009b; Orlicky *et al.,* 2010; Ceccarelli *et al.,* 2011). Similarly, another way could be *(ii) the modulation of specific deubiquitinases* by small molecules that can enhance the deubiquitination of mutant polyUb chains such as UBB^{+1} or normal polyUb to prevent the inhibition of proteasome by the burden of such mutant/normal polyUb chains (Park *et al.,* 2009). Apart from allosteric drugs, other ways could be *(iii) the post-translational modifications of the UPS components,* including phosphorylation, acetylation, SUMOylation, and neddylation to modulate their activities. For instance, phosphorylation of Parkin (an E3 ligase) modulated its ligase activity contributing to the unfolded protein response in SHSY5Y cells (Yamamoto *et al.,* 2005). Likewise, Neddylation, i.e. Nedd8 (an Ubl) mediated modification of Cullin-1 enhanced the activity of an ubiquitin E3 ligase SCF^{skp2} towards the proteasomal degradation of p27 (Morimoto *et al.,* 2000). Likewise, acetylation, SUMOylation and phosphorylation of MDM-2 have imparted it stability and aided in the enhancement of p53 ubiquitination (Miyauchi *et al.,* 2002; Nihira *et al.,* 2017).

Moreover, *(iv) the selective target based specific ubiquitin-like (UBL) modifications* can aid in the therapeutics of neurodegenerative disorders; since, Ubl modifications such as ISGlation, FATtylation of pathogenic proteins has shown interesting results in toxic protein clearance. For instance, FAT10 (an Ubl) is identified to bind and modulate the solubility of poly glutamine proteins revealing it as a therapeutic agent for Huntington's disease (Nagashima *et al.,* 2011). Other Ubls like ATG8 and ISG15 have reported to facilitate the autophagic clearance of toxic proteins in association with p62/SQSTM1 binding and IFN-γ induction respectively (Pankiv *et al.,* 2007;

Nakashima *et al.,* 2015). While some Ubls like SUMOylation reported to enhance the neurodegeneration processes within a cell (Mishra *et al.,* 2013). Further approaches could be *(v) the administration of deubiquitinases and DUB regulators* for their intrinsic role in regulating synapse development and functions. For instance, a DUB called UCHL1 is well identified for their roles in synaptic plasticity and thus preventing the neurodegeneration (Kowalski and Juo, 2012). Another DUB, USP14 has implicated in the regulation of hippocampal short-term synaptic plasticity (Walters *et al.,* 2014). Recently, a study demonstrated the importance of WD repeat proteins WDR20, WDR40, and WDR48 in regulating the expression and catalytic activity of DUBs for controlling the synaptic plasticity and thus neuronal health (Hodul *et al.,* 2017). Another potential approach could be *(vi) the activation of proteasome with suitable drugs,* which may be achieved through any of the following ways. Upregulation of 19S and 20S complex assembly; eliciting the catalytic activity of 20S core proteasomal unit; triggering unfolding of aggregated proteins via modulating the chaperonic activity of either chaperonins or ATPase subunit of 19S complex; and by stimulating the recognition of ubiquitinated protein aggregates (Upadhya and Hegde, 2005). In this favor, a study has demonstrated to curb the level of Aβ upon resveratrol treatment by stimulating its proteasome-mediated degradation (Marambaud *et al.,* 2005). Another study reported a small-molecule bicuculline stimulating calcium/calmodulin-dependent protein kinase II (CaMKII) mediated proteasomal activity to modulate synaptic connections in neurons (Djakovic *et al.,* 2009). Likewise, another small-molecule IU (1-[1-(4-fluorophenyl)-2, 5-dimethylpyrrol-3-yl]-2-pyrrolidin-1-ylethanone) boosted proteasome activity by ameliorating USP14 mediated suppression of ubiquitinated conjugate's degradation (Lee *et al.,* 2010). In addition, a natural occurring compound Sulforaphane is reported to induce proteasomal activity via triggering a transcriptional factor Nrf2 to govern mHtt degradation (Liu *et al.,* 2014). In similar manner, Forskolin, Rolipram and other cyclic AMP (cAMP) inducing compounds has shown enhanced proteasomal degradation of protein aggregates in cAMP/PKA pathway dependent manner (Lokireddy *et al.,* 2015; Myeku *et al.,* 2016). Recently, another small-molecule PD169316 has been found to trigger proteasomal activation and thus PROTAC and,

ubiquitin dependent α-synuclein degradation via hampering p38 MAPK pathway (Leestemaker *et al.*, 2017). Further, the potential drugs and leading compounds that have shown promising results in targeting the progression of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease Huntington's disease, Amyotrophic lateral sclerosis, and Multiple sclerosis are summarized in **Table 1.6.** In spite of the advancements in drug discovery, designing of substrate specific drug against such diverse ubiquitination enzymes is a challenging task, since the E2s, E3-RING and HECT domains possess structural similarities and thereby impart non-specificity towards drug's action (Weissman, 2013). In order to solve this problem a recent technology of Proteolysis-Targeting Chimera (PROTAC^{TM}) has evolved, which has taken up by big pharmaceutical companies to target different cancers and neurodegenerative disorders.

Table 1.6: Potential compounds modulating the activity of ubiquitinating enzymes for their therapeutic implications in neurodegenerative disorders

S.No.	Compounds (Pubchem CID)	Impact on UPS	Target UPS Component	Molecular Action	Target Pathology	References
1	Curcumin (969516)	–	APC, HSP70, HSP90	Inhibits APC leading to cell cycle arrest and apoptosis in mylenomas and increase Hsp70 and Hsp90 activity thereby inhibiting or delaying amyloid formation and reduce neuronal death	Alzheimer's disease, Tauopathies, and Huntington's diseases	Lee and Langhans 2012;, Maiti *et al.*, 2014
2	IU1 (675434)	Proteasome activator	USP14	Boosted proteasome activity by ameliorating USP14 mediated suppression of ubiquitinated conjugate's degradation including tau	Alzheimer's Disease	Lee *et al.*, 2010, Kiprowska *et al.*, 2017
3	Lactacystin (9794358)	Proteasome inhibitor	β1, β2, β5 Proteasomal subunit	Affect β-APP maturation in human cells and induce HSPs to play a protective role	Alzheimer's Disease	Yew *et al.*, 2005; Checler *et al.*, 2000
4	Resveratrol (445154)	E3 and DUB stimulator	MID1, HRD1, USP10	Induces PP2A activity by targeting MID1 that reduces tau phosphorylation and stimulate proteasome mediated degradation of Aβ and also enhance USP10-mediated deubiquitination of p53 to induce apoptosis	Alzheimer's Disease	Yan *et al.*, 2016; Schweiger *et al.*, 2017
5	Rolipram (5092)	Proteasome stimulator	26S Proteasome	Enhance proteasomal degradation of protein aggregates in cAMP/PKA pathway dependent manner	Alzheimer's Disease	Barad *et al.*, 1998; Lokireddy *et al.*, 2015
6	Thapsigargin (446378)	E3 stimulator	HRD1	Increases expression of HRD1 and XBP-1s that can trigger a compensatory mechanism of lowering BACE-1-mediated Aβ production as a potential therapy	Alzheimer's Disease	Gerakis *et al.*, 2016
7	Clioquinol (2788)	Proteasome inhibitor	20S Proteasome	Trigger 20S proteasome inhibition by clioquinol-copper complex thereby affecting its chymotrypsin-like activity and is also a metal protein-attenuating compound that disrupt the interaction between metals and the Aβ peptide in the brain and also down regulates mutant Htt level in HD models	Alzheimer's Disease, Huntington's Disease, Cancer	Nguyen *et al.*, 2005; Jenagaratnam *et al.*, 2006; Chen *et al.*, 2007

S.No.	Compounds (Pubchem CID)	Impact on UPS	Target UPS Component	Molecular Action	Target Pathology	References
8	Bicuculline (10237)	Proteasome activator and UPS activator	Rpt6 (19S Proteasome regulatory subunit) and other UPS component	Stimulate calcium/calmodulin-dependent protein kinase II (CaMKII) mediated proteasomal activity stimulate AMPARs ubiquitination to modulate synaptic connections in neurons	Alzheimer's Disease, Parkinson's Disease	Djakovic *et al.*, 2009; Widagdo *et al.*, 2017
9	PDTC (Pyrrolidine dithiocarbamate) (65351)	Proteasome inhibitor	Immunoproteasome	Immunoproteasome inhibitor that play protective role by acting as NF-κB inhibitor, antioxidant, and Akt, Nrf2 pathway inducer that improve spacial learning in AD and improved motor functions in PD	Alzheimer's Disease, Parkinson's Disease	Malm *et al.*, 2007; Abd-El-Fattah *et al.*, 2014
10	Sulforaphane (5350)	Proteasome stimulator	26S Proteasome	Induce proteasomal activity via triggering a transcriptional factor Nrf2 to govern mHtt degradation and to inhibit rotenone-induced locomotor activity deficiency and dopaminergic neuronal loss. It also up-regulate p75 neurotrophin receptors to impart protection against the Aβ burden	Alzheimer's Disease, Parkinson's Disease, Huntington's Disease	Liu *et al.*, 2014; Zhou *et al.*, 2016, Zhang *et al.*, 2017
11	Pimozide (16362)	DUB inhibitor	USP1	Role in treatment of psychotic disorders and have shown potential in stabilizing neuromuscular transmission and prolonging survival in the disease	Amyotrophic Lateral Sclerosis, Schizophrenia	Sultana and McMonagle 2000; Patten *et al.*, 2017
12	Betulinic acid (64971)	Proteasome activator	20S β5 proteasome subunit	Activates the chymotrypsin-like activity of the proteasome and associated with the enhanced GABAergic activity, Prevent neurobehavioural activity in Alzheimer's disease	Autism, Alzheimer's Disease	Huang *et al.*, 2007, Navabi *et al.*, 2018
13	6OHDA (4624)	E3 inducer	ZNRF1	Induces ZNFR1 E3 ligase activity by EGFR-mediated phosphorylation	Parkinson's Disease	Wakatsuki *et al.*, 2015
14	Cyclosporin A (5284373)	Proteasome inhibitor	20S β5 proteasome subunit	Role in proteasomal inhibition and prevention of NF-kB activation and also prevents α-synuclein mediated mitochondrial dysfunctions	Parkinson's Disease	Meyer *et al.*, 1997; Bir *et al.*, 2014
15	Delta-9-Tetrahydrocannabinol (Δ9-THC) (5321846)	DUB Stimulator	USP3	Its antioxidant effects and increase in the level of USP3 exerts a direct neuroprotective effect in cellular models of Parkinson's disease	Parkinson's Disease	Carroll *et al.*, 2012

S.No.	Compounds (Pubchem CID)	Impact on UPS	Target UPS Component	Molecular Action	Target Pathology	References
16	NC-043 (CAS#1053172-87-4)	DUB inhibitor	USP30	Antagonizes Parkin-dependent mitophagy in cells	Parkinson's Disease	Sorrentino *et al.*, 2018
17	Nicotine (89594)	E2, Proteasome, DUB stimulator	UBE2J2, Proteasome subunits, USP16, USP34	Stimulates nicotinic acetylcholine receptors with alterations in the level of several ubiquitin conjugating enzyme, proteasomal activity and deubiquitinases USP16, USP34 that have shown reduced disease progression, improved symptoms and/or decreased L-dopa-induced dyskinesias	Parkinson's Disease	Quik *et al.*, 2018
18	PD169316 (4712)	Proteasome activator	26S Proteasome	Trigger proteasome for PROTAC and ubiquitin dependent α-synuclein degradation by p38 MAPK inhibition	Parkinson's Disease	Leestemaker *et al.*, 2017
19	Forskolin (47936)	Proteasome inhibitor	26S proteasome	Enhance proteasomal degradation of protein aggregates (mutant FUS, SOD1, TDP43, Tau) in cAMP/PKA pathway dependent manner	Parkinson's Disease, Amyotrophic lateral sclerosis	Lokireddy *et al.*, 2015
20	Doxycycline (54671203)	E3 inhibitor	HUWE1	Decreases expression of Huwe1 that stabilizes MYC-associated protein MIZ1 causing inhibition of MYC function in tumor cells and reshapes α-synuclein oligomers into off-pathway, high-molecular-weight species that do not evolve into fibrils preventing its toxic effects	Parkinson's Disease, Cancer	Peter *et al.*, 2014; Gonzalez-Lizarraga *et al.*, 2017
21	Benzamil (108107)	UPS activator	–	Rescue acid sensing ion channel (ASIC) dependent acidotoxicity and led to enhancement of UPS activity	Huntington's Disease	Wong *et al.*, 2008
22	Trehalose (7427)	E3 stimulator	CHIP	Bind to expanded polyglutamines and stabilizing the partially unfolded polyglutamine-containing protein and increase expression level of CHIP to protect against polyglutamine mediated toxicity	Huntington's Disease	Fernandez-Estevez *et al.*, 2014; Casarejos *et al.*, 2014
23	Mitoxantrone (4212)	DUB inhibitor	USP11	Target ubiquitin-specific peptidase 11 and act as immunosuppressive drug protective in cancer and multiple sclerosis	Multiple sclerosis, Cancer	Cocco and Marrosu 2014

1.7.2 Clinical applications of chimera construct to combat neurodegenerative diseases

The selective protein degradation achieved by proteolysis targeting chimeras (PROTACs) has revolutionized the therapeutic avenues towards a variety of cancers and neurodegenerative diseases like AD and PD. The current PROTAC technology employed the use of hetero bifunctional molecules, including a ligand for an intracellular target protein and a recruiting group for an ubiquitin E3 ligase linked by a linker (Lai and Crews 2017). These chimeras facilitate the ubiquitination of target proteins by specific E3 ligases and their subsequent degradation in the proteasome **(Figure 1.6A).** The specialties of these PROTACs are their ability to cross blood-brain-barrier and their routes of administration, including oral, intravenous, subcutaneous, and intrathecal. The PROTAC technology is based on the innovative research of Dr. Craig Crews at Yale University, which later been translated by the Arvinas biopharmaceutical company. Many oncoprotein, including androgen (AR) and estrogen receptors (ER), estrogen-related receptor alpha (ERRα) and bromodomain containing protein 4 (BRD4) have been targeted for degradation by PROTAC for the therapeutic intervention in prostate and breast cancers (Lu *et al.,* 2015; Neklesa *et al.,* 2017; Flanagan *et al.,* 2018). Moreover, Arvinas also explored the PROTACs potential for targeting neurodegenerative diseases like Alzheimer's and Parkinson's disease. For instance, they demonstrated the 50% reduction of tau proteins in the hippocampus upon direct injection of tau-directed PROTAC protein degraders. Further, they reported tau- and α-synuclein targeted PROTAC protein degraders to cross the blood-brain barriers *in-vivo,* signifying their therapeutic potential against AD and PD (http://arvinas.com/therapeutic-programs/next-generation-targets/). Different pharmaceutical companies, including Genentech, Pfizer and different academicians have shown interest in this area and made collaborations with Arvinas. Other industries like Astex pharmaceuticals are working on the CLIPTACs protein degradation technology i.e. in-cell click-formed proteolysis targeting chimeras **(Figure 1.6B).** This technique employed two cell-permeable ligands that undergo a cycloaddition reaction to form a CLIPTAC inside the cell and thus facilitate ubiquitination and subsequent proteasomal degradation of target proteins- BRD4 and ERK1/2 (Lebraud *et al.,* 2016). Likewise, another company,

Captor Therapeutics is working to develop a molecular repair platform, i.e. low molecular compounds capable of reconstituting interactions between ubiquitin E3 ligases and their target proteins to employ UPS for target protein degradation. Similarly, other groups at LifeSensors are working to identify the biomarkers for PD and other NDDs based on their TUBEs technology, i.e. pulling of significant amounts of ubiquitinated proteins from patient samples **(Figure 1.6C).** This would enable them to identify relative fraction of ubiquitinated proteins, i.e. ubiquitination pattern of specific proteins to deduce the precise biomarkers for the complex diseases. In this manner, the pharmaceutical companies are exploring the starring role of proteolysis in neurodegeneration, which would enable us to develop the potential future therapies avoiding non-specific side effects with enhanced drug efficacies against neurodegenerative disorders.

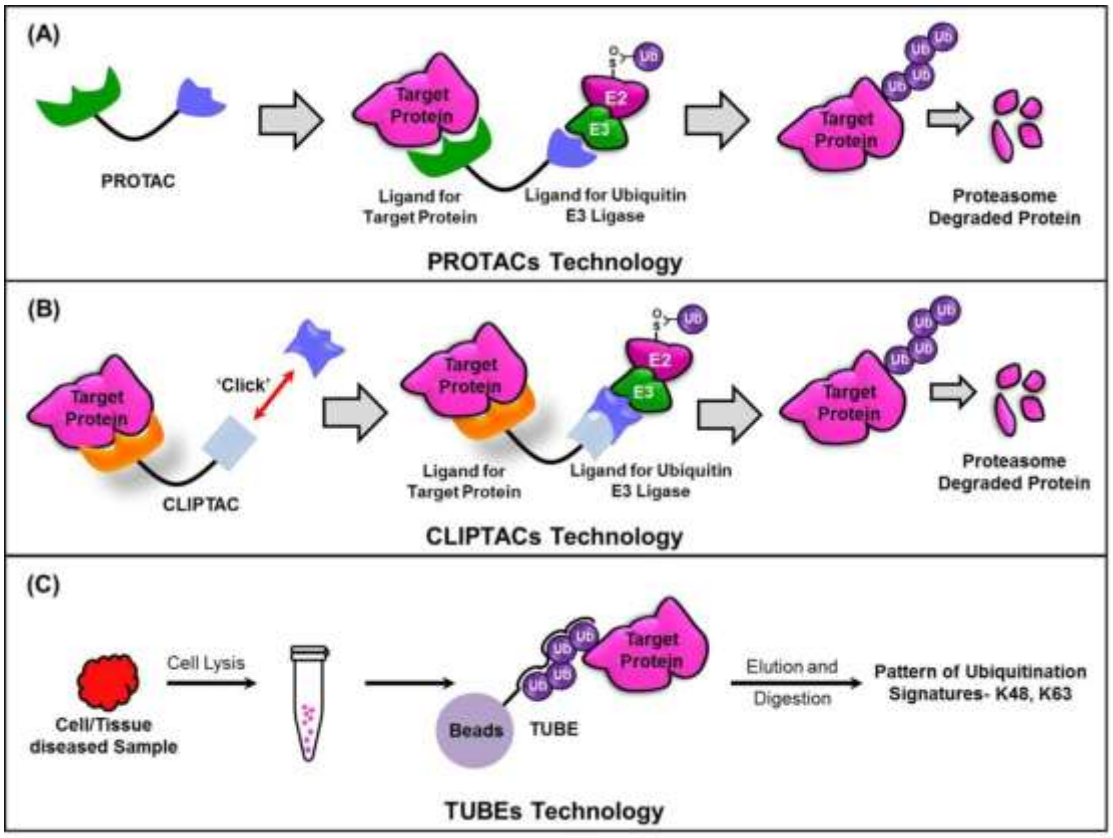


Figure 1.6: Recent technologies for targeted protein degradation and ubiquitination research: (A) PROTACs Technology- This technique employs proteolysis targeting chimeras containing ligands for both target protein and E3 ligase to recruit E3 ligase to the target protein for its proteasomal degradation. **(B) CLIPTACs Technology-** This technique employs two-cell permeable ligands, which

undergo a cycloaddition reaction to form a "In-cell click-formed proteolysis targeting chimeras" with ligands for both target protein and E3 ligase and proteasomally degrades the target protein. **(C) TUBEs Technology**- This technique employs the high-affinity poly-ubiquitin binding matrix, i.e. Tandem Ubiquitin Binding Entities to identify unique patterns of ubiquitination using K48 and K63 selective TUBEs.

1.8 THESIS RATIONALE

Understanding the role of protein clearance in the brain has numerous implications in a variety of neurodegenerative disorders, including but not limited to, Alzheimer's and Parkinson's disease. Interestingly, their pathologies have shown the presence of toxic Aβ, Tau, and α-synuclein aggregates in their advanced stages, suggesting some overlap in their molecular mechanisms (Crews *et al.,* 2009; Lim *et al.,* 2018; Zhang *et al.,* 2018). In this regard, some researchers have reported the central role of amyloid-beta precursor protein in the progression of both diseases. For instance, AβPP being a progenitor for amyloid (Aβ) formation plays a pathogenic role in AD, while its amyloid intracellular domain (AICD) is associated with mitochondrial dysregulation and related neurotoxicity in PD (Chen *et al.,* 2017; Goiran *et al.,* 2018). Moreover, it is triggered to be highly expressed under diseased conditions. Therefore, elucidating the underlying molecular mechanism of AβPP processing, trafficking, degradation and turnover is of great importance. One such highly studied means of protein regulation and degradation includes ubiquitination (a post-translational modification), which was unknown for AβPP. Therefore, the main-focus of the studies described in this thesis was to investigate the ubiquitination pattern of AβPP and thus to rescue its associated burden of Aβ, Tau, and α-synuclein deposits in AD and PD. The aim and specific objectives of this thesis are as follows.

1.9 AIM AND OBJECTIVES OF PRESENT STUDY

Aim: To investigate the clearance of toxic proteins in Alzheimer's and Parkinson's disease at AβPP paradigm

Objectives:

[1] To identify the potential ubiquitination sites in AβPP and study the functional impact of lysine residues on AβPP ubiquitination.

[2] To elucidate the ubiquitination mechanism of AβPP and their regulatory ubiquitination enzymes to rescue toxic protein burden in neurons.

[3] To determine the criteria of lysine selection for AβPP ubiquitination and to identify the AβPP's regulatory partners governing the disease pathogenesis.

[4] To investigate the molecular basis of neurotoxicity caused by pathological proteins, and characterize their functional partners involved in the pathology of AD and PD.

[5] To investigate the molecular crosstalk between Alzheimer's and Parkinson's disease facilitating the clearance of Aβ, Tau, and α-synuclein.

1.10 OUTLINE OF THE THESIS

The dynamics of UPS machinery for the clearance of toxic metabolites in neurodegenerative disorders involving the brain signaling and physiology, proteasome architecture, UPS enzymes- their mutational aberrancies and clinical applications are reviewed in **Chapter I.** Further, the series of experiments and methodologies employed to accomplish the specific objectives of this study are described in **Chapter II.**

The first and second objectives were studied in **Chapter III** in bioinformatics-studies demonstrating the potential ubiquitination sites in AβPP with lysine 351 as the most promising site for ubiquitination. These lysine residues were crucial for imparting stability to the AβPP, since their mutations with other positive charged histidine and arginine residues were highly unstable. Moreover, most of the lysine mutations were susceptible to the diseases and were crucial for AβPP processing and other post-translational modifications such as sumoylation, acetylation, methylation, and

glycosylation. Further, the studies revealed the possibility of lysine residues in non-covalent interactions for ubiquitin positioning during the ubiquitination process. Towards this end, reported an array of ubiquitin E1 activating and E2 conjugating enzymes with Park2 and STUB1 as the potential E3 ligases governing the ubiquitination of AβPP.

The third objective was studied in **Chapter IV,** where we provided more insight into the mechanism by which ubiquitin E3-ligase determines the selection of lysine for ubiquitination. This study revealed the conservation of polar uncharged glutamine and polar negatively charged glutamate indicating their propensity for hydrogen bonds and salt bridges respectively at the ubiquitination sites. This study also revealed the incidence of alpha helical conformations at the ubiquitination sites in comparison to the loop or beta strands. Moreover, this chapter discusses the interacting partners of AβPP and their pathophysiological role in the disease along with the aggregation sites of amyloid-beta peptides.

Finally, the fourth and fifth objectives were addressed in **Chapter V** that demonstrated the molecular basis of $A\beta_{40/42}$, Tau and α-synuclein aggregation followed by mapping of their interacting partners in the pathology of Alzheimer's and Parkinson's disease. Further, this chapter illustrates the molecular crosstalk between AD and PD followed by the characterization of their regulatory ubiquitinational enzymes governing the clearance of Aβ, Tau and α-synuclein.

Chapter VI discusses the results presented in this thesis and addresses future research questions and directions. In addition, it conveys overall significance of findings and their biological implications.

1.11 PLAN OF WORK

The present work is divided into different sections to attain the objectives of our study:

I. Identification of potential ubiquitination sites in AβPP

A. Sequence identity analysis of AβPP and ubiquitin lysine sites.

B. Sequence similarity analysis of AβPP and ubiquitin lysine sites.

C. Ubiquitin-AβPP lysine peptides docking.

D. Machine learning techniques for identifying ubiquitination sites.

II. Functional studies of lysine's impact on AβPP ubiquitination

A. AβPP structure modeling and refinement.

B. Investigation of AβPP stability upon lysine mutations.

C. Physico-chemical property analysis for AβPP ubiquitination.

D. Examination of lysine sites crucial for AβPP modifications.

E. Mutational analysis of lysine residues for disease susceptibility.

III. Elucidation of the ubiquitination mechanism of AβPP

A. AβPP-Ubiquitin interaction prediction and interface residue analysis.

B. Prediction of ubiquitination enzymes interactional network for AβPP.

IV. Identification of the molecular basis of lysine selection for AβPP ubiquitination

A. Ubiquitination site predictions for Aβ, AβPP, and ubiquitin.

B. Determination of structural selectivity of lysine's ubiquitination.

C. Lysine site conserved residue analysis.

V. Investigation of the molecular basis of Aβ, Tau, and α-synuclein aggregation

A. Secondary structural determination.

B. Macromolecular structure design and hydrophobicity annotation.

VI. Characterization of the functional partners of Aβ, Tau, and α-synuclein involved in the pathology of AD and PD

A. Identification of interacting partners with functional association network tool.

B. Protein interaction network analysis for AD and PD.

VII. Investigation of the molecular crosstalk between AD and PD governing the clearance of Aβ, Tau, and α-synuclein

A. Identification of the ubiquitin E3 ligases interacting with Aβ, Tau, and α-synuclein and their interacting partners.

B. Identification of the ubiquitin E2 conjugating enzymes and ubiquitin E1 activating enzymes to the corresponding ubiquitin E3 ligase.

Chapter II

Materials and Methods

CHAPTER II

MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter summarizes the detailed information of various tools and techniques that were employed to determine the potential ubiquitination sites in AβPP, their ubiquitination mechanism, their lysine specificity, molecular basis of pathological proteins- Aβ, Tau, and α-synuclein aggregation, and their ubiquitinational enzymes governing their clearance in Alzheimer's and Parkinson's disease.

2.2 AβPP STRUCTURE MODELING

The AβPP structure has modeled with a protein homology/analogy recognition tool Phyre^2. It included the assembly of homologous sequences by multiple sequence alignments of query sequence with their sequence homologs and their consequent secondary structure prediction by PSI-pred pooled query hidden Markov model. Further, fold library scanned for crude backbone construction based on top scoring alignments. Furthermore, the loop was modeled for correcting insertions and deletions in the models followed by side chain placement to backbone to obtain a final protein structure model (Kelley *et al.,* 2015). Here, six templates (c3ktmB, c3dxeB, c2yszA, d1rw6a, c1amlA, c2lp1A) are employed to model the AβPP protein structure based on heuristics method to maximize the confidence, percentage identity and alignment coverage to the queried sequence.

2.2.1 AβPP domain analysis

The protein domain analysis was done to identify the functional sites in the AβPP with MOTIF Search tool. It extracted domain results based on the matched bit score for AβPP protein sequence and each domain found in Pfam library with an E-value cut-off of 0.001 (Finn *et al.,* 2016).

2.2.2 Structure refinement simulations

The predicted 3D-structure of AβPP was refined with an atomic-level, high-resolution protein-structure refinement tool- ModRefiner (Xu and Zhang 2011). We performed the conformational search for the backbone and side chain atoms to simulate the

predicted model to their native state in terms of backbone topology, side chain positioning and hydrogen bonds directed by combined physics- and knowledge-based force-field energy calculations.

2.2.3 AβPP structural validations through PROVE, ERRAT, VERIFY 3D and RAMPAGE

The reliability of 3D-atomic models of predicted AβPP validated through the stereo chemical quality measurements of modeled proteins with different structural validation programs. ***PROVE*** analyzed the volume-based quality of protein crystal structure by computing the statistical Z-score deviations of the atomic volumes from their standard values (Pontius *et al.,* 1996). The buried atoms less than 1% passed the structural quality test while greater than 5% implied the structural irregularities. ***ERRAT*** determined overall quality factor by identifying the statistical differences between the patterns of non-bonded atomic interactions (ordered vs. randomized distribution) and the error functions of the predicted model with the statistics of highly refined structures (Colovos and Yeates 1993). ***VERIFY 3D*** compared 3D atomic model compatibility with its own primary sequence (1D) associated structural class assigned based on their environment and location (Luthy *et al.,* 1992). ***RAMPAGE*** geometrically validated C-alpha neighboring residues of modeled AβPP (Lovell *et al.,* 2003), which allowed the detection of C-beta bond angle distortions. Moreover, it defined favored and allowed phi-psi regions for glycine, proline and pre-proline residues to validate the accuracy of the model.

2.3 IDENTIFICATION OF POTENTIAL UBIQUITINATION SITES IN AβPP

The important lysine residues for AβPP ubiquitination was identified with four different approaches, including (i) the sequence identity between AβPP and ubiquitin lysine sites, (ii) sequence similarity between AβPP and ubiquitin lysine sites, (iii) ubiquitin-ubiquitin and ubiquitin-AβPP lysine peptides docking, and (iv) machine learning based ubiquitination site predictions.

2.3.1 Sequence identity analysis

Pairwise sequence alignment was performed using BioEdit Sequence Alignment Editor Software (Hall 1999) using BLOSUM 62 substitution matrix. Further, it calculated the identity scores among the 21 window-size central lysine's neighboring residues of ubiquitin and AβPP sequences.

2.3.2 Sequence similarity analysis

Conserved sequence analysis of amino acid residue neighboring lysine sites were performed by aligning the 21 window-size multiple lysine site sequences in AβPP by the BioEdit Sequence Alignment Editor Software (Hall 1999) at different thresholds to identify the important residues common to both ubiquitin and AβPP critical for the ubiquitination.

2.3.3 Ubiquitin-AβPP lysine peptides docking

The protein-peptide interactions of ubiquitin protein and AβPP lysine peptides were modeled by flexible docking approach using CABS-dock web server. It executed simulation searching for the binding sites in the receptor protein allowing full flexibility to the peptides being docked (Ciemny *et al.*, 2017). For ubiquitin-AβPP peptide docking, 21 window-size peptide sequences were prepared by taking 10 amino acid residues on either side of the lysine sites from AβPP protein as test set and from ubiquitin protein as control set respectively. Then, ubiquitin protein (PDB ID: 1UBQ) is flexibly docked with both the test set peptides, and the control set peptides, to identify the potential lysine for ubiquitination.

2.3.4 Machine learning techniques - UbiSite, BDM-PUB, hCKSAAP_UBSITE, UbPred, UbiPred

The potential ubiquitination sites for AβPP was predicted by diverse machine learning tools, including UbiSite, BDM-PUB, CKSAAP, UbPred and UbiPred on the basis of the experimentally verified ubiquitination site datasets. ***UbiSite*** employed maximal dependence decomposition method (specificity-level 'High' at 95%), and support vector machine based on the hybrid features for the large-scale ubiquitin-conjugation site data to predict ubiquitination sites (Huang *et al.*, 2016). ***BDM-PUB*** employed Bayesian Discriminant analysis (at balanced cut off) between high specificity and high sensitivity to apply a probabilistic approach for ubiquitination pattern recognition

(Xue *et al.,* 2006). ***hCKSAAP_UBSITE*** utilized SVM classifiers based diverse amino acid pattern and propensities trained by logistic regression to predict the ubiquitination sites in AβPP (Chen *et al.,* 2013). ***UbPred*** relied upon a random forest based prediction model trained on experimentally verified ubiquitination sites to predict the ubiquitination pattern in AβPP (Radivojac *et al.,* 2010). ***UbiPred*** employed SVM to predict the ubiquitination sites based on the principle of the informative physicochemical property mining algorithm (Tung and Ho 2008).

2.4 FUNCTIONAL STUDIES OF LYSINE'S IMPACT ON AβPP UBIQUITINATION

The refined AβPP structure was incorporated with both positive (Arginine (R), Histidine (H)) and negative (Aspartic acid (D), Glutamic acid (E)) charged mutations at their lysine sites by Pymol software (DeLano 2002). The above mutations were studied for their impact on AβPP stability, ubiquitination, modifications and disease susceptibility.

2.4.1 Investigation of AβPP stability upon lysine mutations

The refined AβPP model and mutated AβPP (Lysine (K)→Arginine(R)/ Histidine(H)/ Aspartic acid(D)/ Glutamic acid(E)) analyzed for their total force field energies by Swiss PDB viewer 4.0.2 software (Guex *et al.,* 2009). The variations in the total force field energies were estimated to observe the impact of lysine mutations on the amyloid-beta precursor protein's stability.

2.4.2 Physico-chemical property analysis for AβPP ubiquitination

The physico-chemical properties of amyloid beta, AβPP and ubiquitin proteins were computationally determined from their peptide sequences by ExPASy server tool ProtParam (Gasteiger *et al.,* 2005). It computed various physico-chemical properties, including the atomic and amino acid compositions along with the count of positively and negatively charged residues, the instability index, aliphatic index, and the grand average of hydropathicity of the desired proteins. The physico-chemical properties of AβPP were compared with ubiquitin to investigate the crucial parameters important for the ubiquitination.

2.4.3 Examination of lysine sites crucial for AβPP modifications

The molecular mechanisms associated with the pathogenic amino acid substitutions in AβPP were identified by a machine-learning tool MutPred (Pejaver *et al.,* 2017). It quantified the pathogenicity of mutations based on the probabilistic modeling of a large repertoire of structural-functional alterations, including the disruptions in structure, stability, macromolecular binding, and post-translational modification sites in amino acid sequences.

2.4.4 Mutational analysis of lysine residues for disease susceptibility

The functional impact of the introduced mutations was studied with different mutation analysis tools, including PANTHER (Tang and Thomas 2016), SNAP2 (Hecht *et al.,* 2015), Polyphen2 (Adzhubei *et al.,* 2010), Pmut (Ferrer-Costa *et al.,* 2005), PhD-SNP (Capriotti *et al.,* 2006) and SIFT (Sim *et al.,* 2012). The obtained results were transformed into numerical values to analyze them on a stacked bar graph. For instance, results with "Probably Benign," "Neutral," "Tolerated" were assigned with '0' numerical value and "Possibly Damaging" with '1' numerical value. Similarly, results with "Probably Damaging," "Effect," and Not tolerated" were assigned with "2" numerical value and the threshold was taken '6' i.e. more than half prediction tools to predict high confidence lysine having disease susceptibility.

2.5 CHARACTERIZATION OF THE UBIQUITINATION PATTERN OF AβPP

The ubiquitination pattern of AβPP was investigated through the combination of multiple studies, including (i) the AβPP-ubiquitin interaction studies, and (ii) the prediction of ubiquitination enzyme's interactional network for AβPP or Aβ clearance in Alzheimer's disease biology.

2.5.1 AβPP-Ubiquitin interaction prediction and interface residue analysis

The interactions among the different domains of AβPP and ubiquitin proteins were predicted by the protein-protein interaction prediction server-PSOPIA (Murakami and Mizuguchi 2014). It compared the sequence similarities to a known interacting protein pair, statistical propensities of the domain-domain interactions, and the sum of edge weights along the shortest path between the homologous proteins in a PPI network.

Moreover, the potential ubiquitin interacting domains of AβPP (PDB IDs: 4JFN_A, 2FKL_A, 1AAP_A, 3UMK_A, 1IYT_A, 2LP1_A) were docked with ubiquitin (PDB ID: 1UBQ) by rigid body docking approach using Fast Fourier Transformation methodology of GRAMM-X software (Tovchigrechko and Vakser 2006). Furthermore, their docked interface residues having distance < 4.5Å were identified by Pymol software (DeLano 2002) to identify the key lysine residues important for ubiquitin and AβPP interactions.

2.5.2 Prediction of ubiquitination enzyme's interactional network for AβPP

The potential ubiquitination enzymes regulating the AD biology of Aβ and AβPP proteasomal clearance was identified by determining the interaction among all the ubiquitin E1-activating enzymes, E2-conjugating enzymes, E3-ligating enzymes and deubiquitinating enzymes with amyloid forming proteins- AβPP, β-secretases and γ-secretases. Further, the protein-protein interactional network among the identified proteins was designed by functional protein-association network prediction STRING tool (Szklarczyk *et al.*, 2015).

2.6 IDENTIFICATION OF THE BASIS OF LYSINE SELECTION FOR AβPP UBIQUITINATION

The key lysine sites for ubiquitination were predicted for Aβ, AβPP and Ubiquitin and their structural incidences were determined to check the protein's structural conformation selectivity for lysine ubiquitination. This is followed by the identification of conserved amino acids at key lysine sites crucial for its selectivity for protein ubiquitination.

2.6.1 Ubiquitination site predictions for $A\beta_{40/42}$, AβPP, and Ubiquitin

The ubiquitination sites in $A\beta_{42}$, ubiquitin and AβPP has been predicted with the help of machine learning tools UbPred (http://www.ubpred.org/) and UbiPred (http://e045.life.nctu.edu.tw/ubipred/). Both are highly potent sequence based prediction tools to identify the promising ubiquitination sites utilizing the random forest-method for UbPred (Radivojac *et al.*, 2010) and support vector machine for UbiPred (Tung and Ho 2008).

2.6.2 Determination of structural selectivity of lysine's ubiquitination

The secondary structure of AβPP has been determined by the help of PSIPRED protein-structure prediction server available at http://bioinf.cs.ucl.ac.uk/psipred/ (Buchan *et al.,* 2013). The obtained information about the secondary structure of AβPP protein was compared with the prediction results of the lysine's ubiquitination information obtained from Ub-site prediction tools- UbPred and UbiPred. The corresponding secondary structure, i.e. alpha helix, beta sheet and turn/loop have been compared with its Ub-informative sites, and comparative structural selectivity has been determined.

2.6.3 Lysine site conserved residue analysis

The lysine site conserved residues were identified by multiple sequence alignments of 21-window size lysine containing sequence at the centre from ubiquitin and AβPP protein using ClustalW MSA tool (Thompson *et al.,* 1994). The 21-window size sequence has been designed by taking 10 residues at both ends of a lysine residue. Further, the obtained alignment was annotated with the help of a Bioedit sequence-alignment editor tool, and the conservation has been identified and shown with color-based shading of sequence identity and sequence similarity (Hall 1999). Furthermore, the overall methodology employed along with the tools and techniques in the study of AβPP ubiquitination biology has been illustrated in **Figure 2.1.**

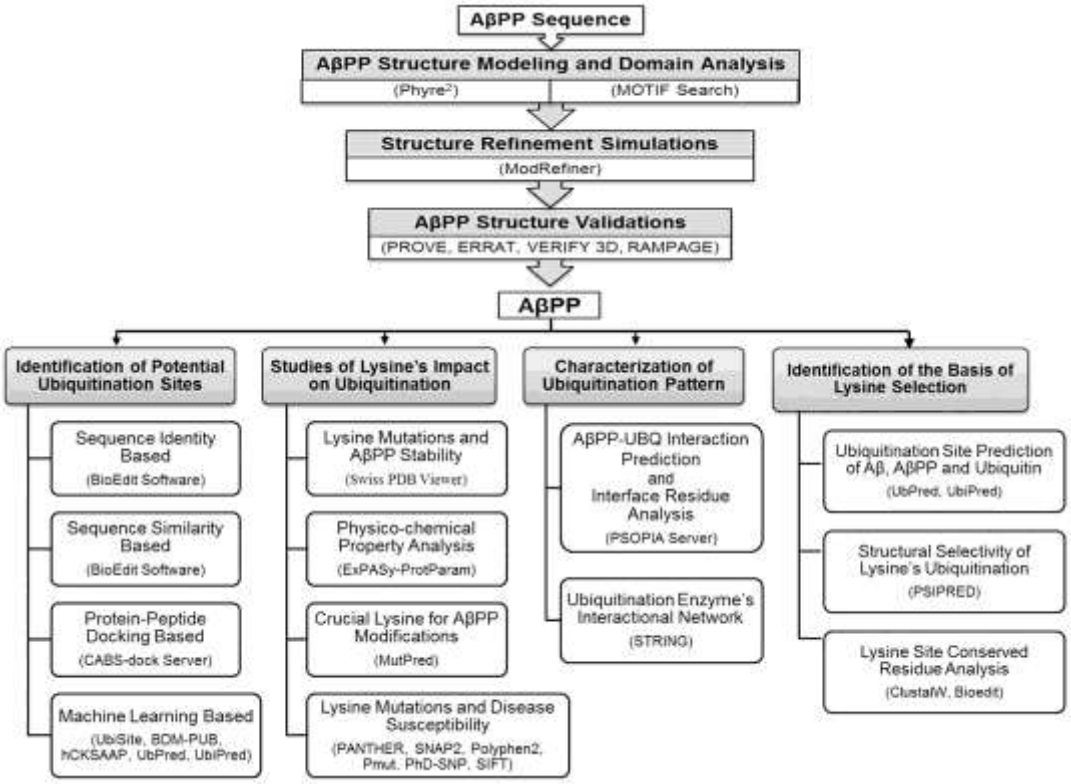


Figure 2.1: Flow chart depicting the tools and techniques employed for the study of AβPP ubiquitination.

2.7 INVESTIGATION OF MOLECULAR BASIS OF $A\beta_{40/42,}$ TAU, AND α-SYNUCLEIN AGGREGATION

The available sequences for the neurotoxic proteins were analysed for their secondary structures, and their present monomer and fibrillar structures were annotated for their hydrophobic residues responsible for aggregation.

2.7.1 Structural determination of $A\beta_{40/42,}$ Tau, and α-Synuclein

The peptide sequence of $A\beta_{40/42,}$ Tau and α-Synuclein were obtained from the protein data bank (RCSB PDB: www.rcsb.org) (Berman *et al.*, 2000) and processed for the determination of its secondary structure by Dictionary of protein secondary structure (DSSP) database (Touw *et al.*, 2015).

2.7.2 Macromolecular structure design and hydrophobicity annotation

The macromolecular monomeric and fibrillar structures of $A\beta_{40/42,}$ Tau and α-Synuclein proteins have been analysed for the hydrophobic or aggregation prone

residues. These sites were annotated to the available 3D-structures with help of NGL viewer (http://proteinformatics.charite.de/ngl) (Rose and Hildebrand 2015) and Pymol software (DeLano WL 2002).

2.8 CHARACTERIZATION OF THE FUNCTIONAL PARTNERS OF $A\beta_{40/42,}$ TAU, AND α-SYNUCLEIN INVOLVED IN THE PATHOLOGY OF AD AND PD

The top interacting partners of the neurotoxic proteins have predicted and analysed for their role in disease pathogenesis.

2.8.1 Prediction of interacting partners of AβPP, Tau, and α-Synuclein

The interacting partners of AβPP, Tau and α-Synuclein were determined by functional protein-association network tool called STRING, online available at https://string-db.org/. The top interactors were identified with high confidence at the threshold of interaction score ≥0.700, and the network were generated without clustering and evidence based upon text mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence (Jensen *et al.*, 2009).

2.8.2 Protein interaction network analysis for Alzheimer's and Parkinson's disease

Top hundred interacting partners of the AβPP, Tau, and α-Synuclein proteins were mapped on the pathways for Alzheimer's and Parkinson's disease from KEGG Pathways database and analysed for their functional association with the disease pathogenesis (Kanehisa *et al.,* 2017).

2.9 INVESTIGATION OF THE MOLECULAR CROSSTALK BETWEEN AD AND PD GOVERNING CLEARANCE OF $A\beta_{40/42,}$ TAU, AND α-SYNUCLEIN

The combined AD and PD related top interactors of AβPP, Tau, and α-Synuclein was iterated for their interactions with topmost hundred interacting partners. Those proteins that qualified their AD and PD incidence were selected and identified for their interactions with AβPP, Tau, and α-Synuclein at a high confidence threshold ≥0.7 with STRING tool, i.e. a Search Tool for the Retrieval of Interacting Genes/Proteins based on the evidence from text mining, experiments, databases, co-

expression, neighborhood, gene fusion and co-occurrence (Szklarczyk et al., 2015). The interacting partner's prediction was followed by the Venn diagram analysis. Thus, the obtained proteins common to both Alzheimer's and Parkinson's disease, and were interacting with AβPP, Tau, and α-Synuclein, were screened as the key marker for AD-PD crosstalk.

2.9.1 Identification of ubiquitination enzymes regulating the clearance of $A\beta_{40/42}$, Tau, and α-Synuclein

The potential ubiquitination enzymes regulating the biology of Aβ, AβPP, Tau and α-Synuclein proteasomal clearances were identified by determining the interaction among all the ubiquitin E1-activating enzymes, E2-conjugating enzymes, E3-ligating enzymes and deubiquitinating enzymes with AD-PD crosstalk proteins- AβPP, CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2, SNCA, and UBB at different confidences. Further, the protein-protein interactional network among the identified proteins was designed by functional protein-association network prediction STRING tool (Szklarczyk *et al.*, 2015).

2.9.2 Functional annotation of the AD-PD cross-talk and Ubiquitination markers

The analysis of the biological processes, reactome pathways, molecular functions, and the protein domains of the predicted AD-PD cross talk markers and the UPS enzymes- E1s, E2s, E3s, and DUBs were preformed with the help of a functional enrichment analysis tool "FunRich" version 3.1.3 (Pathan *et al.,* 2015). It is a tool for the enrichment and interaction network analysis of genes and proteins based on data mining from the available databases, including FunRich, Uniprot, Reactome and Custom. The Unirot and reactome databases have been explored to obtain the best scoring results at very high significant P value, i.e. $P<0.001$.

Chapter III

Integrated Mechanism of Lysine351, PARK2 and STUB1 in AβPP Ubiquitination

CHAPTER III

INTEGRATED MECHANISM OF LYSINE-351, PARK2, AND STUB1 IN AβPP UBIQUITINATION

3.1 ABSTRACT

Intracellular accumulation of aggregated β-amyloid, misfolded and non-functional proteinopathy is the hallmark feature in Alzheimer's disease (AD). There are several mechanisms to clear the amyloid burden in a cell, including transcytosis across blood-brain barrier, immune mediated, lysosomal pathway associated autophagy, enzymatic degradation by insulin degrading enzyme / neprilysin, and the proteasomal pathway. Among them, the ubiquitin proteasome system (UPS) is playing a critical role to prevent the intracellular β-amyloid deposition and to clear off the cellular burden in association with ubiquitin E3 ligase enzymes in AD. For ubiquitination, lysine moiety in a protein, acts like a docking site for the attachment of ubiquitin molecule and different lysine residues act differently in this reaction. Therefore, it is pertinent to understand and link the role of diverse lysine residues along with it effector molecules, for instance, E3 ligases PARK2 and STUB1 in the ubiquitination cascade. Herein, we (i) modeled AβPP structure, determined its topologies, and studied the impact of lysine residues in AβPP stability. (ii) We reported K351 as the most promising target for AβPP ubiquitination; (iii) investigated the plausible role of lysine residues in non-covalent interactions mediated ubiquitin positioning in the ubiquitination. (iv) We detected conserved amino acids that are crucial for AβPP ubiquitination; (v) identified the key ubiquitination enzymes and their interaction network playing major role in the ubiquitination of AβPP.

3.2 INTRODUCTION

Alzheimer's disease (AD) is characterized by the aberrant protein accumulation and aggregates such as amyloid-beta (Aβ), leading to the development of senile plaques in the brain (Singh *et al.,* 2016). In this regard, the amyloid cascade hypothesis affirms the Aβ deposition as an early pathogenic event in the progression of AD (Serrano-Pozo *et al.,* 2011). The intra- and extra-cellular amyloid beta ($Aβ_{40/42}$) deposits perturb the synaptic transmission between neurons and trigger the memory and cognitive

decline in AD patients (Shankar and Walsh 2009). Here, Aβ and its precursor AβPP are the central players in the pathology of AD whose levels are regulated by the protein quality control; ubiquitin proteasome system (UPS). There are other mechanisms that regulate Aβ clearance, including enzymatic pathways utilizing insulin degrading enzyme and neprilysin (Jha *et al.,* 2015), immune-mediated trans-cytosis across the blood-brain barrier (Storck *et al.,* 2016), and the lysosomal pathways through autophagy (Baranello *et al.,* 2015). However, the ubiquitination pathway is central to regulate the level of proteins involved in all these pathways; therefore, it has a great importance in the cellular biology for the clearance of amyloid beta. The ubiquitination process involves the selection of key lysine residues of target protein by E3 ligases for ubiquitin attachment. This ubiquitin ligation at key lysine and their type of poly-ubiquitin chain determines the cellular fate of the target protein (Suryadinata *et al.,* 2013). For instance, studies have identified K6 poly-Ub-chain to trigger DNA repair responses and K33 poly-Ub-chain to initiate stress responses. Moreover, K63 poly-Ub-chain to govern DNA repair, endocytosis and inflammatory responses while K11, K27, K29 and K48 poly-Ub-chain to elicit proteasomal degradation (Li and Ye 2008; Dammer *et al.,* 2011).

In this manner, UPS greatly influences the Aβ production through AβPP ubiquitination and through proteasomal degradation of their regulatory enzymes (β- and γ-secretases). Moreover, there is increasing evidence of non-functional UPS i.e. ubiquitinating and deubiquitinating enzymes and the proteasomal subunits that are responsible for the altered Aβ clearance in AD patients (Gong *et al.,* 2016). However, there are numerous reports depicting the ubiquitination of Aβ or AβPP *in-vitro*, ex-vivo and *in-vivo* but their exact site for ubiquitination remained unknown for most of the cases **(Table 3.1)**. Moreover, the AβPP ubiquitination research is at the preliminary stage, where much needs to be investigated to unravel the mystery of amyloid burden mitigation. Therefore, identification of the key lysine residues and the ubiquitination components are crucial for determining the mechanism behind Aβ clearance. In this regard, we have identified the key lysine residues having great potential for AβPP ubiquitination. Moreover, the importance of these key lysine residues in AβPP processing, non-covalent interactions with ubiquitin and other

functions have also studied. Further, we modeled the 3D structure of holo-AβPP to investigate the impact of key lysine residues on AβPP stability and their mutational disease susceptibility. Nevertheless, we have also identified the potential ubiquitination enzymes- E1s, E2s, E3s and DUBs and their complex interplay in the ubiquitination process of AβPP protein. In summary, this work has demonstrated the AβPP ubiquitination mechanism and Aβ clearance to provide novel therapeutic targets against Alzheimer's disease.

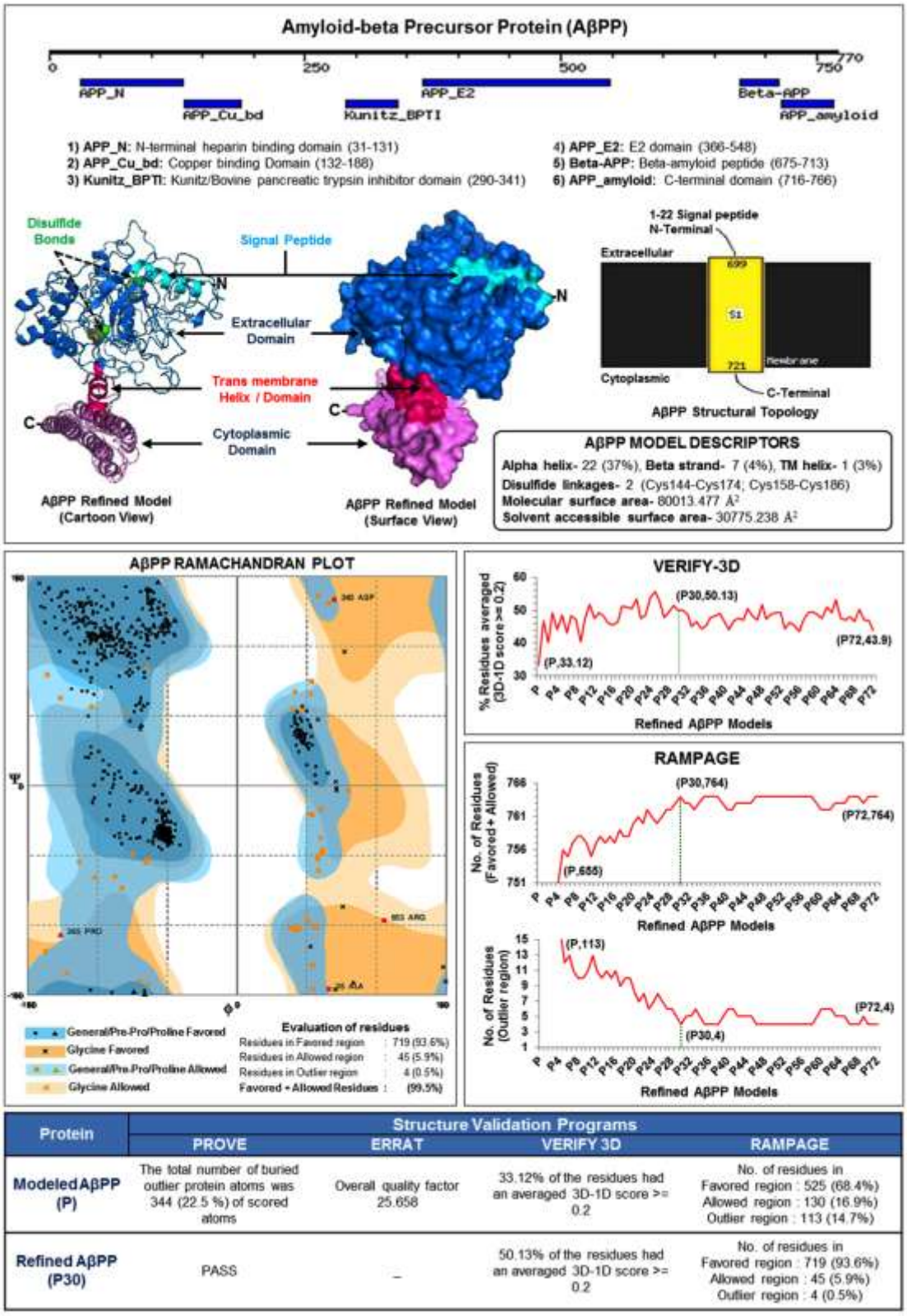
Table 3.1: Experimental studies reporting the ubiquitination of AβPP and Aβ to rescue the amyloid burden in AD

S.No.	Experimental Models	UPS Enzymes	Ubiquitination Sites	Ubiquitination Effects	Reported Studies
1	AβPP/PS1 Transgenic mice	Parkin	–	AβPP degradation	Hong *et al.*, 2014
2	Human Neuroblastoma M17 Cells, Rat Brains		–	Aβ degradation	Burns *et al.*, 2009
3	SHSY5Y Cells, Human and Transgenic Mouse (Parkin K/O) Brain Samples		–	Aβ/AβPP degradation	Rosen *et al.*, 2010; Kumar *et al.*, 2012
4	AβPP23/PS45 Mice	UCHL1	–	Regulate AβPP degradation	Zhang *et al.*, 2014
5	Crbn-KO Mouse Brains	CRL4	K676(AβPP695) / K751(AβPP770)	AβPP interactions with other proteins	Del Prete *et al.*, 2016
6	HeLa Cells, 2xTg Mice (FBL2/AD1) / 3xTg Mice	FBL2	K649-651(AβPP695) / K724-726(AβPP770)	AβPP metabolism, Endocytic trafficking	Watanabe *et al.*, 2012; Morel *et al.*, 2013
7	Hippocampal Neurons, Fbxo2 KO Mice	Fbxo2	–	AβPP processing and degradation	Atkin *et al.*, 2014
8	N2a Cells, P0 Mice Brain	–	K612(AβPP695) / K687(AβPP770)	Endosomal sorting of AβPP	Williamson *et al.*, 2017
			K624(AβPP695) / K699(AβPP770)		
9	SHSY5Y Cells, ddY Mice	HRD1	–	AβPP degradation	Kaneko *et al.*, 2010
10	CHO Cells	HRD1	–	AβPP degradation	Jung *et al.*, 2015
11	SHSY5Y Cells, Human Brain Sample	CHIP	–	AβPP degradation	Kumar *et al.*, 2007
12	Primary Neuron Culture	–	–	Aβ degradation	Lopez Salon *et al.*, 2003
13	Rat Cortical Neurons	–	–	Aβ/AβPP degradation	Favit *et al.*, 2000
14	PC12 Cells	Ubiquilin-1	K688(AβPP695) / K763(AβPP770)	AβPP biosynthesis, trafficking, and degradation	El Ayadi *et al.*, 2012
15	SHSY5Y Cells	–	–	AβPP degradation	Scuderi *et al.*, 2014

3.3 RESULTS

3.3.1 3D-model of Amyloid-beta precursor protein (AβPP)

The three-dimensional structure of AβPP is predicted to analyze the potential lysine residues important for their ubiquitination, since its full experimental structure was lacking. The structural topology of the modeled AβPP has identified an N-terminal signal peptide MLPGLALLLLAAWTARALEVPT (1-22), an extracellular region (1-698), a trans-membrane helix S1 (699-721) and a cytoplasmic region (722-770). Further, domain analysis of full length AβPP (770aa) revealed six functional domains namely (i) N-terminal domain, (ii) Copper binding domain, (iii) Protease inhibitor domain, (iv) E2 domain, (v) Aβ peptide domain, and (vi) C-terminal domain. The combined results suggest that the first four domains parsed in the extracellular region and the Aβ peptide in the trans-membrane region while the C-terminal domain in the cytoplasmic region. Moreover, the modeled structure was spanned by 22 alpha helices (37%), 7 beta-strands (4%) and one trans-membrane alpha helix (3%). In addition, the tertiary structure of the AβPP model is enriched with two disulfide linkages one at cysteine144-cystein174 and another at cysteine158-cysteine186 that are very important for their stability. Further, the structural validation of the refined model by PROVE analysis passed their structural quality test with less than 1% buried outlier atoms. Similarly, the sequence information based tertiary structure; i.e. 3D-1D score >=0.2 was found to be more than 50% for our refined model as per the verify-3D tool. Moreover, the geometrical conformation of the refined model analyzed by RAMPAGE server was found to be greater than 99.5% for the favored and allowed region residues except four amino acids, which included Alanine 35, Aspartic acid 360, Proline 365, and Arginine 653. The refined model of AβPP along with their molecular descriptors and structural refinement simulations is shown in **Figure 3.1.** Further, the structure validation scores of modeled and refined models obtained by verify 3D, and RAMPAGE can be accessed from **Supplementary Table S3.1.**



Protein	Structure Validation Programs			
	PROVE	ERRAT	VERIFY 3D	RAMPAGE
Modeled AβPP (P)	The total number of buried outlier protein atoms was 344 (22.5 %) of scored atoms	Overall quality factor 25.658	33.12% of the residues had an averaged 3D-1D score >= 0.2	No. of residues in Favored region : 525 (68.4%) Allowed region : 130 (16.9%) Outlier region : 113 (14.7%)
Refined AβPP (P30)	PASS	–	50.13% of the residues had an averaged 3D-1D score >= 0.2	No. of residues in Favored region : 719 (93.6%) Allowed region : 45 (5.9%) Outlier region : 4 (0.5%)

Figure 3.1: AβPP modeling and structural refinement. (A) AβPP model: The modeled AβPP (shown in cartoon and surface view) represented an N-terminal signal peptide (Cyan in color), Trans-membrane helix/domain (Hot pink in color) and a C-terminal region (Purple in color) along with the disulfide bonds (Green/Yellow in color). The domain analysis identified

six domains, including N-terminal, copper binding, protease inhibitor, E2, beta-amyloid and C-terminal domain spanned across the full-length 770aa protein. The structural topology of the predicted model has identified a membrane spanning trans-membrane helix S1 at 699 to 721 amino acid residues with an extracellular N-terminal (1-699) carrying signal peptide (1-22) and a cytoplasmic C-terminal (721-770). The structural analysis of the predicted model revealed 22 alpha helices, 7 Beta-sheets and 1 Trans-membrane helix and 2 disulfide linkages (Cys144-Cys174 and Cys158-Cys186). Moreover, the solvent accessible surface area (30775.238 angstrom^2) was less than the molecular surface area (80013.477 angstrom^2) of the model due to inaccessibility of the solvent in the trans-membrane region. **(B) Structural refinement of AβPP-** The modeled AβPP was refined with structural refinement simulations up to 72 cycles (designated P1, P2…P72) and validated for the refinements by different structural validation programs- PROVE, ERRAT, Verify-3D and RAMPAGE. **(C) AβPP Ramachandran plot-** evaluated 99.5% residues in geometrical (ϕ-ψ) favored (719 residues (93.6%)) + allowed residues (45 (5.9%)) regions while only four residues Ala35, Asp360, Pro365, and Arg653 ~0.5% in the outlier region; other structural validation programs, including PROVE and ERRAT identified to PASS the modeled structure for their overall quality. **(D) Verify-3D-** The 3D atomic model compatibility assessment revealed the refinement of modeled AβPP from zero cycles (P)- 33.12% to 72 cycles (P72)- 43.9% residues, with 50.13% residues of the best obtained model P30 at 30th cycle showing 2D-3D structural compatibility. **(E) RAMPAGE-** The geometrical validation of C-alpha neighboring residues revealed the refinement from zero cycles (P) - 655 (favored + allowed residues) and 113 (outlier residues) to 72 cycles (P72) - 764 (favored + allowed residues) and 4 (outlier residues). The best geometrical configuration with minimum simulations was achieved at 30th cycle (P30) signifying it as a best-predicted model.

3.3.2 K351 is the most promising target for AβPP ubiquitination

The potential lysine sites, which are favorable for the ubiquitination of AβPP, are identified by four different methodologies, including sequence identity, sequence similarity, protein-peptide docking and machine learning techniques. Since, ubiquitin is the pre-eminent protein that is ubiquitinated the most in any cellular processes due to the poly-ubiquitination phenomenon; therefore, its lysine site features are of great importance to unravel the ubiquitination mystery. These informative sites of ubiquitin are utilized to deduce the potential lysine sites in AβPP by above-mentioned methodologies.

3.3.2.1 Sequence identity based potential lysine in AβPP

The sequence identity between the 21 window-size peptide sequence with central lysine at seven lysine sites of ubiquitin and forty-one lysine sites of AβPP are determined to infer the promising ubiquitination sites in AβPP. The sequence-identity of at least three amino acids, i.e. identity score >0.2 was taken as the threshold, which

identified 26 key lysine sites in AβPP including K60, K66, K99, K106, K132, K134, K155, K161, K351, K363, K377, K393, K395, K401, K503, K510, K522, K568, K601, K662, K670, K687, K699, K724, K725, and K726 as favorable sites for the ubiquitination **(Figure 3.2A).** Among them, these seven K60, K66, K351, K363, K601, K662, and K687 lysine sites displayed the higher identity scores. For detailed sequence identity scores obtained for each pairwise sequence alignment, the **Supplementary Table S3.2** can be explored.

3.3.2.2 Sequence similarity based potential lysine in AβPP

The sequence conservation analysis among lysine neighboring residues in ubiquitin revealed the conservation of hydrophobic leucine (L), isoleucine (I), valine (V) and polar negatively charged aspartic acid (D), glutamic acid (E) at 57% similarity threshold. Further, the conservation in ubiquitin at 42% similarity threshold provided the majority of informative amino acid residues in common with the amyloid-beta precursor protein that are crucial for the ubiquitination process. These conserved residues included the hydrophobic methionine (M), leucine (L), isoleucine (I), and valine (V), polar negatively charged glutamic acid (E), and polar uncharged glutamine (Q). Moreover, in AβPP only glutamic acid (E) and glutamine (Q) is conserved at the higher threshold of 34% sequence similarity, which depicted K224, K351, K377, K393, K401, K503, K510, K522, K662, K724, K751 and K763 as potential lysine sites **(Figure 3.2B).**

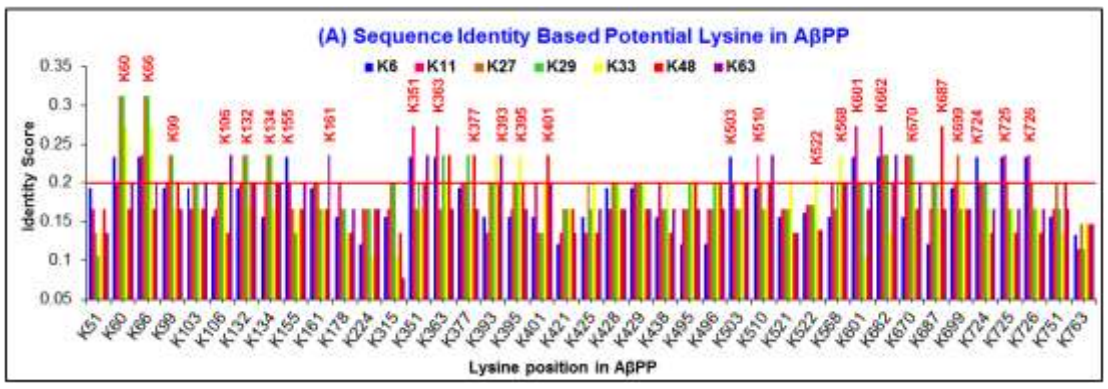


Figure 3.2A: Potential lysine prediction for ubiquitination in AβPP based on sequence identity. The sequence identity scores between 21 window size polypeptides carrying central lysine (K6-Blue, K11-Pink, K27-Brown, K29-Green, K33-Yellow, K48-Red, K63-Purple in ubiquitin and (K51, K60, K66, K99, K103, K106, K132, K134, K155, K161, K178, K224,

K315, K351, K363, K377, K393, K395, K401, K421, K425, K428, K429, K438, K495, K496, K503, K510, K521, K522, K568, K601, K662, K670, K687, K699, K724, K725, K726, K751, K763) in AβPP is plotted on a bar graph. The identity scores greater than 0.2 represented the conservation of more than two amino acid residues in the aligned sequences, signifying the presence of potential lysine for ubiquitination based on sequence identity (shown in Red) - K60, K66, K99, K106, K132, K134, K155, K161, K351, K363, K377, K393, K395, K401, K503, K510, K522, K568, K601, K662, K670, K687, K699, K724, K725, K726.

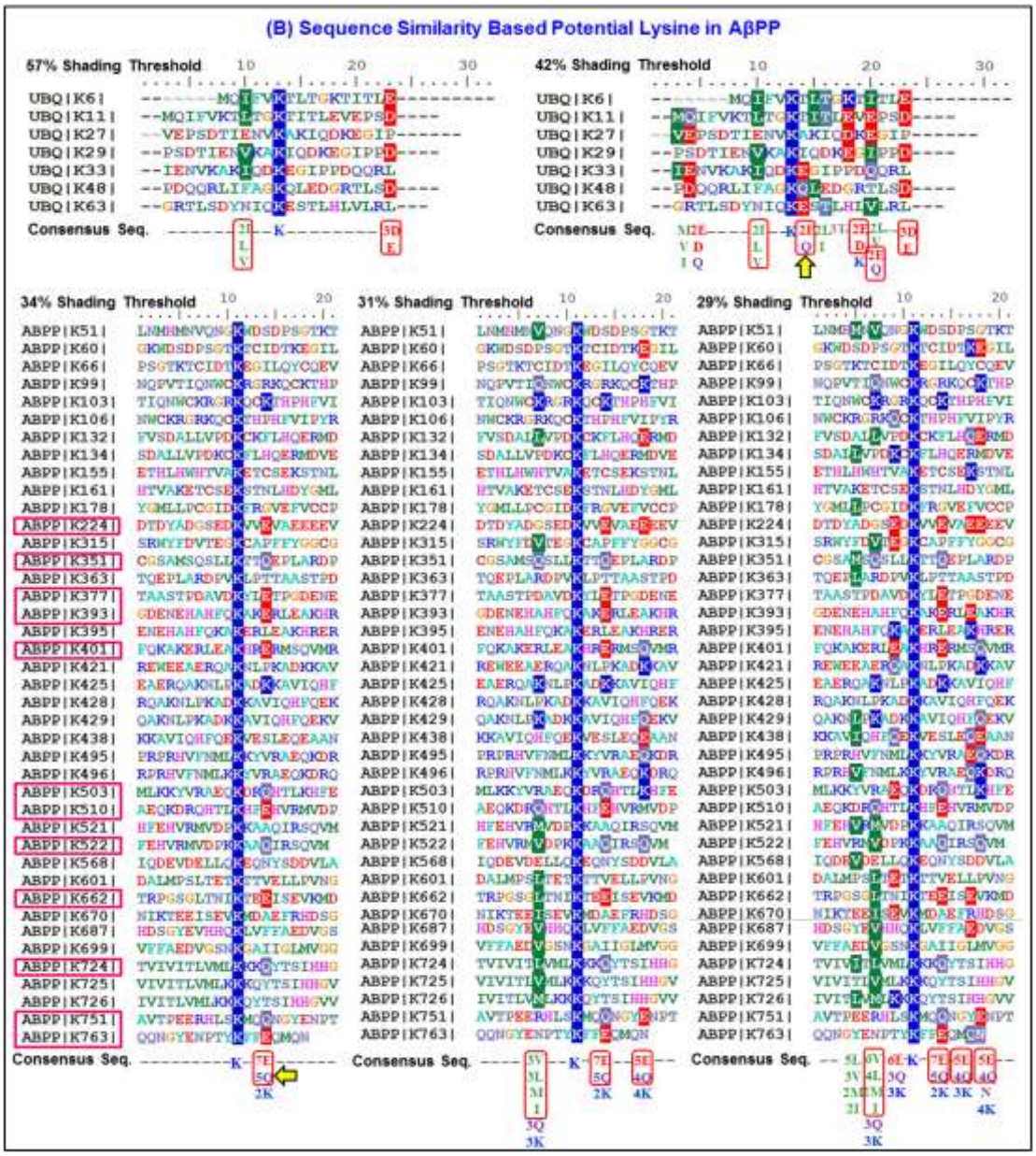


Figure 3.2B: Potential lysine prediction for ubiquitination in AβPP based on sequence similarity. The lysine neighboring amino acid conservation analysis between 21 window size polypeptides carrying central lysine has identified the conservation of Asp(D), Glu(E), Ile(I), Leu(L), Val(V) at 57% similarity threshold and Asp(D), Gln(Q), Glu(E), Ile(I), Leu(L),

Met(M), Thr(T), Val(V) at 42% similarity threshold in ubiquitin while the conservation of Gln(Q), Glu(E) at 34% similarity threshold; Gln(Q), Glu(E), Ile(I), Leu(L), Met(M), Val(V) at 31% similarity threshold and Asn(N), Gln(Q), Glu(E), Ile(I), Leu(L), Met(M), Val(V) at 29% similarity threshold in AβPP. The amino acid residues conserved at the common sites of both ubiquitin and AβPP is enclosed by red rounded rectangles signifying the critical amino acids for ubiquitination, including Glu (E), Gln (Q), Val (V), Leu (L), Met (M), Ile (I). Further, on the basis of best conserved amino acids, i.e. glutamic acid and glutamine common to both ubiquitin and AβPP (marked by yellow arrow), the potential lysine for ubiquitination is identified and encircled in pink rectangles, including K224, K351, K377, K393, K401, K503, K510, K522, K662, K724, K751 and K763.

3.3.2.3 Ubiquitin-AβPP lysine site peptide docking based potential lysine in AβPP

The affinity of lysine specific ubiquitin-ubiquitin interaction in the poly-ubiquitination process is taken as the principal to investigate the potential lysine sites in AβPP important for ubiquitination. The flexible docking of ubiquitin with the best conformations of ubiquitin's seven lysine peptides K6, K11, K27, K29, K33, K48, and K63 resulted in the average root-mean square deviation (Avg. RMSD) of 2.92, 5.31, 4.92, 3.88, 4.49, 1.96, and 4.63 respectively. Further, the obtained docking results of ubiquitin with ubiquitin and AβPP lysine peptides are summarized in **Table 3.2.** Interestingly, the Avg. RMSD of K48 was minimal, i.e. 1.96 showing the best affinity for K48 linked poly-ubiquitination than the K11 site, which had highest Avg. RMSD of 5.31 implying the least propensity of K11 linked poly-ubiquitination. Here, the minimal Avg. RMSD, i.e. best binding affinity than the K11 (5.31) was taken as the threshold to find the best propensity ubiquitination sites in AβPP. The 21 potential sites were identified with good propensities for ubiquitination, including K51, K60, K99, K132, K161, K178, K351, K393, K401, K425, K495, K496, K503, K510, K521, K522, K568, K687, K724, K725, and K726 **(Figure 3.3A).** Among them, the lowest Avg. RMSD of 0.906538 was obtained for the K687 site in AβPP or corresponding K16 site in Aβ showing best potential for ubiquitination.

Table 3.2: Ubiquitin and AβPP lysine peptides docking scores with ubiquitin protein using CABS-dock

Ubiquitin						
S.No.	**Ubiquitin Lysine Site**	**21 residues key lysine sequence**	**Cluster Density**	**Average RMSD**	**Max RMSD**	**No of elements**
1	K6	MQIFV**K**TLTG**K**TITLE	26.338	2.92353	19.4007	77
2	K11	MQIFV**K**TLTG**K**TITLEVEPSD	23.1489	5.31343	23.9976	123
3	K27	VEPSDTIENV**K**A**K**IQD**K**EGIP	27.6251	4.92306	25.651	136
4	K29	PSDTIENV**K**A**K**IQD**K**EGIPPD	38.8683	3.88491	23.829	151
5	K33	IENV**K**A**K**IQD**K**EGIPPDQQRL	27.5913	4.49417	17.6504	124
6	K48	PDQQRLIFAG**K**QLEDGRTLSD	24.0229	1.95647	7.09756	47
7	K63	GRTLSDYNIQ**K**ESTLHLVLRL	25.5963	4.64911	26.4507	119

AβPP						
S.No.	**AβPP Lysine Site**	**21 residues key lysine sequence**	**Cluster Density**	**Average RMSD**	**Max RMSD**	**No of elements**
1	K51	LNMHMNVQNG**K**WDSDPSGT**K**T	21.5806	5.09718	27.0102	110
2	K60	G**K**WDSDPSGT**K**TCIDT**K**EGIL	26.0199	4.3044	24.3425	112
3	K66	PSGT**K**TCIDT**K**EGILQYCQEV	19.0914	9.00931	24.3529	172
4	K99	NQPVTIQNWC**K**RGR**K**QC**K**THP	41.1493	3.57236	14.4164	147
5	K103	TIQNWC**K**RGR**K**QC**K**THPHFVI	24.9614	6.08941	22.8975	152
6	K106	NWC**K**RGR**K**QC**K**THPHFVIPYR	19.9487	9.17352	24.6746	183
7	K132	FVSDALLVPD**K**C**K**FLHQERMD	34.6382	3.0602	18.6577	106
8	K134	SDALLVPD**K**C**K**FLHQERMDVE	17.7294	7.38886	30.4831	131
9	K155	ETHLHWHTVA**K**ETCSE**K**STNL	18.8584	9.22667	27.4895	174
10	K161	HTVA**K**ETCSE**K**STNLHDYGML	25.5239	3.44775	14.9316	88
11	K178	YGMLLPCGID**K**FRGVEFVCCP	31.9959	4.53183	19.5177	145
12	K224	DTDYADGSED**K**VVEVAEEEEV	21.3651	7.58246	19.827	162
13	K315	SRWYFDVTEG**K**CAPFFYGGCG	28.418	5.38391	18.2749	153
14	K351	CGSAMSQSLL**K**TTQEPLARDP	26.6421	4.42907	21.7612	118
15	K363	CGSAMSQSLL**K**TTQEPLARDP	22.2974	9.59752	30.1062	214
16	K377	TAASTPDAVD**K**YLETPGDENE	30.3162	5.50861	30.4716	167
17	K393	GDENEHAHFQ**K**A**K**ERLEA**K**HR	34.6566	4.09735	12.2452	142
18	K395	ENEHAHFQ**K**A**K**ERLEA**K**HRER	26.0746	6.36634	25.9803	166
19	K401	FQ**K**A**K**ERLEA**K**HRERMSQVMR	23.396	4.2315	18.9197	99
20	K421	REWEEAERQA**K**NLP**K**AD**KK**AV	21.1402	10.4067	31.0374	220
21	K425	EAERQA**K**NLP**K**AD**KK**AVIQHF	32.8454	3.83616	27.7128	126
22	K428	RQA**K**NLP**K**AD**K**AVIQHFQE**K**	24.0608	5.94329	22.5957	143
23	K429	QA**K**NLP**K**AD**KK**AVIQHFQE**K**V	24.0077	6.70617	28.9284	161
24	K438	**KK**AVIQHFQE**K**VESLEQEAAN	37.1462	6.38019	28.0285	237
25	K495	PRPRHVFNML**KK**YVRAEQ**K**DR	22.7752	4.1712	14.4324	95

AβPP						
S.No.	**AβPP Lysine Site**	**21 residues key lysine sequence**	**Cluster Density**	**Average RMSD**	**Max RMSD**	**No of elements**
26	K496	RPRHVFNMLKKYVRAEQKDRQ	39.035	4.73934	17.5878	185
27	K503	MLKKYVRAEQKDRQHTLKHFE	26.2182	4.61512	15.6449	121
28	K510	AEQKDRQHTLKHFEHVRMVDP	26.9482	5.19515	20.7974	140
29	K521	HFEHVRMVDPKKAAQIRSQVM	20.0593	5.23448	19.223	105
30	K522	FEHVRMVDPKKAAQIRSQVM	29.0115	4.10182	18.1209	119
31	K568	IQDEVDELLQKEQNYSDDVLA	36.6961	2.9431	28.6506	108
32	K601	DALMPSLTETKTTVELLPVNG	19.4168	11.5879	26.6436	225
33	K662	TRPGSGLTNIKTEEISEVKMD	22.9797	8.39872	29.6782	193
34	K670	NIKTEEISEVKMDAEFRHDSG	27.64	8.53834	32.0268	236
35	K687	HDSGYEVHHQKLVFFAEDVGS	40.8146	0.906538	1.81447	37
36	K699	VFFAEDVGSNKGAIIGLMVGG	19.2171	9.62683	22.5839	185
37	K724	TVIVITLVMLKKKQYTSIHHG	20.3169	5.1189	28.5382	104
38	K725	VIVITLVMLKKKQYTSIHHGV	29.4156	2.00574	19.6385	59
39	K726	IVITLVMLKKKQYTSIHHGVV	31.7439	4.75682	19.5129	151
40	K751	AVTPEERHLSKMQQNGYENPT	21.713	11.0533	30.6823	240
41	K763	QQNGYENPTYKFFEQMQN	23.4377	5.54662	28.2897	130

3.3.2.4 Machine learning ubiquitination tools based potential lysine in AβPP

The different machine learning approaches, including maximal dependence decomposition, Bayesian discriminant analysis, random forest models, and support vector machine classifiers based tools (UbiSite, BDM-PUB, CKSAAP, UbPred and UbiPred) have been employed to determine the potential lysine sites in AβPP for ubiquitination based on experimentally verified ubiquitination site datasets. The predicted ubiquitination sites and scores obtained by UbiSite, BDM-PUB, CKSAAP, UbPred and UbiPred tools are summarized in **Table 3.3.** Moreover, the stacked bar graph of the ubiquitination prediction scores vs. AβPP lysine sites are plotted to identify the potential lysine in AβPP as shown in **Figure 3.3B.** The best ubiquitination aggregate scores were obtained for K351 and K377, i.e. 3.19 and 2.47 respectively with a maximal prediction by four tools- UbiSite, BDM-PUB, UbPred and UbiPred.

Table 3.3: Ubiquitination prediction scores of Ubiquitination-site prediction tools

AβPP Key Lysine Site	Ubiquitination Site Prediction Tools					Aggregate Scores
	UbiSite	BDM-PUB	CKSAAP	UbPred	UbiPred	
K51	_		_	0.71	_	0.71
K60	_	0.9	_	0.72	_	1.62
K103	_	2.15	_	_	0.54	2.69
K134	_	0.48	_	_	_	0.48
K155	_	_	_	0.65	_	0.65
K161	_	1.56	_	0.7	_	2.26
K224	_	0.62	_	0.97	0.56	2.15
K351	0.510765	0.98	_	0.79	0.91	3.190765
K363	0.544687	0.62	_	0.64	0.67	2.474687
K377	_	0.59	_	0.94	0.69	2.22
K393	_	0.48	_	0.86	_	1.34
K395	_	_	_	0.88	_	0.88
K401	_	0.98	_	_	0.6	1.58
K421	_	1.47	_	_	_	1.47
K425	_	1.74	_	_	_	1.74
K428	_	1.41	_	_	0.57	1.98
K429	_	1.79	_	_	0.61	2.4
K438	_	_	_	0.89	_	0.89
K495	_	_	_	_	0.61	0.61
K496	_	_	0.751	_	0.57	1.321
K503	_	_	_	_	0.71	0.71
K510	_	_	0.7805	_	0.69	1.4705
K521	_	_	_	_	0.72	0.72
K522	_	0.34	_	_	0.66	1
K568	_	_	_	0.62	_	0.62
K601	_	1.16	_	0.81	0.71	2.68
K662	_	_	_	0.81	0.56	1.37
K670	_	_	_	_	0.55	0.55
K699	_	0.61	_	_	_	0.61
K724	_	0.53	_	_	_	0.53
K751	0.569523	_	_	0.74	0.59	1.899523
K763	_	_	0.7883	0.7	0.54	2.0283

Further, these potential lysine residues were classified into different confidence levels- very high, high, medium, low and very low based on the evidence from ubiquitination prediction tools and their verification by other methods, including sequence identity, sequence similarity and flexible protein-peptide docking. For

instance, the potential ubiquitination sites predicted by at least three ubiquitination prediction tools and is verified by at least any two other methods are classified as "Very High" confidence, while those verified by any one of the other methods is classified as "High" confidence. In a similar way, the potential ubiquitination sites predicted by any two-ubiquitination prediction tools that are verified by at least any one of the other methods is categorized into "Medium" confidence. Additionally, the potential ubiquitination sites predicted by any one of the ubiquitination prediction tools, which is verified by at least, any two of the other methods are assigned as "Low" confidence whereas if verified by any one of the other methods, then classified as "Very Low" confidence. The key lysine residues important for the ubiquitination of AβPP with predicted confidence is listed in **Table 3.4.** Moreover, the Venn diagrams comprehensively reviewed the predicted ubiquitination sites and illustrated the logical relations between the key ubiquitination sites in AβPP **(Figure 3.3C).** The Venn diagram analysis clearly depicted K351 as the most promising ubiquitination site at high confidence; K393, K401, K510, and K522 at medium confidence, and K503 and K724 at low confidence.

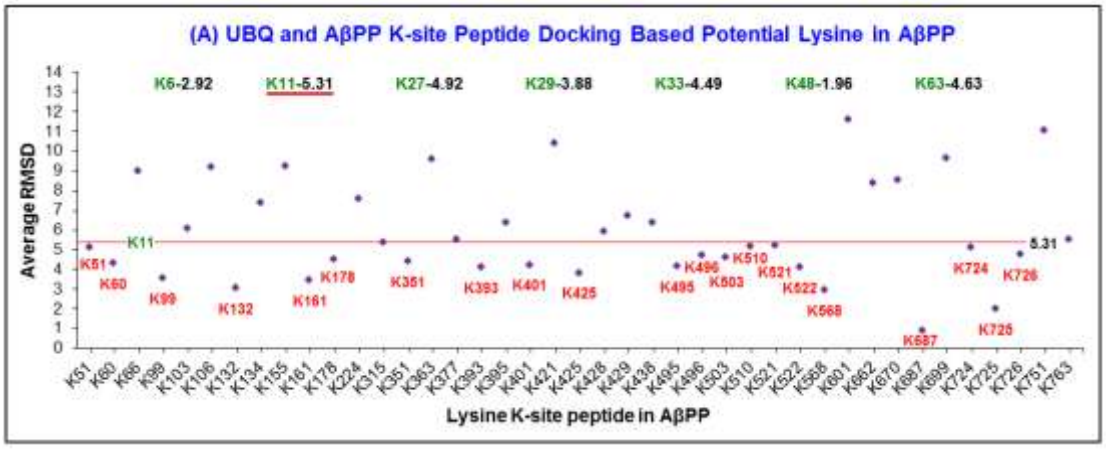


Figure 3.3A: Potential lysine prediction in AβPP based on Ubiquitin and K-AβPP site peptide docking. The average root-mean square deviation (Avg. RMSD) graph of the docked 21-window size K-site AβPP peptide with ubiquitin is dot plotted and is compared with the Avg. RMSD of the docked 21-window size K-site Ubiquitin peptide with the ubiquitin protein. The Avg. RMSD of K-11 ubiquitin, i.e. 5.31 is taken as the threshold to identify the potential ubiquitination sites in AβPP (K51, K60, K99, K132, K161, K178, K351, K393, K401, K425, K495, K496, K503, K510, K521, K522, K568, K687, K724, K725, and K726) with lower Avg. RMSD values.

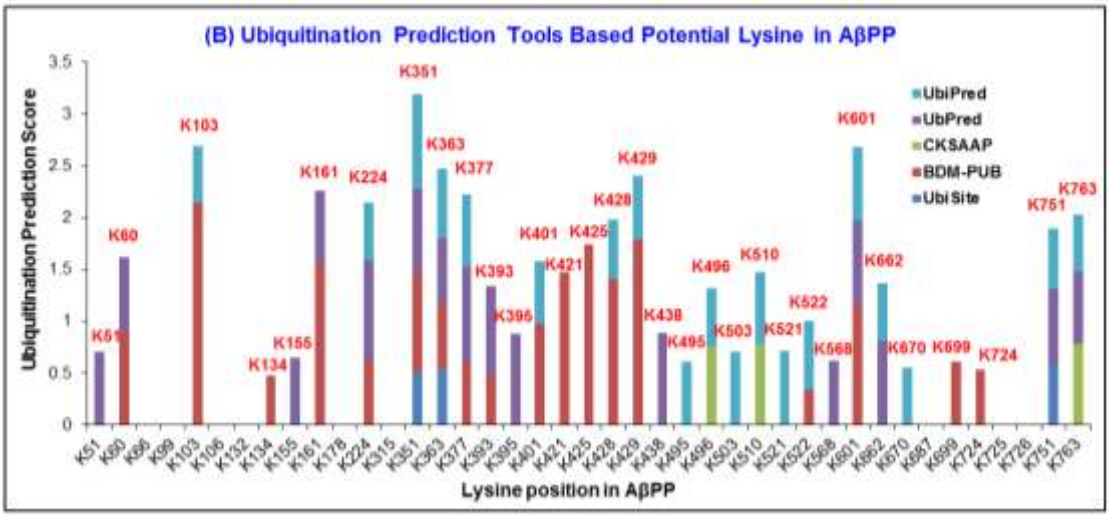


Figure 3.3B: Potential lysine prediction in AβPP based on the ubiquitination prediction tools. The machine learning based ubiquitination prediction tools-UbiPred (Light Blue), UbPred (Purple), CKSAAP (Green), BDM-PUB (Dark Red), and UbiSite (Blue) has predicted potential ubiquitination sites in AβPP including K51, K60, K103, K134, K155, K161, K224, K351, K363, K377, K393, K395, K401, K421, K425, K428, K429, K438, K495, K496, K503, K510, K521, K522, K568, K601, K662, K670, K699, K724, K751, and K763.

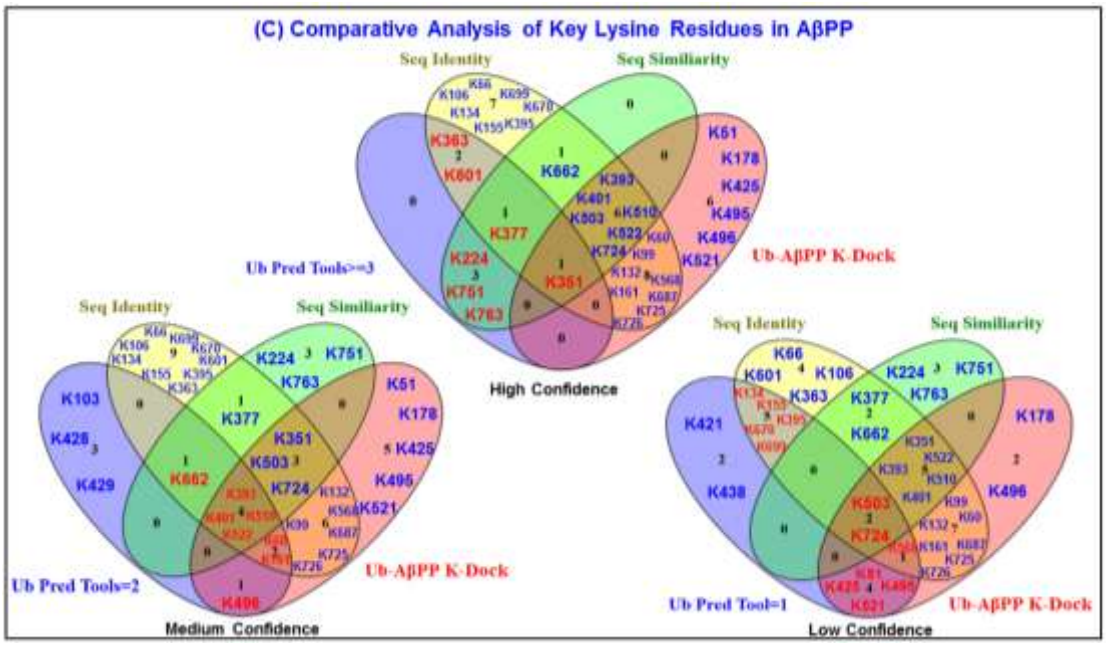


Figure 3.3C: Comparative analysis of the predicted ubiquitination sites. The comparison of the potential ubiquitination sites predicted by all the methods has revealed the most

potential ubiquitination sites which are predicted by at least three ubiquitination prediction tools and verified by at least, any two other methods are K351 and K377 (marked Red) with very high confidence, while verified by any one of the other methods is K224, K363, K601, K751, and K763 (marked Red) with high confidence. Similarly, the potential ubiquitination sites predicted by any two ubiquitination prediction tools and verified by at least any one of the other methods is K60, K161, K393, K401, K496, K510, K522, and K662 (marked Red) with medium confidence. Likewise, the potential ubiquitination sites predicted by any one of the ubiquitination prediction tools and is verified by at least any two of the other methods are K503, K568, and K724 (marked Red) with low confidence, while verified by any one of the other methods is K51, K134, K155, K395, K425, K495, K521, K670, and K699 (marked Red) with very low confidence.

Table 3.4: Predicted confidence of the key ubiquitination sites in AβPP

Key Lysine Sites	Potential lysine (K) in AβPP for ubiquitination based upon						Predicted Confidence
	Ubiquitination prediction tools			Amino Acid Sequence Identity Matrix	Conserved Similar Amino Acid Residues	Ubiquitin and AβPP K-site peptide docking	
	1 Tool	2 Tools	≥ 3 Tools	Sites >0.2 Sequence Identity Score	@34% Similarity Threshold	Sites < 5.31 Average RMSD Scores	
K51							Very Low
K60							Medium
K134							Very Low
K155							Very Low
K161							Medium
K224							High
K351							Very High
K363							High
K377							Very High
K393							Medium
K395							Very Low
K401							Medium
K425							Very Low

Key Lysine Sites	Potential lysine (K) in AβPP for ubiquitination based upon						Predicted Confidence
	Ubiquitination prediction tools			Amino Acid Sequence Identity Matrix	Conserved Similar Amino Acid Residues	Ubiquitin and AβPP K-site peptide docking	
	1 Tool	2 Tools	≥ 3 Tools	Sites >0.2 Sequence Identity Score	@34% Similarity Threshold	Sites < 5.31 Average RMSD Scores	
K495							**Very Low**
K496							**Medium**
K503							**Low**
K510							**Medium**
K521							**Very Low**
K522							**Medium**
K568							**Low**
K601							**High**
K662							**Medium**
K670							**Very Low**
K699							**Very Low**
K724							**Low**
K751							**High**
K763							**High**

3.3.3 Lysine residues are crucial for AβPP stability, ubiquitination, and other functions

The mutational studies on AβPP revealed the importance of lysine residues in AβPP processing and ubiquitination. When we mutated the key lysine (Polar; positively charged; basic amino acid; pK=10.5) with other polar; positively charged; basic amino acids- Arginine (pK=12.5), Histidine (pK=6.0) and polar; negatively charged; acidic amino acids- Aspartate (pK=3.9) and Glutamate (pK=4.2), we observed some interesting effects on the internal potential energy of mutated AβPP summarized in **Table 3.5.** We found that only arginine had imparted site specific stability to even more than half of the predicted ubiquitination sites, including K51, K60, K134, K161, K224, K393, K401, K425, K496, K510, K521, K522, K699, K724, and instability at the rest site of AβPP **(Figure 3.4A).** Moreover, the lysine residues at high confidence ubiquitination sites were found intolerant of all mutations against the stability of AβPP. Apart from arginine, histidine was observed to greatly affect the stability of AβPP followed by glutamate and aspartate. However, the effect of glutamate on AβPP stability was less than the arginine and histidine, but it affected nearly all the ubiquitination sites **(Figure 3.4A).** Further, the detailed internal potential energies obtained for the modeled and mutated AβPP; and the mutational impact of lysine mutations on their total potential energies can be inferred from **Supplementary Tables S3.3** and **S3.4** respectively.

Table 3.5: Effect of lysine mutation on total potential energy of AβPP

Confidence Level	Mutational Site	ΔE Total (KJ/mol) ($E_{Mutation}$-$E_{No\ Mutation}$)			
		Mutation Type			
		Lys(K)-Arg(R)	Lys(K)-His(H)	Lys(K)-Asp(D)	Lys(K)-Glu(E)
Very High	K351	5876.014	222.42	4.633	61.496
	K377	1194.364	62.217	15.471	14.557
High	K224	-238.418	35.037	17.395	20.996
	K363	1026.26	9837.761	33.565	13848.825
	K601	2083620.973	16503.902	11.123	8.77
	K751	2473.202	165.922	-0.629	38950.161
	K763	4890663.473	6062.487	6.621	156472.582

Confidence Level	Mutational Site	ΔE Total (KJ/mol) ($E_{Mutation}$-$E_{No\ Mutation}$)			
		Mutation Type			
		Lys(K)-Arg(R)	Lys(K)-His(H)	Lys(K)-Asp(D)	Lys(K)-Glu(E)
Medium	K60	-236.179	106.526	48.961	34.491
	K161	-184.562	7.19899E+11	27.44	12.717
	K393	-234.58	74.135	23.825	20.399
	K401	-194.841	86.977	52.08	62.194
	K496	-239.636	59.368	20.416	12.114
	K510	-240.533	616957.348	21.922	17.953
	K522	-231.222	90.121	34.071	29.496
	K662	765427662.5	68410	226.784	601.836
Low	K503	487.243	70.155	13.244	173.287
	K568	1.21228E+11	51.993	23.744	23.338
	K724	-245.779	23655.275	35.864	19.457
Very Low	K51	-228.095	0	14.649	26.098
	K134	-131.127	17.27	7.151	9.86
	K155	1569.943	31.192	10.395	143.977
	K395	17638.653	237.323	36.303	11.287
	K425	-231.168	64.469	24.192	29.85
	K495	331718094.5	153.008	309.342	7639277.973
	K521	-232.041	49.873	27.586	24.532
	K670	2799.446	139.305	39005702.47	1061.897
	K699	-235.707	4256364558	23.08	24.27

Further, the disease susceptibility of lysine mutations was checked through the mutational analysis tools-PANTHER, SNAP2, Polyphen2, PMut, Phd-SNP and SIFT. It revealed that mutations had an effect on all the sites, but they had a very least effect on the high confidence ubiquitination sites in comparison with the medium and low confidence sites. The highly intolerant mutations that were most susceptible to the diseases are shown in **Figure 3.4B.** In addition, the detailed results for the mutation associated disease susceptibility predicted by PANTHER, SNAP2, Polyphen2, PMut, PhD-SNP, and SIFT can be accessed through **Supplementary Table S3.5.** Further, we analyzed the physico-chemical and amino acid compositional analysis of ubiquitin, Aβ42 peptides and AβPP to understand the crucial factors that determine the ubiquitination of a protein. Interestingly we found that total number of positively and negatively charged residues were in same proportion in ubiquitin along with the aliphatic index- 100 and GRAVY- -0.489 that could be the decisive factor for ubiquitin to be the top ubiquitination protein. Moreover, some amino acid

compositions including arginine, asparagine, aspartate, glutamate, phenylalanine, proline, and serine were found in the comparable range ± 1% in ubiquitin, Aβ42 peptides and AβPP **(Figure 3.4C)**. Further, lysine sites in AβPP was investigated for their role in the other cellular processes and functions by MutPred, which has been outlined in **Table 3.6.** The collective results depicted that apart from ubiquitination; lysine residues are also crucial for protein stability, modifications and other functions.

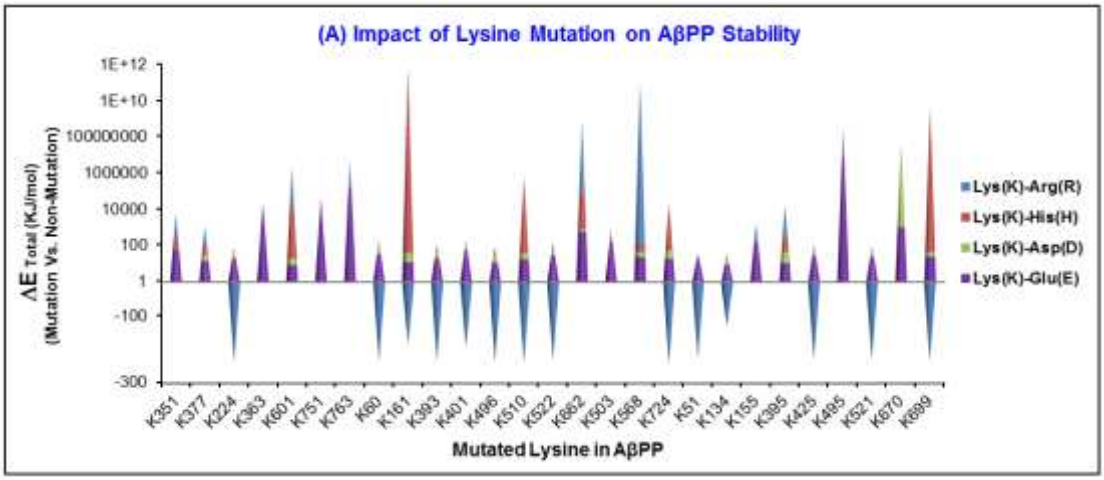
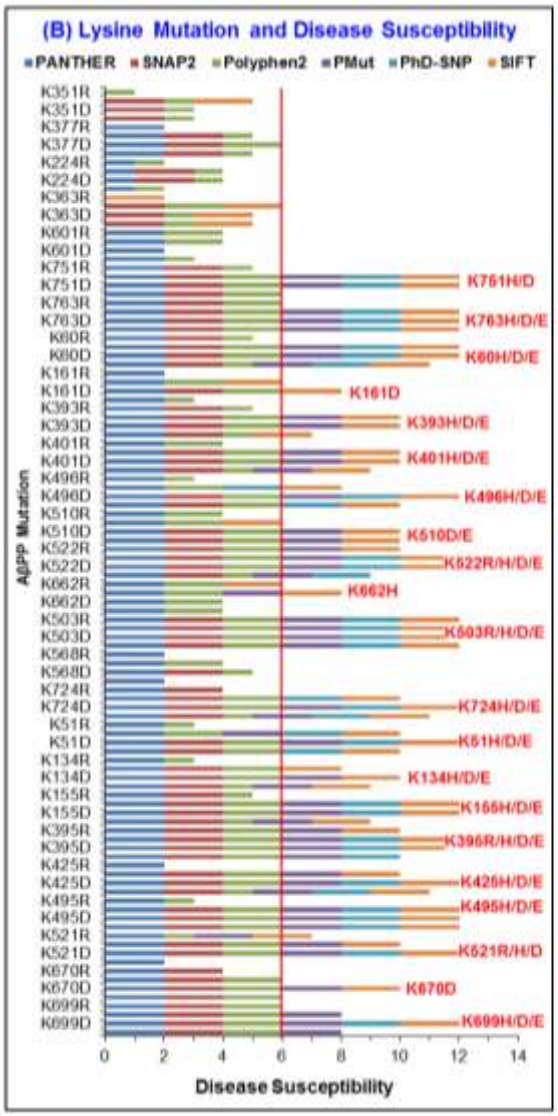


Figure 3.4A: Mutational analysis of lysine residues depicting the impact of lysine mutation on AβPP stability. The total energy change in AβPP upon Lysine mutations has identified the prominent effect of Lysine-Arginine and Lysine-Histidine mutations as the most detrimental to AβPP stability in comparison with Glutamic and Aspartic acid. Here, nearly 14 Lysine-Arginine mutations, including K51, K60, K134, K161, K224, K393, K401, K425, K496, K510, K521, K522, K699, and K724 are reported to increase the stability of AβPP by reducing their total energy in KJ/mol.



(C) Physico-chemical Properties and Amino acid Composition

Physico-chemical Properties	Ubiquitin	Aβ42	AβPP
Number of amino acids	76	42	770
Molecular weight (KDa)	8.56484	4.5141	86.94325
Theoretical pI	6.56	5.31	4.73
Total number of negatively charged residues (Asp + Glu)	11	6	142
Total number of positively charged residues (Arg + Lys)	11	3	78
Instability index	36.06 (Stable)	18.17 (Stable)	40.69 (Unstable)
Aliphatic index	100	97.38	73.18
Grand average of hydropathicity (GRAVY)	-0.489	0.205	-0.584

Amino acid residues	Amino acid % Composition		
	Ubiquitin	Aβ42	AβPP
Ala (A)	2.60%	9.50%	8.20%
Arg (R)	5.30%	2.40%	4.80%
Asn (N)	2.60%	2.40%	4.00%
Asp (D)	6.60%	7.10%	6.50%
Cys (C)	0.00%	0.00%	2.30%
Gln (Q)	7.90%	2.40%	4.70%
Glu (E)	7.90%	7.10%	11.90%
Gly (G)	7.90%	14.30%	4.90%
His (H)	1.30%	7.10%	3.20%
Ile (I)	9.20%	7.10%	3.10%
Leu (L)	11.80%	4.80%	7.30%
Lys (K)	9.20%	4.80%	5.30%
Met (M)	1.30%	2.40%	3.10%
Phe (F)	2.60%	7.10%	2.70%
Pro (P)	3.90%	0.00%	4.50%
Ser (S)	3.90%	4.80%	4.50%
Thr (T)	9.20%	0.00%	6.50%
Trp (W)	0.00%	0.00%	1.20%
Tyr (Y)	1.30%	2.40%	2.60%
Val (V)	5.30%	14.30%	8.40%

Figure 3.4: Mutational analysis of lysine residues for AβPP ubiquitination- (B) Lysine mutation and disease susceptibility. The investigation by various mutation analysis tools, including PANTHER, SNAP2, Polyphen2, Pmut, PhD-SNP, and SIFT have identified the key lysine sites where mutations are susceptible to disease pathogenesis and are marked with red color taking the threshold of predictions by more than three tools for best accuracy. The highly disease susceptible mutations include K51H/D/E, K60H/D/E, K134H/D/E, K155H/D/E, K161D, K393H/D/E, K395R/H/D/E, K401H/D/E, K425H/D/E, K495H/D/E, K496H/D/E, K503R/H/D/E, K510D/E, K521R/H/D, K522R/H/D/E, K662H, K670D, K699H/D/E, K724H/D/E, K751H/D, K763H/D/E; **(C) Physico-chemical properties and Amino acid composition.** The physico-chemical properties of Ubiquitin showed equal no of positive and negative charged residues, with low hydrophobicity and high stability, while Aβ42 showed high hydrophobicity and stability in comparison to AβPP low hydrophobicity and instability. Amino acid compositional analysis revealed the comparable percentage within 1% range of Ubiquitin with Aβ42 and AβPP including Arginine, Asparagine, Aspartic acid, Glutamic acid, Phenylalanine, Proline, and Serine.

Table 3.6: Importance of lysine residues in AβPP ubiquitination, processing and other functions

AβPP Mutational Site (Ubiquitination Confidence)		**Affected molecular mechanisms (P-values ≤ 0.05)**		**Affected Motifs**		**Pathogenic Score**
K351 (Very High)	**Lys(K)-His(H)**	Loss of O-linked glycosylation (T353)	Loss of Proteolytic cleavage (Q347)	GSK3, CK2, PIKK Phosphorylation site and FHA Phosphopeptide ligand		0.647
	Lys(K)-Asp(D)		Gain of Proteolytic cleavage (Q347)			0.658
	Lys(K)-Glu(E)					0.629
K377 (Very High)	**Lys(K)-Asp(D)**	Gain of Phosphorylation and Sulfation (Y378); Loss of Acetylation, SUMOylation, and Ubiquitylation (K377); Gain of Proteolytic cleavage (D376); Altered Coiled coil		–		0.517
K224 (High)	**Lys(K)-His(H)**	Loss of SUMOylation and Ubiquitylation (K224); Loss of Proteolytic cleavage (D219); Altered Coiled coil and Trans membrane protein		–		0.7
	Lys(K)-Asp(D)			CK2 Phosphorylation site, SUMO Interaction site		0.755
	Lys(K)-Glu(E)					0.722
K601 (High)	**Lys(K)-His(H)**	Loss of SUMOylation (K601); Altered Trans membrane protein		NEK2 Phosphorylation site and FHA Phosphopeptide ligand	–	0.611
	Lys(K)-Asp(D)				SUMO Interaction site	0.601
	Lys(K)-Glu(E)					0.567
K751 (High)	**Lys(K)-His(H)**	Loss of Allosteric site (R747); Altered Metal binding; Altered Trans membrane protein	Altered Ordered and Disordered interface	–		0.737
	Lys(K)-Asp(D)		–			0.713
	Lys(K)-Glu(E)					0.705
K763 (High)	**Lys(K)-Arg(R)**	Loss of N-terminal acetylation (M768); Altered Disordered interface		Tyrosine based sorting signal, Phosphotyrosine binding ligand, PKC Phosphorylation site, Amyloidogenic glycoprotein intracellular domain		0.601
	Lys(K)-His(H)	Loss of Methylation (K763); Gain of N-terminal acetylation (M768)	Altered Metal binding; Altered Ordered and Disordered interface			0.829
	Lys(K)-Asp(D)					0.839
	Lys(K)-Glu(E)		Gain of Sulfation (Y762)			0.832
K60 (Medium)	**Lys(K)-Arg(R)**	Loss of Relative solvent accessibility; Altered Trans membrane protein; Loss of Disulfide linkage (C62)	Gain of ADP-ribosylation (K60);	GSK3, CK1 Phosphorylation site, FHA Phosphopeptide ligand, N-myristoylation site	–	0.509
	Lys(K)-His(H)		–			0.812
	Lys(K)-Asp(D)		Altered Metal binding; Loss of Loop		CK2 Phosphorylation site	0.797
	Lys(K)-Glu(E)	Loss of Relative solvent accessibility; Altered Trans membrane protein; Loss of Loop				0.79

AβPP Mutational Site (Ubiquitination Confidence)		**Affected molecular mechanisms (P-values ≤ 0.05)**		**Affected Motifs**		**Pathogenic Score**
K161 (Medium)	**Lys(K)-His(H)**	Gain of Loop; Loss of Disulfide linkage (C158); Altered Trans membrane protein	Altered Metal binding; Loss of Relative solvent accessibility	GSK3, CK1, PKC Phosphorylation site, FHA Phosphopeptide ligand		0.619
	Lys(K)-Asp(D)		–			0.635
	Lys(K)-Glu(E)					0.629
K393 (Medium)	**Lys(K)-His(H)**	Altered Coiled coil; Loss of Acetylation (K393); Loss of Helix	–	–		0.783
	Lys(K)-Asp(D)					0.782
	Lys(K)-Glu(E)		Gain of SUMOylation (K395)			0.737
K401 (Medium)	**Lys(K)-Arg(R)**	Loss of SUMOylation and Acetylation (K401); Altered Disordered interface	–	–		0.525
	Lys(K)-His(H)		Loss of Helix			0.798
	Lys(K)-Asp(D)					0.774
	Lys(K)-Glu(E)					0.782
K496 (Medium)	**Lys(K)-His(H)**	Loss of Acetylation (K496); Altered Disordered interface		–		0.613
	Lys(K)-Asp(D)					0.544
	Lys(K)-Glu(E)					0.516
K510 (Medium)	**Lys(K)-Arg(R)**	Altered Metal binding; Altered DNA binding; Loss of Allosteric site (R505); Loss of Acetylation (K510); Altered Disordered interface	–	PKC Phosphorylation site		0.524
	Lys(K)-His(H)		Loss of Helix			0.814
	Lys(K)-Asp(D)					0.801
	Lys(K)-Glu(E)		–			0.806
K522 (Medium)	**Lys(K)-His(H)**	Loss of Helix; Altered Disordered interface; Altered Coiled coil		–		0.773
	Lys(K)-Asp(D)					0.745
	Lys(K)-Glu(E)					0.754
K662 (Medium)	**Lys(K)-Arg(R)**	Gain of ADP-ribosylation (K662)	Loss of SUMOylation, Acetylation, Ubiquitylation (K662); Altered Trans membrane protein	GSK3, CK1 Phosphorylation site, SUMOylation site, FHA Phosphopeptide ligand	–	0.541
	Lys(K)-His(H)	Loss of Phosphorylation (S667)				0.808
	Lys(K)-Asp(D)	Gain of Phosphorylation (S667)			CK2 Phosphorylation site	0.813
	Lys(K)-Glu(E)	Loss of Phosphorylation (S667)				0.803

AβPP Mutational Site (Ubiquitination Confidence)		**Affected molecular mechanisms (P-values ≤ 0.05)**		**Affected Motifs**	**Pathogenic Score**
K503 (Low)	**Lys(K)-Arg(R)**	Altered Disordered interface; Loss of Acetylation (K503); Altered DNA binding; Loss of Allosteric site (R505)	_	PKB Phosphorylation site	0.571
	Lys(K)-His(H)		Altered Metal binding; Loss of Helix	LATS Kinase Phosphorylation site	0.805
	Lys(K)-Asp(D)			_	0.815
	Lys(K)-Glu(E)		Loss of Helix	TRAF2 Binding site	0.786
K568 (Low)	**Lys(K)-His(H)**	Altered Coiled coil; Loss of Helix; Loss of Phosphorylation (Y572); Altered Trans membrane protein; Loss of N-linked glycosylation (N571)	Loss of Sulfation (Y572)	_	0.608
	Lys(K)-Asp(D)		Gain of Sulfation (Y572)		0.629
	Lys(K)-Glu(E)				0.621
K724 (Low)	**Lys(K)-His(H)**	Altered Trans membrane protein; Altered DNA binding; Altered Metal binding; Gain of Pyrrolidone carboxylic acid (Q727)	Altered Disordered interface	LATS Kinase Phosphorylation site	0.641
	Lys(K)-Asp(D)		–	Clathrin box, SUMO Interaction site	0.648
	Lys(K)-Glu(E)				0.638
K51 (Very Low)	**Lys(K)-Arg(R)**	Altered Trans membrane protein; Loss of Relative solvent accessibility; Loss of Ubiquitylation (K51)	_	Integrin Binding site	0.511
	Lys(K)-His(H)		Altered Ordered interface	_	0.773
	Lys(K)-Asp(D)		Altered Ordered interface; Altered Metal binding		0.778
	Lys(K)-Glu(E)		_		0.736
K134 (Very Low)	**Lys(K)-His(H)**	Altered Metal binding; Altered Trans membrane protein	Loss of Relative solvent accessibility	_	0.744
	Lys(K)-Asp(D)		_		0.769
	Lys(K)-Glu(E)		Gain of SUMOylation (K132)		0.726
K155 (Very Low)	**Lys(K)-His(H)**	Altered Metal binding; Altered Trans membrane protein	Loss of Helix; Loss of Disulfide linkage (C158)	CK2 Phosphorylation site, FHA Phosphopeptide ligand	0.815
	Lys(K)-Asp(D)		Gain of Disulfide linkage (C158)		0.801
	Lys(K)-Glu(E)		Loss of Disulfide linkage (C158)		0.811

AβPP Mutational Site (Ubiquitination Confidence)		**Affected molecular mechanisms (P-values ≤ 0.05)**		**Affected Motifs**	**Pathogenic Score**
K395 (Very Low)	**Lys(K)-His(H)**	Loss of Helix; Loss of Acetylation(K393); Altered Disordered interface; Loss of SUMOylation(K395)		–	0.751
	Lys(K)-Asp(D)	Altered Coiled coil; Loss of Helix; Loss of Acetylation and SUMOylation (K393); Altered Disordered interface			0.709
	Lys(K)-Glu(E)				0.73
K425 (Very Low)	**Lys(K)-His(H)**	Altered Coiled coil; Loss of Helix; Altered Disordered interface; Loss of Acetylation (K425); Gain of Ubiquitylation (K428)		–	0.506
	Lys(K)-Asp(D)				0.527
K495 (Very Low)	**Lys(K)-His(H)**	Altered Disordered interface; Loss of Acetylation (K495); Altered Coiled coil		–	0.779
	Lys(K)-Asp(D)	Altered Disordered interface; Gain of Acetylation (K496)	–		0.781
	Lys(K)-Glu(E)		Altered Coiled coil		0.731
K521 (Very Low)	**Lys(K)-His(H)**	Loss of Helix; Altered Coiled coil	–	–	0.729
	Lys(K)-Asp(D)		Altered Disordered interface		0.72
	Lys(K)-Glu(E)				0.731
K670 (Very Low)	**Lys(K)-His(H)**	Altered Trans membrane protein; Loss of Acetylation (K670)	Loss of Phosphorylation (S667)	–	0.655
	Lys(K)-Asp(D)		Gain of Phosphorylation (S667)	CK2 Phosphorylation site	0.642
	Lys(K)-Glu(E)				0.634
K699 (Very Low)	**Lys(K)-His(H)**	Altered Trans membrane protein; Loss of Ubiquitylation (K699);	Loss of GPI-anchor amidation (N698)	PKC Phosphorylation site, N-myristoylation site	0.621
	Lys(K)-Asp(D)		Gain of GPI-anchor amidation (N698); Altered Metal binding		0.661
	Lys(K)-Glu(E)		Gain of GPI-anchor amidation (N698)		0.641

GSK3-Glycogen synthase kinase-3; **CK1-**Casein kinase-1; **CK2-**Casein kinase-2; **PIKK-**Phosphatidylinositol 3-kinase-related kinase; **FHA-**Forkhead-associated domain; **SUMO-**Small Ubiquitin-like Modifier; **NEK2-**NIMA Related Kinase 2; **PKB-**Protein Kinase B; **PKC-**Protein Kinase C; **LATS-**Large tumor suppressor kinase 1; **TRAF2-**TNF receptor-associated factor 2

3.3.4 Plausible role of lysine in ubiquitin positioning and conjugation during AβPP ubiquitination

The non-covalent interactions of ubiquitin with different proteins such as human DNA repair proteins, insulin-degrading enzyme, SUMO protein, are well known for modulating their activity or correctly orienting ubiquitin for lysine specific conjugation (McKenna *et al.,* 2001; Saric *et al.,* 2003; Ouyang *et al.,* 2015). These interactions can also impart stability to the ubiquitin-protein conjugates in the ubiquitination process (Nick *et al.,* 2014). In this regard, we predicted the interaction among all the domains of AβPP and ubiquitin and examined them for the lysine mediated polar interactions among their interacting residues. The ubiquitin-AβPP interaction predictions reported that ubiquitin has nearly same interactive affinity towards all the domains of AβPP except Aβ peptide. The prediction of ubiquitin-AβPP interaction was highest on the basis of homologous protein interactional network (S_{Net}), then by the statistical domain-domain interactions (S_{Dom}) and sequence similarity based interacting protein pairs (S_{Seq}) shown in **Figure 3.5A.** Further, lysine investigation in non-covalent electrostatic, polar interactions revealed the presence of lysine interactions with N-terminal domain (K66, K99), Copper binding domain (K155), E2 domain (K447), Aβ peptide (K28), and C-terminal domain of AβPP (K687). Apart from AβPP, ubiquitin's lysine residues, including K6, K11, K33, K48, and K63 was also involved in the polar interactions **(Figure 3.5B).** The presence of lysine as interacting residues provided a clue for their role in ubiquitin positioning or ubiquitin-AβPP conjugation but further researches are required for clear understanding. These informative residues are the foundations for future avenues of AβPP ubiquitination mechanistic research.

(A) Prediction of AβPP – Ubiquitin Interaction

Ubiquitin (1UBQ) interactions with	S_{Seq}	S_{Dom}	S_{Net}	S_{All}
APP Full Length	0.3537	0.5146	0.6359	0.5577
N-Terminal APP (4JFN_A)	0.3613	0.5146	0.6359	0.5555
Cu-binding APP (2FKL_A)	0.3537	0.5146	0.6359	0.5577
Protease inhibitor APP (1AAP_A)	0.3613	0.5146	0.6359	0.5555
E2 Domain APP (3UMK_A)	0.3537	0.5146	0.6359	0.5577
Aβ-42 of APP (1IYT_A)	0	0	0	0
C-Terminal APP (2LP1_A)	0.3537	0.5146	0.6359	0.5577

(B) Important Lysine for AβPP – Ubiquitin Interactions

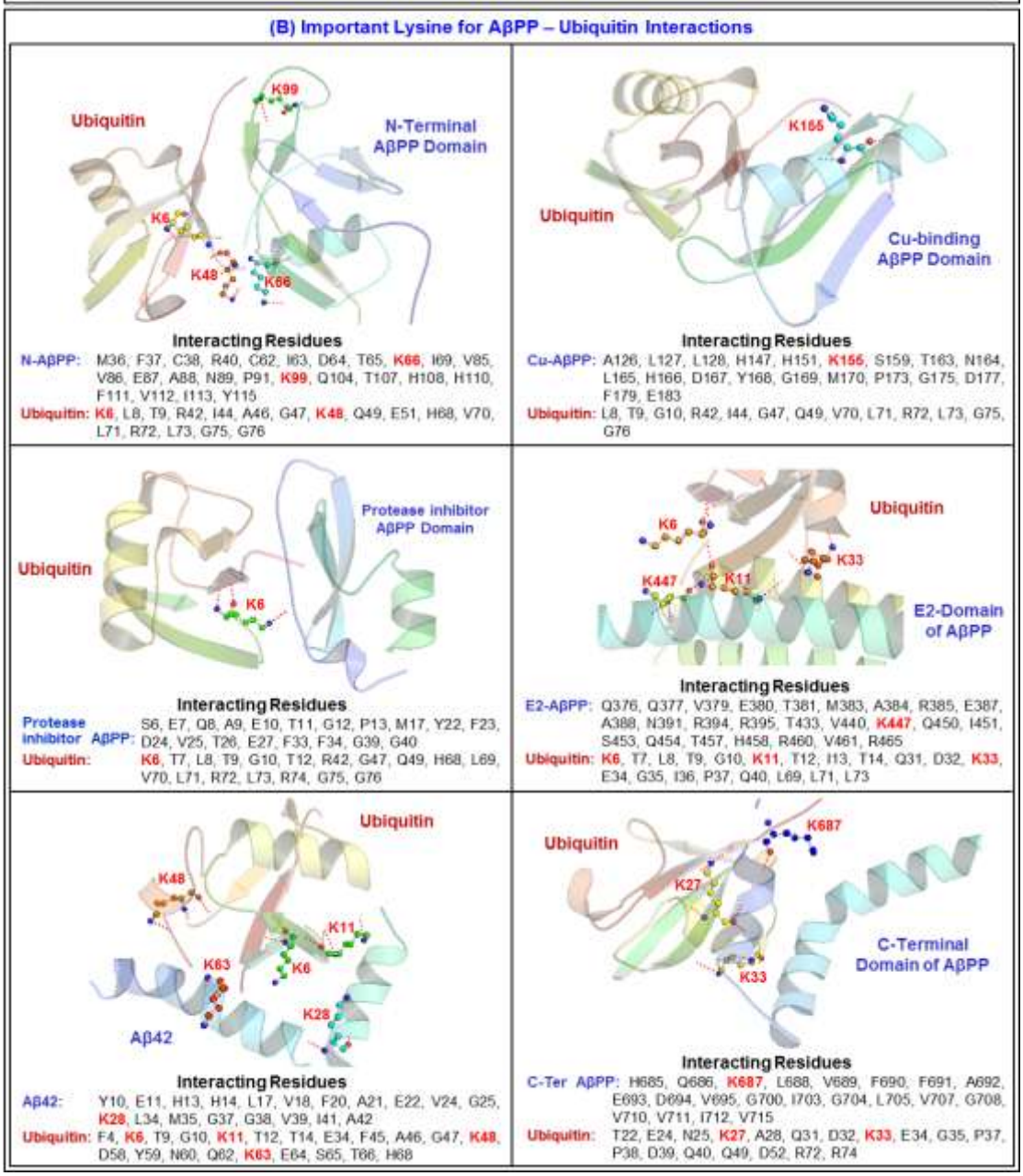


Figure 3.5: Lysine residues in Ubiquitin and Amyloid-beta precursor protein interactions- (A) Prediction of AβPP–Ubiquitin interactions: The strongest evidence for AβPP – Ubiquitin interactions were reported by S_{Net} i.e. sum of edge weights along the shortest path between homologous proteins in a protein-protein interaction network followed

by overall interactions (S_{All}), statistical propensities of domain-domain interactions (S_{Dom}), and sequence similarities to a known interacting protein pair (S_{Seq}); **(B) Important lysine for AβPP – Ubiquitin interactions:** The AβPP – Ubiquitin interactions revealed the role of diverse lysine residues in the interaction of different AβPP domains and Ubiquitin, including K6, K11, K33, K48 and K63 in ubiquitin and K66, K99, K155, K447, K687 (K16 in Aβ), and K699 (K28 in Aβ) in Amyloid beta precursor protein.

3.3.5 Interaction network of ubiquitination proteins for AβPP clearance

The accumulation of well-known pathogenic amyloid-beta can be regulated by the clearance of its precursor AβPP protein through ubiquitination. However, ubiquitination is a well-defined process, but the association of different ubiquitination E1s, E2s, E3s and deubiquitinating enzymes (DUBs) always remained an interesting field of research for target specific protein clearance. Here, we investigated the interaction network of E1s, E2s, E3s and DUBs enzymes for the clearance of AβPP in AD biology. The interaction network identified ubiquitin E3 ligases- PARK2 and STUB1 to be associated with AβPP ubiquitination while deubiquitination by the USP25 and UCHL1 **(Figure 3.6A).** The ubiquitination of other enzymes that govern the synthesis of Aβ peptides are also crucial to restrict them. Here, certain E3 ligases like FBXO2 is reported to regulate the ubiquitination of BACE1; TRIM13 to regulate PSENEN; CDH1 to regulate PSEN1 and NCSTN, while TRIM55 and SART1 to regulate the ubiquitination of NCSTN. Likewise, specific deubiquitinase enzymes, including USP25 and UCHL are found to regulate AβPP, USP8 to BACE1, and USP39 to NCSTN respectively **(Figure 3.6A).** Further, examinations on the potential E1 activating and E2 conjugating enzymes were done to identify the pre-processors of AβPP ubiquitin ligation by Park2 and STUB1 E3 ligases. We reported an array of ubiquitin E1 activating enzymes UBA1, UBA6, UBA7, and ATG7 that can possibly activate specific ubiquitin E2 conjugating enzymes, including UBE2A, UBE2B, UBE2G1, UBE2J1, UBE2K, UBE2L3, UBE2L6, UBE2N, UBE2Q1, UBE2S, UBE2U, and UBE2Z to conjugate lysine to Park2 **(Figure 3.6B)**, while additional UBE2I, UBE2T, and UBE2W enzymes to conjugate lysine to STUB1 **(Figure 3.6C).** The PPI network provided us the substantial information about the ubiquitination enzymes associated with the clearance of AβPP in the Alzheimer's disease biology that can be further explored for their therapeutic prospects.

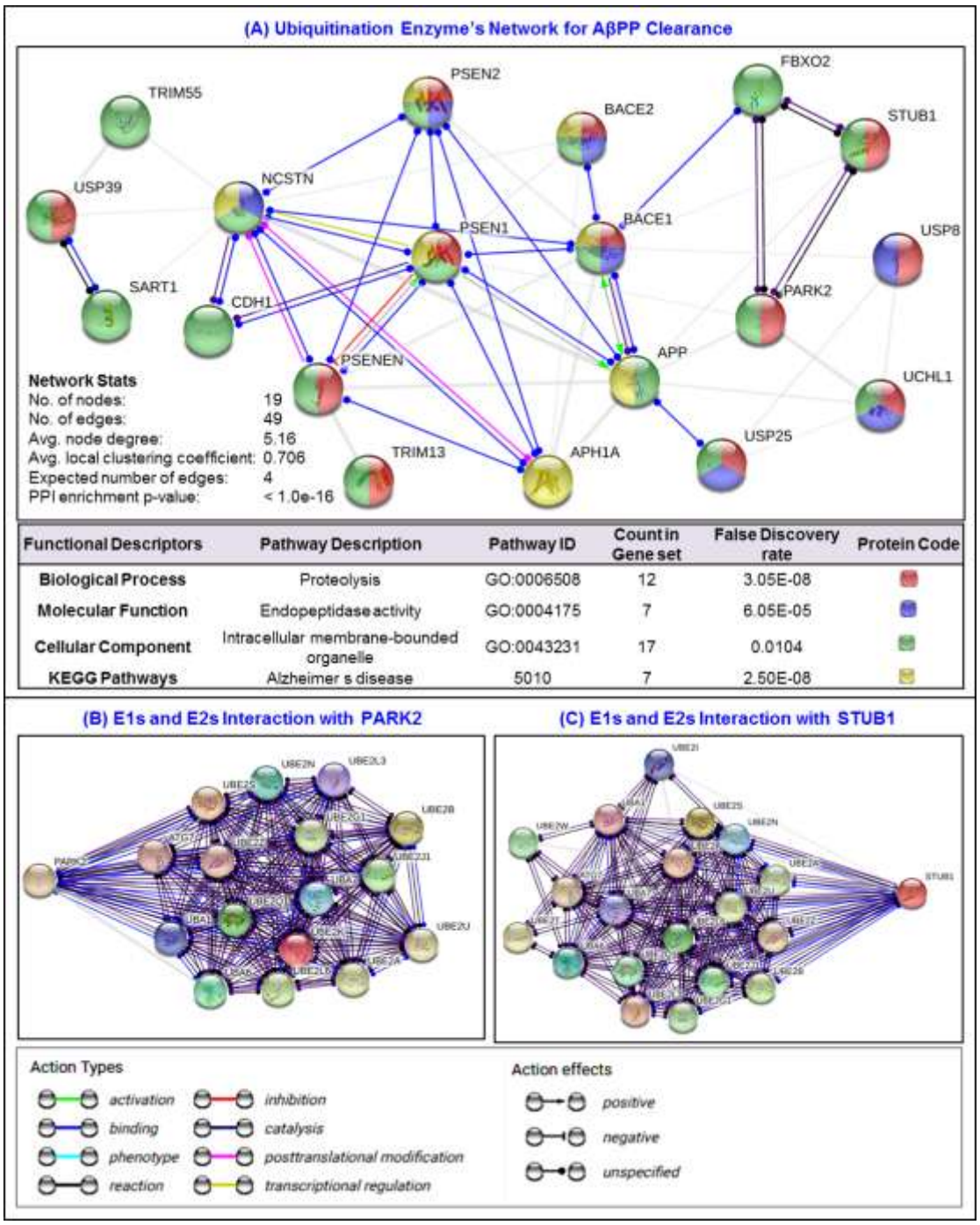


Figure 3.6: Protein-Protein interaction network- (A) Ubiquitination enzyme's network for AβPP: The interaction network of AβPP and amyloid cascade proteins (BACE1, BACE2, PSEN1, PSEN2, PSENEN, NCSTN, APH1A) with the ubiquitin E3 ligases are shown in molecular action view. Ubiquitin E3 ligases- Park2 and STUB1 along with deubiquitinases- USP25 and UCHL1 interact with AβPP to regulate its ubiquitination process. Other E3s, including FBXO2, CDH1, TRIM13, TRIM55, and SART1 along with deubiquitinases USP8, USP25, USP39, and UCHL1 regulate the ubiquitination of amyloid cascade enzymes β-secretase (BACE1) and γ-subunit complex (PSEN1, PSNEN, NCSTN). The network's whole genome statistical analysis identified the interacting proteins associated with the proteolysis

process, endopeptidase activity, intracellular membrane-bound organelle cellular component and Alzheimer's disease pathway; **(B) E1s and E2s interaction with PARK2:** The network identified different ubiquitin E2 conjugating enzymes- UBE2A, UBE2B, UBE2G1, UBE2J1, UBE2K, UBE2L3, UBE2L6, UBE2N, UBE2Q1, UBE2S, UBE2U, UBE2Z; and ubiquitin E1 activating enzymes- UBA1, UBA6, UBA7, ATG7 associated with the conjugation of lysine to Park2; **(C) E1s and E2s interaction with STUB1:** The network identified different ubiquitin E2 conjugating enzymes- UBE2A, UBE2B, UBE2G1, UBE2I, UBE2J1, UBE2K, UBE2L3, UBE2L6, UBE2N, UBE2Q1, UBE2S, UBE2T, UBE2U, UBE2W, UBE2Z; and ubiquitin E1 activating enzymes- UBA1, UBA6, UBA7, ATG7 associated with the conjugation of lysine to STUB1. **STUB1**-STIP1 homology and U-box containing protein 1; **PSENEN**-Presenilin enhancer 2 homolog; **CDH1**-Cadherin 1; **UCHL1**-Ubiquitin carboxyl-terminal esterase L1; **AβPP**-Amyloid beta (A4) precursor protein; **USP25**-Ubiquitin specific peptidase 25; **NCSTN**-Nicastrin; **TRIM13**-Tripartite motif containing 13; **USP8**-Ubiquitin specific peptidase 8; **SART1**-Squamous cell carcinoma antigen recognized by T cells; **USP39**-Ubiquitin specific peptidase 39; **BACE1**-Beta-site AβPP-cleaving enzyme 1; **TRIM55**-Tripartite motif containing 55; **PSEN1**-Presenilin 1; **BACE2**-Beta-site AβPP-cleaving enzyme 2; FBXO2-F-box protein 2; **PSEN2**-Presenilin 2; **PARK2**-Parkinson protein 2; **APH1A**-Anterior pharynx defective 1 homolog A.

3.4 DISCUSSION

The structural topology of the modeled AβPP has identified (i) an N-terminal signal peptide of 22AA residues- MLPGLALLLLAAWTARALEVPT and a trans-membrane segment S1 (699-721). Here, positively charged arginine (Arg16) and the N-terminal hydrophobic amino acids are crucial for AβPP's post-translational translocation across the ER membrane. Since, the removal of positively charged amino acid from signal peptide selectively impairs the translocation, while the presence of hydrophobic residues favors it through the lipid bilayer of ER membrane towards the secretory pathway (Guo *et al.,* 2018). (ii) The domain analysis revealed six domains that are contributing towards the functionality of AβPP. Among them four domains, including N-terminal, copper binding, protease inhibitor (Kunitz_BPTI) and E2 domain spans over the extracellular surface region and are responsible for copper binding, protease inhibition, platelet aggregation and hemostasis. However, the proteolytic product of cytoplasmic c-terminal domain acts as a transcriptional regulator in neurons. In addition, the cleavage of their trans-membrane domain is responsible for beta-amyloid peptide formation (Cappai 2014). (iii) The secondary structure analysis identified a higher alpha-helical content ~37% in AβPP indicating their potent ability to tolerate mutations, since helices can accrue more mutations than beta strands without structural distortion due to their higher numbers of inter-residue

contacts (Abrusan and Marsh 2016). (iv) Their tertiary structure revealed two disulfide linkages (cysteine144-cystein174 and cysteine158-cysteine186) that imparted stability to the AβPP. Further, the potential ubiquitination sites in AβPP has identified by adopting the sequence identity, sequence similarity and protein-peptide docking approach using lysine site features of ubiquitin, for being the eminent protein for poly-ubiquitination (Cai and Jiang 2016; Swatek and Komander 2016). The comparative analysis of thus predicted ubiquitination sites with the potential sites identified by the machine learning approaches revealed K351 as most promising ubiquitination sites at high confidence, K393, K401, K510, and K522 at medium confidence, and K503 and K724 at low confidence. Additionally, the site specific conserved amino acids- glutamic acid (E), glutamine (Q), valine (V), leucine (L), isoleucine (I), and methionine (M) was identified neighboring the lysine residue in AβPP. These conserved polar residues Glu(E) and Gln(Q) at the ubiquitination site would display their propensities for salt-bridges while conservation of hydrophobic residues Val(V), Leu(L), Ile(I), Met(M) indicate their propensities for buried contacts signifying their role in the ubiquitination reaction (Fornili *et al.*, 2013). Interestingly, we can comprehend the presence of polar negatively charged Glu(E) amino acids neighboring lysine for their electrostatic interactions among each other in their native folded state (Fallas *et al.*, 2012) while ubiquitination directs lysine's covalent attachment with c-terminal glycine of the ubiquitin molecules that opened the avenues for further validations.

Moreover, the mutational analysis of lysine's impact on AβPP stability revealed only arginine for imparting site-specific stability to the AβPP while rest mutations were detrimental for the structural conformation of AβPP. The stability associated with arginine mutations can be attributed to its highly basic nature and resonating states than lysine that can stabilize the positive charges in protein. The arginine has also shown protein stability results with other proteins (Sokalingam *et al.*, 2012) thereby also indicating their role in structural stability of AβPP. Interestingly, lysine residues at high confidence ubiquitination sites were intolerant of all mutations thereby affecting AβPP stability and signifying their importance in protein's structural and functional regulation. Further, the evaluation of lysine mutations for disease susceptibility indicated their least effect on the high confidence ubiquitination sites

than the other sites. This situation can be extrapolated that ubiquitination may be more selective towards highly conserved or stable lysine sites than the least stable sites as observed by Kim and Hahn, who reported the gain of ubiquitination sites in the highly conserved region of proteins (Kim and Hahn 2012). Further analysis by Mutpred identified lysine residues to play a role in AβPP acetylation, glycosylation, phosphorylation, and SUMOylation apart from the ubiquitination. Additionally, investigations on AβPP-ubiquitin non covalent electrostatic, polar interactions reported K66, K99, K155, K447, K687, and K699 lysine residues having their plausible role in ubiquitin positioning or ubiquitin-AβPP conjugation that is the foundations for further research. Last but not the least; we investigated the interaction network of E1s, E2s, E3s, and DUBs enzymes for the clearance of AβPP in AD biology. The network revealed Parkin and STUB1 to be the key ubiquitin E3 ligases and USP25 and UCHL1 to be the key deubiquitinases directly involved in the ubiquitination of AβPP along with more than a dozen of E2 conjugating and E1 activating enzymes. Instead other E3 ligases such as FBXO2, TRIM13, CDH1, TRIM55, and SART1 are reported to regulate the ubiquitination of BACE1, PSENEN, PSEN1 and NCSTN respectively. Similarly, other deubiquitinases like USP8 and USP39 regulate BACE1 and NCSTN deubiquitination respectively. In summary, the in-depth studies pertaining to the lysine potential in AβPP processing, stability, interaction, ubiquitination, and other functions are provided herein that needed further investigations *in-vivo* at the molecular level to devise novel therapeutic modalities against Alzheimer's disease.

Chapter IV

An in-silico Investigation of Key Lysine Residues and Their Selection for Clearing off Aß and Holo-AßPP through Ubiquitination

CHAPTER IV

AN *IN-SILICO* INVESTIGATION OF KEY LYSINE RESIDUES AND THEIR SELECTION FOR CLEARING OFF Aβ AND HOLO-AβPP THROUGH UBIQUITINATION

4.1 ABSTRACT

Malicious progression of neurodegeneration is a consequence of toxic aggregates of proteins or peptides such as amyloid-beta (Aβ) in case of Alzheimer's disease (AD). These aggregates hinder the electrochemical transmission at neuronal-junctions and thus deteriorate neuronal-health by triggering dementia. Electrostatic and hydrophobic interactions among amino-acid residues are the governing principle behind the self-assembly of such toxic oligomers or agglomerate. Interestingly, lysine residues are crucial for such interactions and for facilitating the clearance of toxic metabolites through the ubiquitination process. The mechanisms behind lysine-selectivity and modifications of target proteins are very intriguing process and an avenue to explore the clearance of unwanted proteins from neurons. Therefore, it is fascinating for the researchers to investigate the role of key-lysine, their selectivity and interactions with other amino acids to clear-off toxic products in exempting the progression of Neurodegenerative disorders (NDDs). Herein, i) we identified the aggregation prone sequence in Aβ40 and Aβ42 as 'HHQKLVFFAE' and 'SGYEVHHQKLVFFAEDVG/KGAIIGLMVGGV' respectively with critical lysine (K) at 16 and 28 for stabilizing the aggregates; ii) elucidated the interaction pattern of AβPP with other AD-related proteins BACE1, APOE, SNCA, APBB1, CASP8, NAE1, ADAM10 and PSEN1 to describe the pathophysiology; iii) found APOE as commonly interacting factor between Aβ and Tau for governing AD pathogenesis, iv) reported K224, K351, K363, K377, K601, K662, K751, and K763 as potential putative lysine for facilitating AβPP clearance through ubiquitination thereby arresting Aβ formation, and v) observed conserved glutamine(Q), glutamic acid(E) and alpha-helical conformation as a crucial factor for lysine selectivity in the ubiquitination of AβPP.

4.2 INTRODUCTION

Alzheimer's disease is a problematic state where human's memory, thinking and behavior get affected, which account for 60-80 per cent dementia cases. Typically, these symptoms develop leisurely without prior notice of its actual onset and worsen over time to the extent of impeding with day-to-day tasks. Moreover, it has become a 6th leading cause of death in the United States with undefined treatment until-date (Alzheimer's Association 2017). The researchers are striving for finding the ways to treat, delay, or prevent the onset of this dreadful disease. The prime suspect for the pathogenic events in Alzheimer's disease (AD) is the senile plaque deposition inside the brain. Here the amyloid-β proteins Aβ40 and Aβ42 are the building blocks of these senile plaques that are produced by the sequential cleavage of amyloid-beta precursor protein (AβPP) by β-site AβPP cleaving enzyme (BACE1) and γ-secretase, a multi-subunit PS1/PS2-containing integral membrane protease (Armstrong 2009). These plaques principally block the communications between the neurons and interfere with the proper functioning of the brain. However, increased Aβ production is attributed to the AβPP gene duplication or base substitutions on AβPP and γ-secretase subunits PS1/PS2, but a majority of AD has exhibited Aβ accumulation without these mutations (Bohm *et al.,* 2015). This observation signifies the role of defective Aβ degradation and clearance in AD pathogenesis. Therefore, facilitating degradation and clearance of Aβ and AβPP could be the potential approach for alleviating the disease symptoms.

In order to facilitate the clearance of Aβ and AβPP, ubiquitination play a cardinal role in their proteasomal degradation by the 26S proteasomal complex with help of its key lysine. The selectivity of pathogenic proteins for degradation is mediated by the specific ubiquitin E3 ligases (UbE3s) that ubiquitinate the substrate protein with the help of its lysine (K) residues. For instance, certain UbE3s such as Parkin, Mdm2 (Mouse double minute 2 homolog), HRD1 (HMG-coA Reductase Degradation 1), CHIP (Carboxy terminus of Hsc70 interacting protein), Gigaxonin and NEDD8 (Neural precursor cell expressed developmentally down-regulated protein 8) are reported in Alzheimer's disease to facilitate the ubiquitination process (Mo *et al.,* 2010; Nomura *et al.,* 2016; Stankowski *et al.,* 2011; Cleveland *et al.,* 2009; Chen *et*

al., 2012). The UbE3 attaches ubiquitin (Ub) molecule to the specific lysine residue and marks it as an identification flag for degradation (David *et al.,* 2011). The ubiquitination pattern of the target protein determines the fate of that protein based on the site of ubiquitination. For instance, K11, K29, K48 and K63 polyubiquitination (PolyU) trigger proteasomal degradation, K6 PolyU triggers DNA repair, K63 PolyU trigger signal transduction and DNA repair while monoubiquitination (MonoU) triggers degradation by endosomal lysosomal pathway (Morris and Solomon 2004; Hayden and Ghosh 2008; Al-Hakim *et al.,* 2008; Bergink and Jentsch 2009; Matsumoto *et al.,* 2010). Therefore, identification of key lysine in a pathogenic protein responsible for its proteasomal degradation is of great importance for unraveling the therapeutic avenues for various neurodegenerative diseases. Moreover, the roles played by respective UbE3s in controlling the ubiquitination pattern of pathogenic proteins are also crucial for their contribution in developing future therapies. Our work has provided the possible insights associated with the ubiquitination of Aβ and AβPP through key lysine residue prediction that could mediate their clearance through proteasomal degradation and depicted the crucial region in $A\beta_{40/42}$ responsible for its aggregation. Moreover, this study deduced the interacting partners of AβPP and their interaction network with their roles in the pathogenesis of disease. Another interesting finding is the crucial lysine in Aβ, ubiquitin and AβPP along with their nearby conserved residues and their structural selectivity, which could serve as an important factor for lysine selection in the ubiquitination process of toxic proteins.

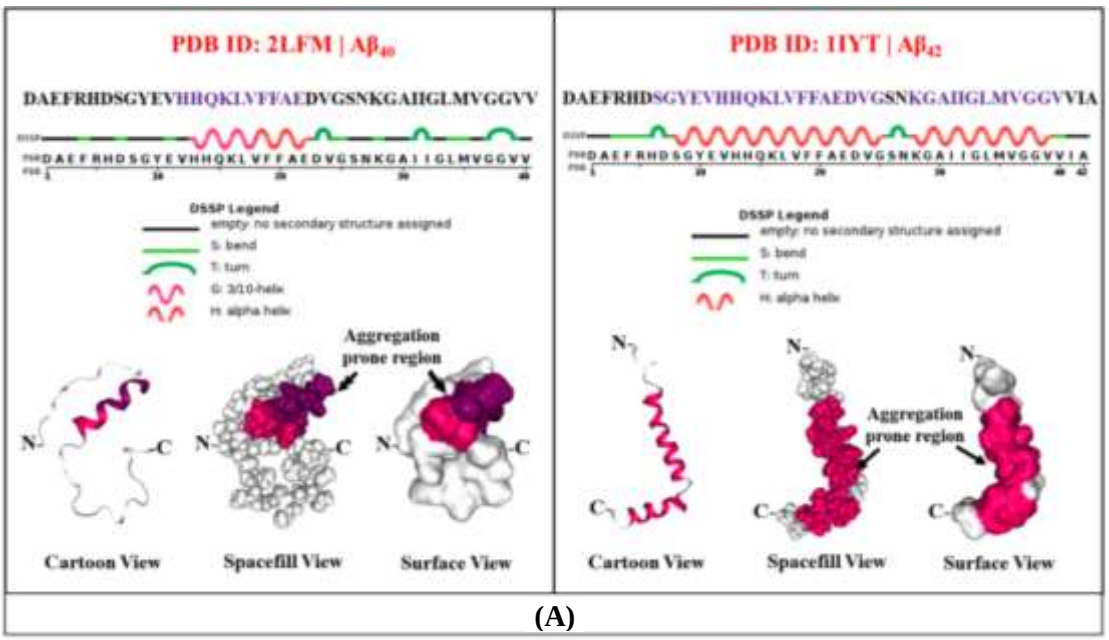
4.3 RESULTS

4.3.1 Lysine residues K_{16} and K_{28} in Aβ aggregation

The Amyloid beta 40 and 42 were analysed for their aggregation sites responsible for fibril formation with the help of a secondary structure prediction tool DSSP. The obtained results have identified one sequence motif HHQKLVFFAE in $A\beta_{40}$ and two sequence motifs SGYEVHHQKLVFFAEDVG and KGAIIGLMVGGV in $A\beta_{42}$. The obtained motif is found to code for both 3-10 helix and alpha helix in $A\beta_{40}$ while only for alpha helix in $A\beta_{42}$ **(Figure 4.1A).** Interestingly these motifs are spanned with lysine residues namely K16 in $A\beta_{40}$ while K16 and K28 in $A\beta_{42}$. These lysine residues

are crucial for imparting self-assembling property to the Aβ sequence via stabilizing the aggregates through their inherent potential to have salt bridges, hydrogen bonding, electrostatic and hydrophobic interactions. Sinha et al. have also reported the role of lysine residues (K16) in Aβ folding, assembly and toxicity (Sinha *et al.,* 2012). In this connection, NMR studies have shown the contribution of hydrophobic interaction and salt bridges in imparting the stability to beta sheets and turns in Aβ folding and assembly (Petkova *et al.,* 2002, 2006). Therefore, disrupting these interactions of Lys via ubiquitination could not only perturb its assembly but also provide an avenue to clear the burden of toxic proteins in the cell.

Moreover, secondary structural analysis results revealed 25% helical (2 helices; 10 residues) regions in $A\beta_{40}$ and 71% helical (2 helices; 30 residues) regions in $A\beta_{42}$ with rest as coils. The higher helical content in $A\beta_{42}$ and additional hydrophobic residues at its C-terminal contribute towards its higher aggregation as reported in familial AD patients and contributes towards toxicity to the neurons (Selkoe and Podlisny 2002; Chang and Chen 2014). Interestingly, the participation of K28 only in the aggregation of $A\beta_{42}$ as proposed by our results also corresponds with the results of Vandersteen *et al.,* who observed higher oligomer accumulation in $A\beta_{42}$ peptides than in $A\beta_{40}$ (Vandersteen *et al.,* 2012).



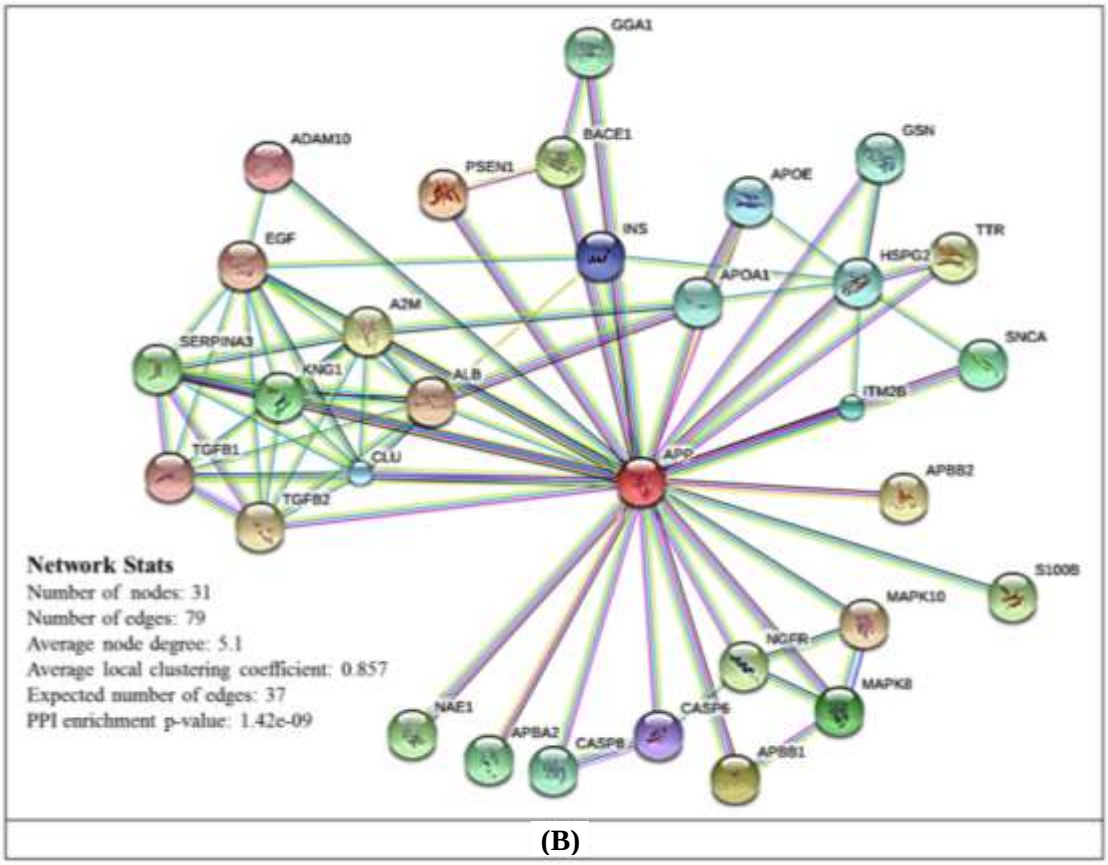
(A)

Figure 4.1A: The primary sequence of $A\beta_{40}$ (PDB ID: 2LFM) and $A\beta_{42}$ (PDB ID: 1IYT)

with secondary and tertiary structure. Primary sequence is highlighted for the aggregation prone sequence. Secondary structure is shown over the sequence based on DSSP prediction. Tertiary structure is shown in its three views, i.e. cartoon, space fill and surface to depict the aggregation prone region responsible for fibril formation.

4.3.2 Functional partners of AβPP with APOE as common interactor of Aβ and TAU

The functional interaction network of amyloid-beta precursor protein is mined to understand the pathophysiology of AD, which has been shown in **Figure 4.1B.** The network revealed 30 potential interacting partners with nine potential AD expressed proteins, i.e. BACE1, APOE, SNCA, APBB1, CASP8, NAE1, ADAM10, PSEN1 and AβPP. These proteins are reported to catalyze the formation of amyloid beta and trigger signaling pathways to activate the defense mechanisms against toxic agglomerate. Further network analysis identified 9 Aβ interacting proteins including BACE1, APOE, APOA1, ITM2B, APBB2, NGFR, APBB1, APBA2 and TGFB2 along with 3 tau-binding proteins, i.e. APOE, SNCA and S100B playing a crucial role in the pathology of Alzheimer's disease. The clustering analysis of the network revealed the proteins involved in various cellular processes like cell activation, extracellular matrix organization, exocytosis, platelet activation and degranulation with Aβ and Tau binding molecular function. Moreover, the comprehensive analysis of AβPP interacting proteins identified APOE as a commonly interacting protein binding with both Aβ and Tau, screening it as a key target for future therapies. Furthermore, the detailed functional roles of interacting protein in the pathophysiology of disease progression with their interaction scores are summarized in **Table 4.1** and the AβPP interacting partners identified by us were consistent with the results obtained by Perreau *et al.* about interaction network of amyloid-beta precursor protein (Perreau *et al.*, 2010).



(B)

Figure 4.1B: Predicted functional partners of AβPP (Amyloid beta Precursor protein). PSEN1-Presenilin 1; APBB1-Amyloid beta (A4) precursor protein-binding, family B, member 1; BACE1-Beta-site AβPP-cleaving enzyme 1; MAPK8-Mitogen-activated protein kinase 8; SNCA- α-Synuclein; ITM2B-Integral membrane protein 2B; CLU-Clusterin; INS-Insulin; CASP6-Caspase 6; ADAM10- ADAM metallopeptidase domain 10; TGFB1-Transforming growth factor, beta 1; EGF-Epidermal growth factor; ALB-Albumin; MAPK10-Mitogen-activated protein kinase 10; TGFB2-Transforming growth factor, beta 2; APBB2-Amyloid beta (A4) precursor protein-binding, family B, member 2; A2M-Alpha-2-macroglobulin; TTR-Transthyretin; NGFR-Nerve growth factor receptor; S100B-S100 calcium binding protein B; NAE1-NEDD8 activating enzyme E1 subunit 1; SERPINA3-Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3; KNG1-Kininogen 1; APBA2-Amyloid beta (A4) precursor protein-binding, family A, member 2; GGA1-Golgi-associated, gamma adaptin ear containing, ARF binding protein 1; CASP8-Caspase 8; GSN-Gelsolin; APOA1-Apolipoprotein A-I; HSPG2-Heparan sulfate proteoglycan 2; APOE-Apolipoprotein E.

Table 4.1: Functional role of AβPP interacting proteins in the pathophysiology of disease

S.No.	Protein	Full Name	Length	Molecular Function	Score
1	AβPP	Amyloid beta Precursor protein	770	N-AβPP binds TNFRSF21 triggering caspase activation and degeneration of both neuronal cell bodies (via caspase-3) and axons (via caspase-6)	Input
2	PSEN1	Presenilin 1	467	Catalytic subunit of g-secretase complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and AβPP	0.999
3	APBB1	Amyloid beta (A4) precursor protein-binding, family B, member 1	708	It acts like an adapter protein that forms transcriptionally active complex with g-secretase-derived amyloid β-precursor protein intracellular domain	0.999
4	BACE1	Beta-site AβPP-cleaving enzyme 1	501	Proteolytically process AβPP and cleaves at N-terminus of the Aβ peptide sequence, between 671-672 residues of AβPP to generate soluble AβPP and corresponding cell-associated C-terminal fragment	0.995
5	MAPK8	Mitogen-activated protein kinase 8	427	Involved in various processes such as cell proliferation, differentiation, migration, transformation and programmed cell death	0.985
6	SNCA	α-Synuclein	140	Involved in the regulation of dopamine release and transport and induces fibrillation of microtubule-associated protein tau	0.984
7	ITM2B	Integral membrane protein 2B	266	It has a regulatory role in the processing of AβPP and acts as an inhibitor of Aβ peptide aggregation and fibrils deposition	0.983
8	CLU	Clusterin	449	Functions as extracellular chaperone which prevents aggregation of nonnative proteins and inhibits formation of amyloid fibrils by AβPP, APOC2, B2M, CALCA, CSN3, SNCA and aggregation-prone LYZ variants (in vitro)	0.979
9	INS	Insulin	110	It decreases blood glucose concentration and increases cell permeability to monosaccharide, amino acids and fatty acid	0.976
10	CASP6	Caspase 6	293	Involved in the activation cascade of capsizes responsible for apoptosis execution	0.975
11	ADAM10	ADAM metallopeptidase domain 10	748	Responsible for the proteolytic release of several cell-surface proteins, including heparin-binding epidermal growth- like factor, ephrin-A2 and for constitutive and regulated α-secretase cleavage of AβPP	0.972

S.No.	Protein	Full Name	Length	Molecular Function	Score
12	TGFB1	Transforming growth factor, beta 1	390	Multifunctional protein that controls proliferation, differentiation and other functions in many cell types	0.972
13	EGF	Epidermal growth factor	1207	EGF stimulates the growth of various epidermal and epithelial tissues in vivo and in vitro and of some fibroblasts in cell culture	0.97
14	ALB	Albumin	609	It's a main protein of plasma that has a good binding capacity for water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones, bilirubin and drugs. Moreover, it functions for the regulation of the colloidal osmotic pressure of blood	0.969
15	MAPK10	Mitogen-activated protein kinase 10	464	It is a serine/threonine-protein kinase involved in various processes such as neuronal proliferation, differentiation, migration and programmed cell death	0.968
16	TGFB2	Transforming growth factor, beta 2	442	It is a cytokine which performs many cellular functions especially during embryonic development	0.964
17	APBB2	Amyloid beta (A4) precursor protein-binding, family B, member 2	759	It modulate the internalization of AβPP	0.963
18	A2M	Alpha-2-macroglobulin	1474	It is able to inhibit all four classes of proteinases by a unique 'trapping' mechanism. This protein has a peptide stretch, called 'bait region' which contains specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced in the protein which traps the proteinase	0.961
19	TTR	Transthyretin	147	It is a thyroid hormone-binding protein that transports thyroxine from bloodstream to the brain	0.961
20	NGFR	Nerve growth factor receptor	427	It plays a role in the regulation of GLUT4 translocation to the cell surface in adipocytes and skeletal muscles in response to insulin. It can mediate cell survival as well as cell death of neural cells	0.961
21	S100B	S100 calcium binding protein B	92	It binds and initiates the activation of STK38 by releasing auto inhibitory intra-molecular interactions within the kinase and its interaction with AGER after myocardial infarction may play a role in myocyte apoptosis by activating ERK1/2 and p53/TP53 signaling	0.96
22	NAE1	NEDD8 activating enzyme E1 subunit 1	534	Activates NEDD8 and involved in regulating cell death	0.959

S.No.	Protein	Full Name	Length	Molecular Function	Score
23	SERPINA 3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	423	Although its physiological function is unclear, but it is known to inhibit neutrophil cathepsin G and mast cell chymase, both of which can convert angiotensin-1 to the active angiotensin-2	0.958
24	KNG1	Kininogen 1	644	Kininogens are inhibitors of thiol proteases and plays an important role in blood coagulation, influence smooth muscle contraction, induce hypotension, natriuresis and diuresis	0.956
25	APBA2	Amyloid beta (A4) precursor protein-binding, family A, member 2	749	Putative function in synaptic vesicle exocytosis by binding to STXBP1, an essential component of the synaptic vesicle exocytosis machinery and may modulate processing of AβPP and hence formation of Aβ	0.956
26	GGA1	Golgi-associated, gamma adaptin ear containing, ARF binding protein 1	639	It plays a role in protein sorting and trafficking between the trans-Golgi network and endosomes	0.954
27	CASP8	Caspase 8	538	It is an apoptosis-related cysteine peptidase and is the most upstream protease of the activation cascade of caspases responsible for the TNFRSF6/FAS mediated and TNFRSF1A induced cell death	0.954
28	GSN	Gelsolin	782	Calcium-regulated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange (end-blocking or capping) and promote the assembly of monomers into filaments (nucleation)	0.951
29	APOA1	Apolipoprotein A-I	267	Participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase	0.951
30	HSPG2	Heparan sulfate proteoglycan 2	4391	Integral component of basement membranes providing fixed negative electrostatic membrane charge, and thus provides a barrier by both size- and charge-selective. It also serves as an attachment substrate for cells and plays essential roles in vascularization	0.949
31	APOE	Apolipoprotein E	317	Mediates the binding, internalization, and catabolism of lipoprotein particles and serve as a ligand for the LDL (apo B/E) receptor and for the specific apo-E receptor (chylomicron remnant) of hepatic tissues	0.948

4.3.3 Potential lysines for triggering ubiquitination

4.3.3.1 Amyloid beta 42

$A\beta_{42}$ peptide sequence has two lysines at position 16 and 28. The respective ubiquitination-site prediction scores by UbPred and UbiPred machine-learning tools were K16: 0.52 and 0.24 while K28: 0.50 and 0.44 respectively. However, computationally both the tools were failed to identify the significant score for ubiquitin attachment but comparatively K28 was shown the more probable scores for ubiquitination **(Figure 4.2A)**, which corresponds with the structural studies suggesting its involvement in intra- or intermolecular contacts while K16 exposed to solvent. Interestingly toxicological studies identified greater impact of K16 on $A\beta_{40}$ toxicity as compared with $A\beta_{42}$ while K28 has a greater impact on folding and assembly of both $A\beta_{40}$ and $A\beta_{42}$ (Lopes *et al.,* 2011; Bera *et al.,* 2016).

4.3.3.2 Ubiquitin

Ubiquitin protein contains seven lysines at positions 6, 11, 27, 29, 33, 48 and 63. The predicted sites for ubiquitination by UbPred and UbiPred machine learning tools were K27 and K33, K48 respectively. The significant score obtained for K27 by UbPred is 0.64 and K33, K48 by UbiPred is 0.53, 0.83 respectively. Comparatively K48 is shown to have the highest probable score for ubiquitination site as shown in **Figure 4.2B.** Moreover, K48 polyubiquitin chain marks a signal for targeting the proteins towards proteasomal degradation. The significant high score for K48 corresponds well for the selection of UbPred and UbiPred machine learning tools for predicting key lysine ubiquitination with higher confidence for proteasomal degradation of target protein.

Figure 4.2: Ubiquitination site predictions using UbPred and UbiPred: (A) Aβ$_{42}$, (B) Ubiquitin

4.3.3.3 Amyloid beta Precursor Protein

AβPP sequence analysis identified 41 lysine residues in total spanned throughout the sequence where UbPred analysis reported 16 potential sites. Among them, 5-sites K224, K377, K393, K395 and K438 are with high confidence, 8-sites K51, K60, K161, K351, K601, K662, K751, and K763 are with medium confidence while 3-sites K155, K363, and K568 are with low confidence **(Figure 4.3A).** The most significant score obtained is for K224, i.e. 0.97. Likewise, 19 potential lysine sites were predicted by UbiPred for ubiquitination, including K103, K224, K351, K363, K377, K401, K428, K429, K495, K496, K503, K510, K521, K522, K601, K662, K670, K751, and K763 **(Figure 4.3B).** Here, the most significant score obtained was for K351, i.e. 0.91. In summary, the ubiquitination site predicted by UbPred and UbiPred has identified 8-sites in common for AβPP ubiquitination, which includes K224, K351, K363, K377, K601, K662, K751, and K763 summarized in **Table 4.2.** Since, there is very little information available regarding the ubiquitinating sites of AβPP, our

in-silico analysis could be a stepping-stone in providing an avenue for identifying the ubiquitination patterns of AβPP for restricting Aβ production.

AβPP

UbPred

>sp|P05067.3|A4_HUMAN RecName: Full=Amyloid beta A4 protein

MLPGLALLLLAAWTARALEVPTDGNAGLLAEPQIAMFCGRLNMHMNVQNGKWDSDPSGTKTCIDTKEGILQYCQEVYPEL
QITNVVEANQPVTIQNWCKRGRKQCKTHPHFVIPYRCLVGEFVSDALLVPDKCKFLHQERMDVCETHLHWHTVAKETCSE
KSTNLHDYGMLLPCGIDKFRGVEFVCCPLAEESDNVDSADAEEDDSDVWWGGADTDYADGSEDKVVEVAEEEEVAEVEEE
EADDDEDDEDGDEVEEEAEEPYEEATERTTSIATTTTTTTESVEEVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFF
YGGCGGNRNNFDTEEYCMAVCGSAMSQSLLKTTQEPLARDPVKLPTTAASTPDAVDKYLETPGDENEHAHFQKAKERLEA
KHRERMSQVMREWEEAERQAKNLPKADKKAVIQHFQEKVESLEQEAANERQQLVETHMARVEAMLNDRRRLALENYITAL
QAVPPRPRHVFNMLKKYVRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQD
EVDELLQKEQNYSDDVLANMISEPRISYGNDALMPSLTETKTTVELLPVNGEFSLDDLQPWHSFGADSVPANTENEVEPV
DARPAADRGLTTRPGSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVITL
VMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN

Output:

Residue	Score	Ubiquitinated
51	0.71	Yes Medium confidence
60	0.72	Yes Medium confidence
66	0.55	No
99	0.10	No
103	0.10	No
106	0.11	No
132	0.41	No
134	0.40	No
155	0.65	Yes Low confidence
161	0.70	Yes Medium confidence
178	0.48	No
224	0.97	Yes High confidence
315	0.45	No
351	0.79	Yes Medium confidence
363	0.64	Yes Low confidence
377	0.94	Yes High confidence
393	0.86	Yes High confidence
395	0.88	Yes High confidence
401	0.24	No
421	0.56	No
425	0.37	No

Residue	Score	Ubiquitinated
428	0.17	No
429	0.13	No
438	0.89	Yes High confidence
495	0.07	No
496	0.10	No
503	0.27	No
510	0.55	No
521	0.44	No
522	0.42	No
568	0.62	Yes Low confidence
601	0.81	Yes Medium confidence
662	0.81	Yes Medium confidence
670	0.58	No
687	0.57	No
699	0.60	No
724	0.16	No
725	0.14	No
726	0.21	No
751	0.74	Yes Medium confidence
763	0.70	Yes Medium confidence

(A)

Figure 4.3A: Ubiquitination sites in AβPP predicted by UbPred tool.

AβPP

UbiPred

>SPIP05067.3IA4 HUMAN RECNAME: FULL=AMYLOID BETA A4 PROTEIN

MLPGLALLLLAAWTARALEVPTDGNAGLLAEPQIAMFCGRLNMHMNVQNGKWDSDPSGTKTCIDTKEGIL
QYCQEVYPELQITNVVEANQPVTIQNWCKRGRKQCKTHPHFVIPYRCLVGEFVSDALLVPDKCKFLHQER
MDVCETHLHWHTVAKETCSEKSTNLHDYGMLLPCGIDKFRGVEFVCCPLAEESDNVDSADAEEDDSDVWW
GGADTDYADGSEDKVVEVAEEEEVAEVEEEEADDDEDDEDGDEVEEEAEEPYEEATERTTSIATTTTTTT
ESVEEVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAMSQSLL
KTTQEPLARDPVKLPTTAASTPDAVDKYLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWEEAERQA
KNLPKADKKAVIQHFQEKVESLEQEAANERQQLVETHMARVEAMLNDRRRLALENYITALQAVPPRPRHV
FNMLKKYVRAEQKDRQHTLKHFEHVRMVDPKKAAQIRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQD
EVDELLQKEQNYSDDVLANMISEPRISYGNDALMPSLTETKTTVELLPVNGEFSLDDLQPWHSFGADSVP
ANTENEVEPVDARPAADRGLTTRPGSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKG
AIIGLMVGGVVIATVIVITLVMLKKKQYTSIHHGVVEVDAAVTPEERHLSKMQQNGYENPTYKFFEQMQN

Position	Sequence	Ubiquitylation	Score	Position	Sequence	Ubiquitylation	Score
51	LNMHMNVQNGKWDSDPSGTKT	N	0.45	**428**	**RQAKNLPKADKKAVIQHFQEK**	**Y**	**0.57**
60	GKWDSDPSGTKTCIDTKEGIL	N	0.28	**429**	**QAKNLPKADKKAVIQHFQEKV**	**Y**	**0.61**
66	PSGTKTCIDTKEGILQYCQEV	N	0.32	438	KKAVIQHFQEKVESLEQEAAN	N	0.43
99	NQPVTIQNWCKRGRKQCKTHP	N	0.49	**495**	**PRPRHVFNMLKKYVRAEQKDR**	**Y**	**0.61**
103	**TIQNWCKRGRKQCKTHPHFVI**	**Y**	**0.54**	**496**	**RPRHVFNMLKKYVRAEQKDRQ**	**Y**	**0.57**
106	NWCKRGRKQCKTHPHFVIPYR	N	0.49	**503**	**MLKKYVRAEQKDRQHTLKHFE**	**Y**	**0.71**
132	FVSDALLVPDKCKFLHQERMD	N	0.35	**510**	**AEQKDRQHTLKHFEHVRMVDP**	**Y**	**0.69**
134	SDALLVPDKCKFLHQERMDVC	N	0.42	**521**	**HFEHVRMVDPKKAAQIRSQVM**	**Y**	**0.72**
155	ETHLHWHTVAKETCSEKSTNL	N	0.44	**522**	**FEHVRMVDPKKAAQIRSQVMT**	**Y**	**0.66**
161	HTVAKETCSEKSTNLHDYGML	N	0.27	568	IQDEVDELLQKEQNYSDDVLA	N	0.42
178	YGMLLPCGIDKFRGVEFVCCP	N	0.21	**601**	**DALMPSLTETKTTVELLPVNG**	**Y**	**0.71**
224	**DTDYADGSEDKVVEVAEEEEV**	**Y**	**0.56**	**662**	**TRPGSGLTNIKTEEISEVKMD**	**Y**	**0.56**
315	SRWYFDVTEGKCAPFFYGGCG	N	0.14	**670**	**NIKTEEISEVKMDAEFRHDSG**	**Y**	**0.55**
351	**CGSAMSQSLLKTTQEPLARDP**	**Y**	**0.91**	687	HDSGYEVHHQKLVFFAEDVGS	N	0.24
363	**TQEPLARDPVKLPTTAASTPD**	**Y**	**0.67**	699	VFFAEDVGSNKGAIIGLMVGG	N	0.44
377	**TAASTPDAVDKYLETPGDENE**	**Y**	**0.69**	724	TVIVITLVMLKKKQYTSIHHG	N	0.17
393	GDENEHAHFQKAKERLEAKHR	N	0.49	725	VIVITLVMLKKKQYTSIHHGV	N	0.23
395	ENEHAHFQKAKERLEAKHRER	N	0.46	726	IVITLVMLKKKQYTSIHHGVV	N	0.23
401	**FQKAKERLEAKHRERMSQVHR**	**Y**	**0.60**	**751**	**AVTPEERHLSKMQQNGYENPT**	**Y**	**0.59**
421	REWEEAERQAKNLPKADKKAV	N	0.31	**763**	**QQNGYENPTYKFFEQMQN**	**Y**	**0.54**
425	EAERQAKNLPKADKKAVIQHF	N	0.48				

(b)

Figure 4.3B: Ubiquitination sites in AβPP predicted by UbiPred tool.

Table 4.2: Key lysine sites commonly predicted by UbPred and UbiPred for ubiquitination

Substrate Protein	Site	Sequence	Ubiquitination status
Amyloid beta Precursor Protein (AβPP)	224	DTDYADGSED**K**VVEVAEEEEV	Yes
	351	CGSAMSQSLL**K**TTQEPLARDP	Yes
	363	TQEPLARDPV**K**LPTTAASTPD	Yes
	377	TAASTPDAVD**K**YLETPGDENE	Yes
	601	DALMPSLTET**K**TTVELLPVNG	Yes
	662	TRPGSGLTNI**K**TEEISEVKMD	Yes
	751	AVTPEERHLS**K**MQQNGYENPT	Yes
	763	QQNGYENPTY**K**FFEQMQN	Yes

4.3.4 Glutamine and Glutamic acid: Key residues conserved at lysine site for ubiquitination

The functionally predicted ubiquitination sites were scanned for the conserved residues, which could be critical for providing lysine selectivity for the ubiquitination process. The multiple sequence analyses of potential ubiquitinating sites in ubiquitin and AβPP revealed the conservation of neutrally charged Glutamine (Q) and negatively charged Glutamic acid (E) (marked with green arrow) in close proximity with the positively charged lysine residue shown in **Figure 4.4.** These conserved residues could serve as the ubiquitin-interacting motif important for imparting lysine selectivity for ubiquitin attachment via providing favorable environment, i.e. suitable charge or interacting potential. Further investigations are required to validate their role in the ubiquitination process of AβPP.

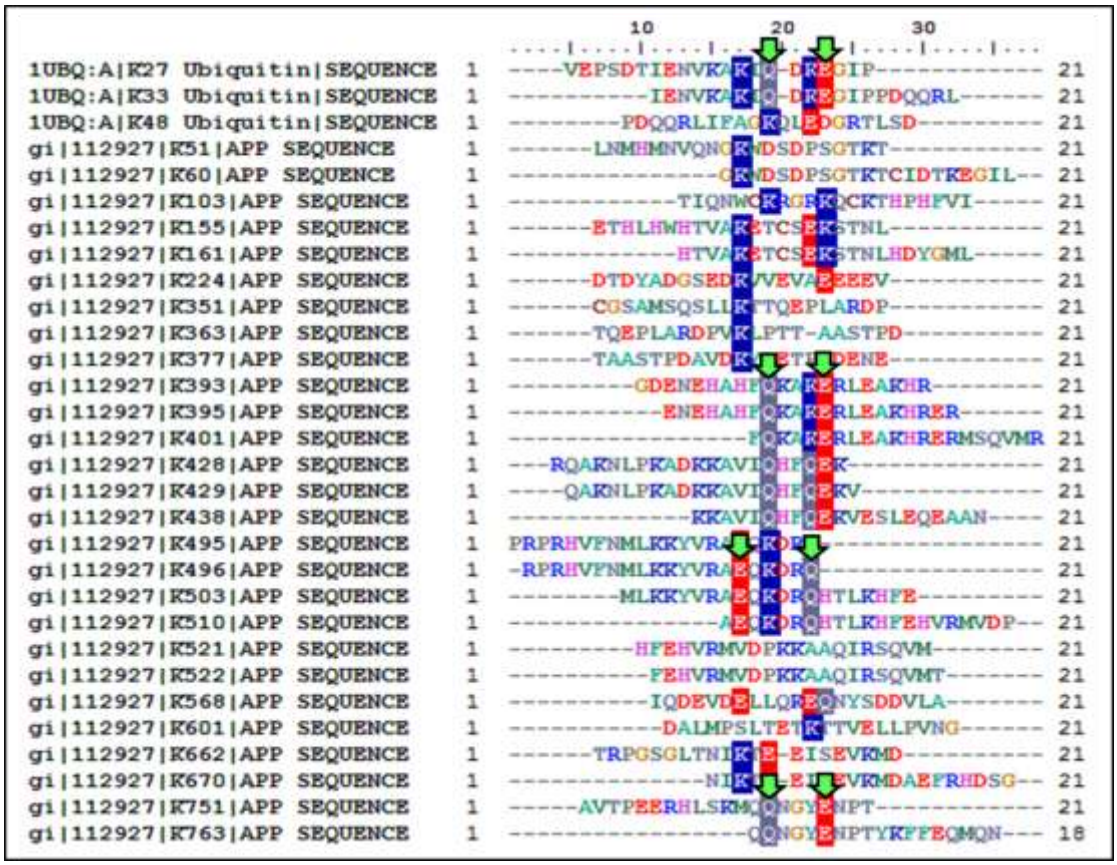


Figure 4.4: Multiple sequence alignment of 21 window-size lysine sites of Ubiquitin and AβPP predicted by UbPred and UbiPred

4.3.5 Alpha helical structural selectivity for lysine's ubiquitination

The potential ubiquitination sites were analysed for their structural selectivity for lysine's recognition and ubiquitin attachment that has been summarized in **Table 4.3.** The structural incidence of the putative ubiquitination sites revealed the presence of both alpha helix and Turn/Loop regions. But close analysis of both UbPred and UbiPred prediction results showed alpha helical region in the majority of the ubiquitination sites while Turn/Loop region in the majority of non-ubiquitination sites. However, the ubiquitination process was not exactly found to be a structure selective, but the results signify the importance of alpha helix in ubiquitination that need further investigations along with the role of ubiquitination in structural detriment to take proteasomal degradation in effect.

Table 4.3: Secondary structures of the non-ubiquitinated and ubiquitinated sites predicted by UbPred and UbiPred

UbPred Sites				UbiPred Sites			
Protein	**Lysine Residue**	**Ubiquitination**	**Secondary Structure**	**Protein**	**Lysine Residue**	**Ubiquitination**	**Secondary Structure**
Ubiquitin	6	No	Beta Strand	Ubiquitin	6	No	Beta Strand
Ubiquitin	11	No	Turn/Loop	Ubiquitin	11	No	Turn/Loop
Ubiquitin	29	No	Alpha Helix	Ubiquitin	27	No	Alpha Helix
Ubiquitin	33	No	Alpha Helix	Ubiquitin	29	No	Alpha Helix
Ubiquitin	48	No	Beta Strand	Ubiquitin	63	No	Turn/Loop
Ubiquitin	63	No	Turn/Loop	AβPP	51	No	Turn/Loop
AβPP	66	No	Turn/Loop	AβPP	60	No	Turn/Loop
AβPP	99	No	Turn/Loop	AβPP	66	No	Turn/Loop
AβPP	103	No	Turn/Loop	AβPP	99	No	Turn/Loop
AβPP	106	No	Turn/Loop	AβPP	106	No	Turn/Loop
AβPP	132	No	Turn/Loop	AβPP	132	No	Turn/Loop
AβPP	134	No	Turn/Loop	AβPP	134	No	Turn/Loop
AβPP	178	No	Turn/Loop	AβPP	155	No	Alpha Helix
AβPP	315	No	Turn/Loop	AβPP	161	No	Turn/Loop
AβPP	401	No	Alpha Helix	AβPP	178	No	Turn/Loop
AβPP	421	No	Turn/Loop	AβPP	315	No	Turn/Loop
AβPP	425	No	Turn/Loop	AβPP	393	No	Alpha Helix
AβPP	428	No	Alpha Helix	AβPP	395	No	Alpha Helix
AβPP	429	No	Alpha Helix	AβPP	421	No	Turn/Loop
AβPP	495	No	Alpha Helix	AβPP	425	No	Turn/Loop
AβPP	496	No	Alpha Helix	AβPP	438	No	Alpha Helix
AβPP	503	No	Alpha Helix	AβPP	568	No	Alpha Helix
AβPP	510	No	Alpha Helix	AβPP	687	No	Turn/Loop
AβPP	521	No	Alpha Helix	AβPP	699	No	Turn/Loop
AβPP	522	No	Alpha Helix	AβPP	724	No	Turn/Loop
AβPP	670	No	Turn/Loop	AβPP	725	No	Turn/Loop
AβPP	687	No	Turn/Loop	AβPP	726	No	Turn/Loop
AβPP	699	No	Turn/Loop				
AβPP	724	No	Turn/Loop				
AβPP	725	No	Turn/Loop				
AβPP	726	No	Turn/Loop				

UbPred Sites				UbiPred Sites			
Protein	**Lysine Residue**	**Ubiquitination**	**Secondary Structure**	**Protein**	**Lysine Residue**	**Ubiquitination**	**Secondary Structure**
Ubiquitin	27	Yes	Alpha Helix	Ubiquitin	33	Yes	Alpha Helix
AβPP	51	Yes	Turn/Loop	Ubiquitin	48	Yes	Beta Strand
AβPP	60	Yes	Turn/Loop	AβPP	103	Yes	Turn/Loop
AβPP	155	Yes	Alpha Helix	AβPP	224	Yes	Alpha Helix
AβPP	161	Yes	Turn/Loop	AβPP	351	Yes	Turn/Loop
AβPP	224	Yes	Alpha Helix	AβPP	363	Yes	Turn/Loop
AβPP	351	Yes	Turn/Loop	AβPP	377	Yes	Turn/Loop
AβPP	363	Yes	Turn/Loop	AβPP	401	Yes	Alpha Helix
AβPP	377	Yes	Turn/Loop	AβPP	428	Yes	Alpha Helix
AβPP	393	Yes	Alpha Helix	AβPP	429	Yes	Alpha Helix
AβPP	395	Yes	Alpha Helix	AβPP	495	Yes	Alpha Helix
AβPP	438	Yes	Alpha Helix	AβPP	496	Yes	Alpha Helix
AβPP	568	Yes	Alpha Helix	AβPP	503	Yes	Alpha Helix
AβPP	601	Yes	Turn/Loop	AβPP	510	Yes	Alpha Helix
AβPP	662	Yes	Turn/Loop	AβPP	521	Yes	Alpha Helix
AβPP	751	Yes	Alpha Helix	AβPP	522	Yes	Alpha Helix
AβPP	763	Yes	Alpha Helix	AβPP	601	Yes	Turn/Loop
				AβPP	662	Yes	Turn/Loop
				AβPP	670	Yes	Turn/Loop
				AβPP	751	Yes	Alpha Helix
				AβPP	763	Yes	Alpha Helix

Although, the ubiquitination is an intriguing process involving the role of a wide range of factors for the successful clearance of toxic proteins, but the obtained results have revealed the facts to an extent. Based on the obtained results, we can hypothesize the ubiquitination mechanism associated with the clearance of Aβ or AβPP **(Figure 4.5)** that requires further researches for better understanding the ubiquitination process.

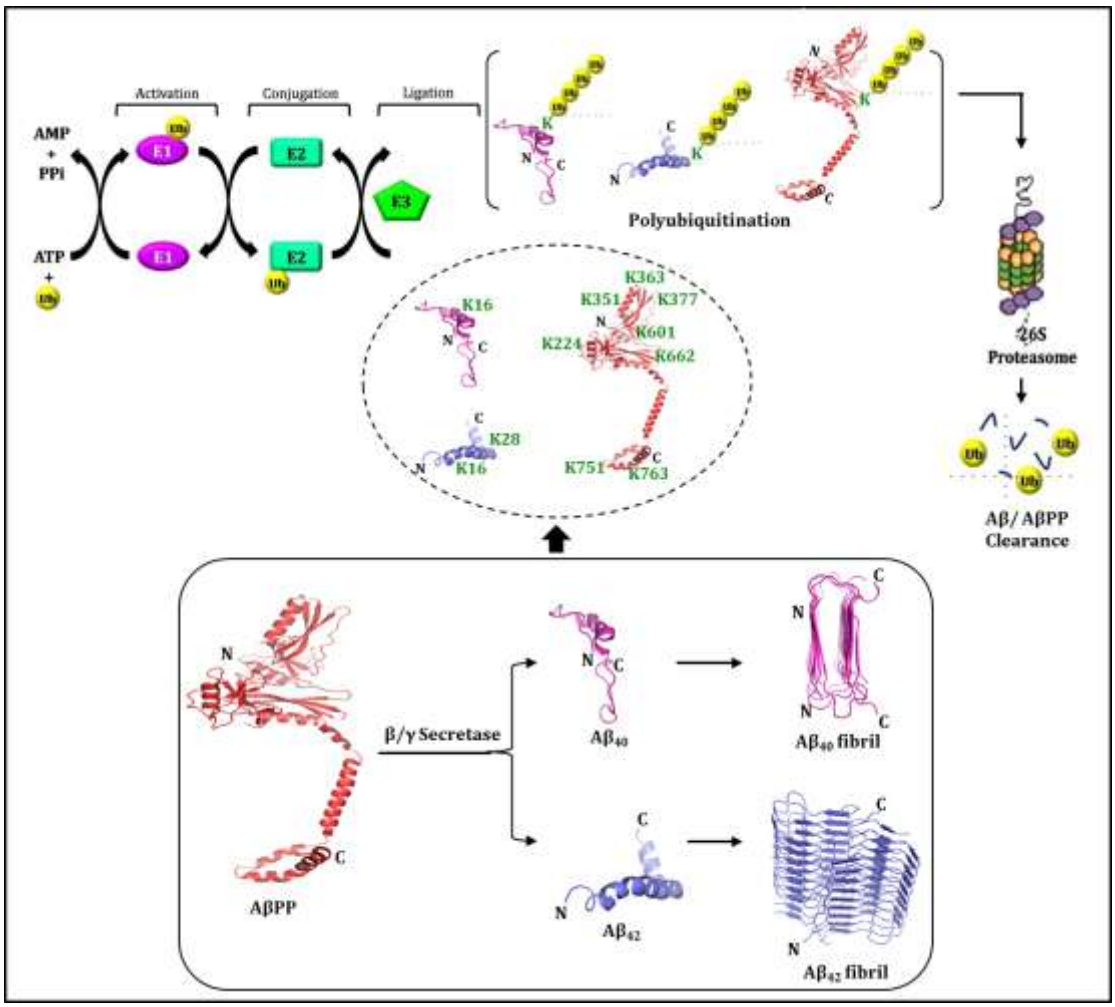


Figure 4.5: Proposed Amyloid beta clearance mechanism. AβPP is enzymatically cleaved by β and γ-secretases to form Aβ plaques, which are further, processed via the ubiquitin proteasome system with help of selective lysine residues. In ubiquitination, E1 ubiquitin-activating enzyme activates the ubiquitin molecules and E2 conjugating-enzyme conjugate this activated ubiquitin either directly to the substrate protein or through conjugation with ubiquitin E3 ligase itself at specific lysine- K16 for $A\beta_{40}$ or K16/K28 for $A\beta_{42,}$ and K224/K351/K363/K377/K601/K662/K751/K763 for AβPP. Thus, polyubiquitinated substrate, e.g. Aβ40/42 or AβPP is processed for proteolytic degradation by 26S proteasome to clear the toxic Aβ plaques.

4.4 DISCUSSION

In spite of intensive research in the pathogenesis of neurodegenerative disorders such as AD, a clear understanding about its treatment remained rudimentary. Undeveloped or unidirectional symptomatic medication approaches showed adverse results in treatment trials. New approaches that can target it at the molecular level could be the best possible method to control the disease progression. Such an effective method is the triggering of ubiquitination, i.e. self-defense mechanism of cell to overcome the protein burden inside it. For that, molecular-level understanding of Aβ or AβPP ubiquitination is necessary for developing the prospective therapeutic agents to address the clearance of such toxic proteins or toxin progenitor proteins for ameliorating the neurodegenerative diseases like AD. Here, the aggregation prone region in Aβ40 and Aβ42 were identified to be HHQKLVFFAE and SGYEVHHQKLVFFAEDVG / KGAIIGLMVGGV respectively. An additional hydrophobic region in Aβ42 could be the plausible explanation for its higher aggregation (Gu and Guo 2013). Moreover, they adopt different conformations that facilitate the aggregation and are responsible for its toxic properties (Sgourakis *et al.,* 2007). Further, both lysines K16 and K28 were present in the aggregation prone region could be suggestive of its role in Aβ aggregation. In this regard, some studies indicated the role of K28 in salt bridge formation with E22 or D23 suggestive of the nucleation events of Aβ assembly (Lazo and Grant 2005). However, significant scores were not obtained for K16 and K28 in Aβ but both lysines were crucial for aggregation and clearance through ubiquitination that requires further studies. Further interaction studies identified 9 AD-related proteins AβPP, BACE1, APOE, SNCA, APBB1, CASP8, NAE1, ADAM10 and PSEN1 along with 9 Aβ binding BACE1, APOE, APOA1, ITM2B, APBB2, NGFR, APBB1, APBA2, TGFB2 and 3 Tau binding proteins APOE, SNCA, and S100B with APOE as commonly interacting partner with Aβ and TAU. Previous studies have also reported APOE to modulate multiple processes in AD, including Aβ deposition, tangle formation, synaptic impairment and neuroinflammation (Yamazaki *et al.,* 2016). Apart from this, we reported glutamine (Q) and glutamic acid (E) as K-site conserved residues that could be crucial for lysine selection in ubiquitination. These amino acids overall impart a

negative charge in proximity to the positively charged lysine residue and could aid in its selection for ubiquitination by the respective ubiquitin E3 ligase enzymes. In addition, the study by Suryadinata *et al.,* supported our finding that has shown the importance of glutamine residue in K48 linked polyubiquitination by Cdc34 ubiquitination enzyme (Suryadinata *et al.,* 2013). Further, lysine site analysis interestingly reported the lysines responsible for ubiquitination were mostly present in alpha helical region, signifying ubiquitination's role in structural disruption while non-ubiquitinated lysine residues were in turn/loop region. Altogether, lysine selection is both sequence and structurally based mechanism for Aβ or AβPP ubiquitination and is crucial for addressing the pathogenesis of AD that has been addressed here to a certain extent. Further researches are required to generate the novel potential therapeutic avenues to treat the malicious progression of AD worldwide.

Chapter V

Aβ, Tau, and α-Synuclein aggregation and integrated role of PARK2 in the regulation and clearance of toxic peptides

CHAPTER V

Aβ, TAU, AND α-SYNUCLEIN AGGREGATION AND INTEGRATED ROLE OF PARK2 IN THE REGULATION AND CLEARANCE OF TOXIC PEPTIDES

5.1 ABSTRACT

Alzheimer's and Parkinson's disease is one of the world's leading causes of death. More than 50 million people throughout the world are suffering with these diseases. They are two distinct progressive neurodegenerative disorders affecting different regions of the brain with diverse symptoms, including memory and motor loss respectively, but with the advancement of diseases, both affect the whole brain and exhibit some common biological symptoms. For instance, more than 50% PD patients develop dementia in their later stages, though it is a hallmark of Alzheimer's disease. In fact, latest research has suggested the involvement of some common pathophysiological and genetic links between these diseases, including the deposition of pathological Aβ, Tau, and α-synuclein in both the cases. Therefore, it is pertinent to diagnose the common pathological biomarkers, their aggregation mechanism, their intricate relationships in the pathophysiology of disease and therapeutic markers to target them. This would enable us to identify novel markers for the early detection of disease and targets for the future therapies. Herein, we investigated the molecular aspects of Aβ, Tau and α-Synuclein aggregation, and characterized their functional partners involved in the pathology of AD and PD. Moreover, we identified the molecular crosstalk between AD and PD associated with their pathogenic proteins- Aβ, Tau and α-Synuclein. Further, we characterized their ubiquitinational enzymes and associated interaction network regulating the proteasomal clearance of these pathological proteins.

5.2 INTRODUCTION

The aggregation of certain misfolded proteins is the chief pathogenic event that evokes neurotoxicity in many neurodegenerative disorders like Alzheimer's and Parkinson's disease (Jellinger 2010). The major structural changes that take place are the rise in β-sheet conformation in the misfolded protein that promotes

oligomerization and amyloid like fibril formation (Relini *et al.,* 2013). Moreover, the aggregation mechanism involves a crucial step of seed-nucleus formation, which provides a surface for a series of misfolding, and protein-protein interaction events to take place that govern oligomer or protofibril formation (Breydo and Uversky 2014). Consequently, aggregated proteins become resistant to proteolysis and cellular clearance that cause chronic endoplasmic reticulum stress, mitochondrial dysfunction, reactive oxygen species formation, intense tissue inflammation and activation of apoptotic pathways leading to the neuronal loss (Rutkowski and Kaufman 2004; Morimoto 2008). Although AD and PD exhibit heterogeneity at genetic and clinical level and affect different regions of the brain, including acetylcholineric neurons in hippocampus and dopaminergic neurons in substantia nigra pars compacta respectively. Recent studies revealed significant similarity in their overlapping role of pathological proteins, including amyloid-β, tau protein, and α-synuclein and suggestive genetic link in their pathogenesis (Jellinger 2012). For instance, above 50 percent of patients, suffering from AD revealed amyloid like alpha-synuclein peptide aggregates (Marsh and Blurton-Jones 2012) while PD patients displayed frequent tau deposits (Lei *et al.,* 2010). Moreover, it has demonstrated that the accumulation of tau proteins could take place with the help of specific strains of alpha-synuclein (Guo *et al.,* 2013). Another study identified that Aβ seeding escalated the formation of big sized α-synuclein oligomers that efficiently hampered neuronal SNARE-mediated vesicle fusion at synaptic junctions (Choi *et al.,* 2015). Apart from proteinopathic similarities, a recent breakthrough suggested possible genetic links between these diseases in their advanced stages. For instance, LRRK2 mutant G2019S mediated phosphorylation of AβPP at T668 promoted APP intracellular domain's (AICD) nuclear translocation and consequent neurotoxicity in PD (Zeng *et al.,* 2018). However, further research is required to establish well-defined pathological links between them. Moreover, these pathogenic proteins differ in their structural and functional biology, but they may share their protein-misfolding events and interact together to aggravate the disease symptoms (Cuanalo-Contreras *et al.,* 2013). Further, they interact with other ubiquitination markers to relieve the proteotoxic burden inside neurons, via refolding or targeting proteins to ubiquitin

proteasome system for degradation (Ciechanover and Kwon 2015). Therefore, elucidation of molecular mechanism behind pathogenic protein aggregation and their interaction with other proteins are crucial for neurobiologist to design better diagnostic and treatment options for such neurodegenerative disorders.

5.3 RESULTS

5.3.1 Hydrophobic interactions are the basis of neurotoxic protein aggregation

The pathological peptides and proteins, including $A\beta_{40}$, $A\beta_{42}$, Tau, and α-synuclein in AD and PD were studied for their aggregation sites to analyze the protein folding dynamics in its diseased state. The analysis of their secondary structures revealed the transformation of their helical conformations into beta strands from their monomer to fibrillar state respectively. Moreover, their tertiary structures revealed the presence of crucial hydrophobic sites responsible for intra- and inter-molecular interactions to govern protein aggregation.

5.3.1.1 Amyloid beta peptide

The amyloid peptide exists in two the most common isoforms in the brain, including $A\beta_{40}$ and $A\beta_{42}$ responsible for the senile plaques in Alzheimer's disease. We reported high content of alpha helical structure in $A\beta_{42}$ isoforms than $A\beta_{40}$. However, $A\beta_{42}$ found to exist in monofibril to difibril state while $A\beta_{40}$ reported upto trifibrilar state in terms of their complexity. Moreover, they shared their aggregation regions rich in hydrophobic residues with some additional amino acids. For instance, the aggregation sites responsible for intra- and intermolecular fibril formation in $A\beta_{40}$ and $A\beta_{42}$ were HQKLVFFAEDV (14-24)/ GAIIGLMVGGVV (29-40) and HQKLVFFAEDVGS (14-26)/ GAIIGLMVGGVVI (29-41). Although the specific aggregation sites for individual fibrillar peptides have shown in **Figure 5.1** that were populated with glycine, alanine, valine, leucine, isoleucine and phenylalanine.

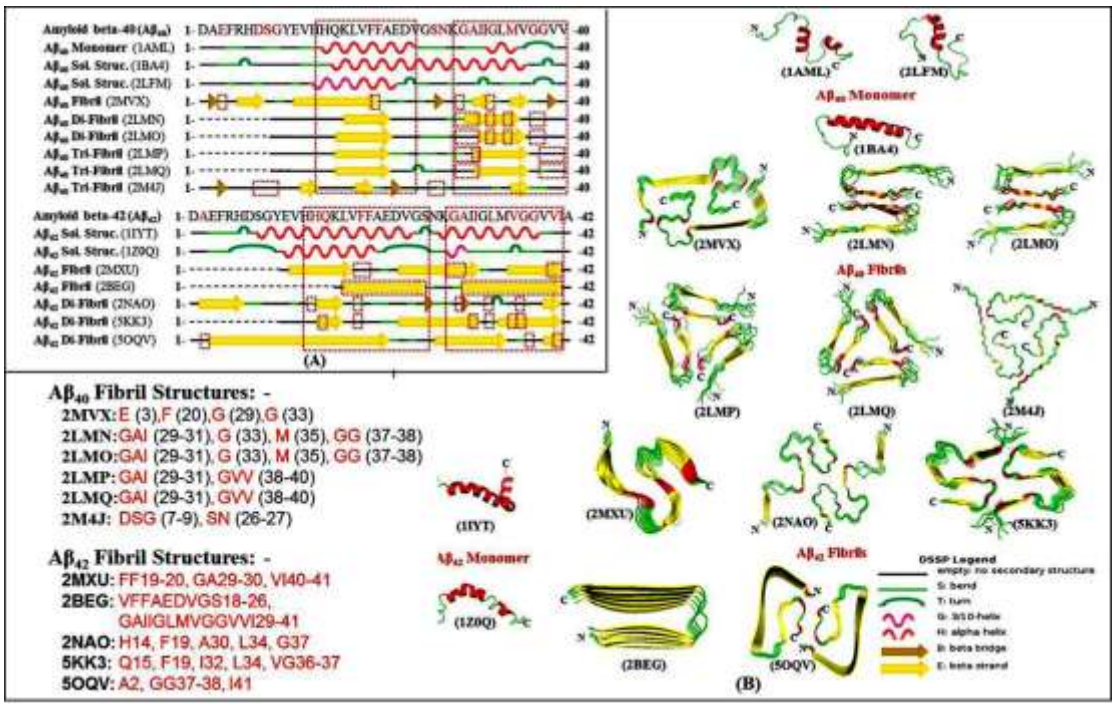


Figure 5.1: Molecular basis of Amyloid beta peptide aggregation- (A) Structural changes in $A\beta_{40}$/$A\beta_{42}$ pathology: Helical conformation of amyloid beta peptide gets distorted into the fibril structure rich in beta strands and few beta bridges. Polypeptide regions, including HQKLVFFAEDV (14-24) and GAIIGLMVGGVV (29-40) are responsible for the intra- and intermolecular fibril formation and consequent aggregation of $A\beta_{40}$ peptides. Similarly, HQKLVFFAEDVGS (14-26) and GAIIGLMVGGVVI (29-41) regions are responsible for intra- and intermolecular fibril formation and consequent aggregation of $A\beta_{42}$ peptides. Moreover, fibril-forming peptides are highlighted in red colour and fibril specific aggregation regions are shown with red rectangles that showed the sequence motif responsible for aggregation of amyloid beta peptide. **(B) 3D Structure of $A\beta_{40}$/$A\beta_{42}$ peptides:** $A\beta_{40}$ monomers (1AML, 2LFM, 1BA4) and $A\beta_{42}$ monomers (1IYT, 1Z0Q) are shown in its cartoon view with aggregation prone region highlighted in red. The reported $A\beta_{40}$/$A\beta_{42}$ fibrils are shown in cartoon view with their aggregation prone regions in red colour respectively. (2MVX: E3, F20, G29, G33; 2LMN: GAI29-31, G33, M35, GG37-38; 2LMO: GAI29-31, G33, M35, GG37-38; 2LMP: GAI29-31, GVV38-40; 2LMQ: GAI29-31, GVV38-40; 2M4J: DSG7-9, SN26-27/ 2MXU: FF19-20, GA29-30, VI40-41; 2BEG: VFFAEDVGS18-26, GAIIGLMVGGVVI29-41; 2NAO: H14, F19, A30, L34, G37; 5KK3: Q15, F19, I32, L34, VG36-37, 5OQV: A2, GG37-38, I41).

5.3.1.2 Microtubule associated Tau protein

The MAPT is a 758 amino acid residue long protein whose microtubule-binding domain spans from 561 to 697 amino acids. It is hydrophilic, unstructured and dynamic in its aggregation. Therefore, it binds to microtubule in a random coil like fashion. The amyloid spine forming TAU (5V5B, 5V5C, 4NP8, 3OVL, 2ON9)

revealed the KVQIINKKLD (591-600), VQIINK (592-597) and VQIVYK (623-628) sequence motif crucial for its aggregation. Moreover, Tau's fibrillar form (6HRE, 5O3O, 5O3L) identified two types of helical filaments, including paired helical and straight helical form. The paired helical filaments reported intra- and inter-molecular interactions with help of VQIVYK (623-628) and GGG (650-652) residues while straight helical filaments with help of VQIVYK (623-628) and Valine (630), and Glycine (640) residues. Moreover, the protein-specific aggregation sites and the structural changes in their secondary structures have shown in **Figure 5.2.**

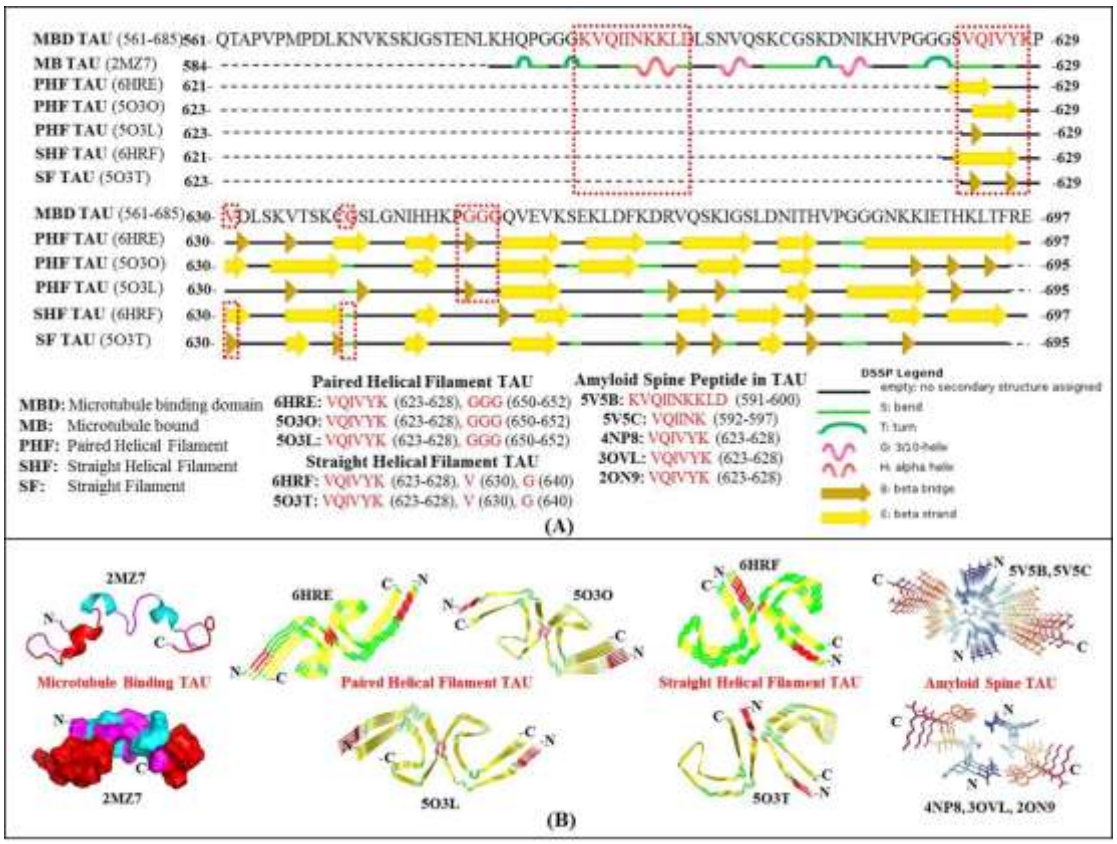


Figure 5.2: Molecular basis of TAU protein aggregation- (A) Structural changes in TAU pathology: Helical conformation of microtubule binding domain is distorted into the filament structure (paired helical/ straight helical/ straight) rich in beta strands and beta bridges. Tripeptide glycine (650-652; Red) is responsible for paired helical filament while valine (630; Red) and glycine (640; Red) are responsible for straight helical filament formation and consequent aggregation. Moreover, amyloid spines forming peptides are highlighted in red colour that showed the sequence motif VQI(IN)/(VY)K responsible for aggregation of TAU protein in Alzheimer's disease. **(B) 3D Structure of TAU protein:** Microtubule binding domain of TAU (2MZ7) is shown in its cartoon and surface view with aggregation prone region highlighted in red. Paired helical filaments of TAU (6HRE, 5O3O, 5O3L) reported in

AD are shown in ribbon/cartoon view with their aggregation prone regions in red colour. Likewise, Straight helical filaments of TAU (6HRF, 5O3T) reported in AD are shown in ribbon/cartoon view with their aggregation prone regions in red colour. Further, amyloid spine forming TAU peptide models KVQIINKKLD and VQIVYK are shown in Licorice view that formed amyloid aggregate with help of interactions between valine and Isoleucine.

5.3.1.3 Alpha-synuclein

The α-synuclein is a small protein of 140 amino acids chief among other isoforms, i.e. β and γ-synuclein. The conformational changes in its native structure take place to form prefibrillar oligomers and consequent fibril formation. It is also reported to attain amyloid like conformations (4R0U, 4R0W, 4RIK, 4RIL), which is formed by GVVHGVTTVA (47-56), TGVTAVA (72-78), VVTGVTA (70-76), AVVTGVTAV (69-77), and GAVVTGVTAVA (68-78) segments. Further, the polypeptide regions, GVVHGVATVAE (47-57) and GAVVTGVTAVA (68-78) is found to interact and form intra- and intermolecular fibril formation that have shown in **Figure 5.3.** These α-synuclein fibrils are rich in beta strands and beta bridges with varied structural conformations that initiate the lewy body formation in Parkinson's disease.

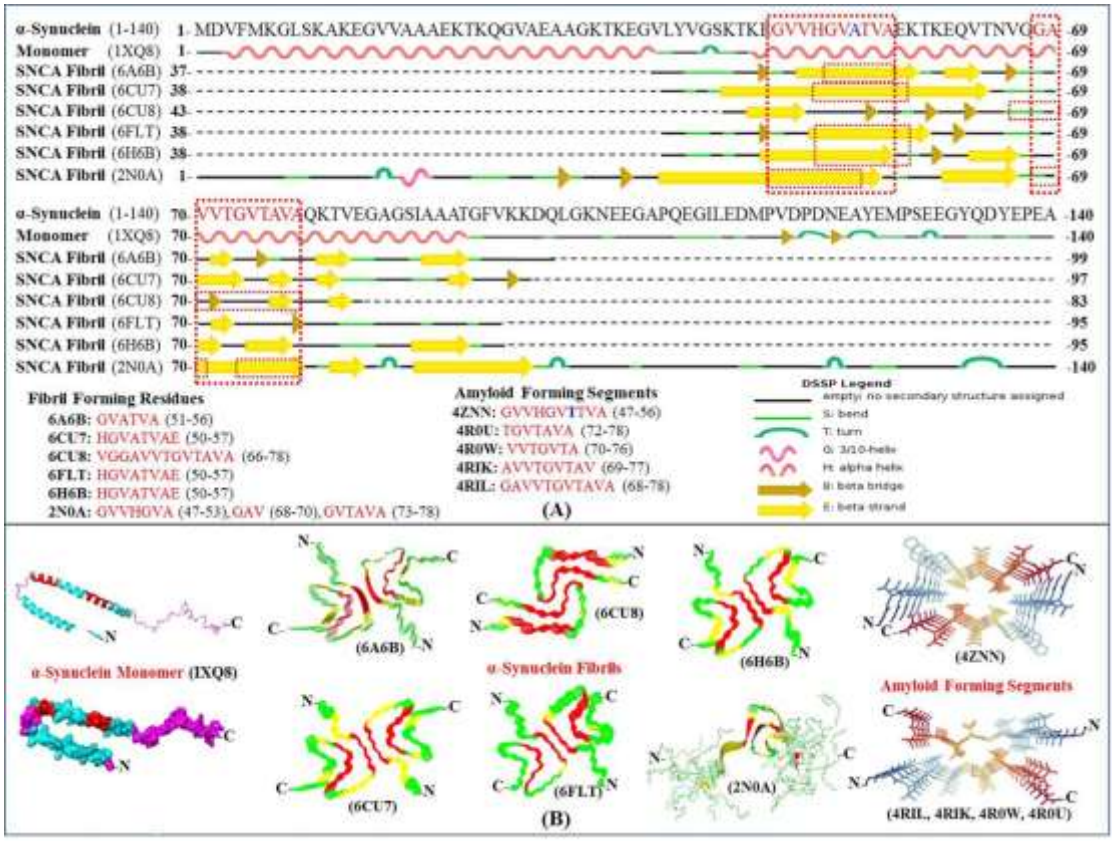


Figure 5.3: Molecular basis of α-Synuclein protein aggregation- (A) Structural changes in α-Synuclein pathology: Helical conformation of synuclein monomer is distorted into the fibril structure rich in beta strands and beta bridges. Polypeptide regions, including GVVHGVATVAE (47-57) and GAVVTGVTAVA (68-78) are responsible for the intra- and intermolecular fibril formation and consequent aggregation of SNCA protein. Moreover, amyloid forming peptides are highlighted in red colour that showed the sequence motif responsible for aggregation of SNCA protein in Alzheimer's disease. Fibril-specific aggregation regions are shown with green rectangles. **(B) 3D Structure of α-Synuclein protein:** SNCA monomer (IXQ8) is shown in its cartoon and surface view with aggregation prone region highlighted in red. Reported alpha synuclein fibrils (6A6B, 6CU7, 6CU8, 6FLT, 6H6B, 2N0A) are shown in ribbon/cartoon view with their aggregation prone regions in red colour. Likewise, Straight helical filaments of TAU (6HRF, 5O3T) reported in AD are shown in ribbon/cartoon view with their aggregation prone regions in red colour. Further, amyloid forming peptide models 4ZNN and 4RIL is shown in Licorice view that formed amyloid aggregate with help of interactions between their intermittent residues, glycine and valine.

5.3.2 Functional partners of Aβ, Tau, and α-Synuclein involved in Alzheimer's and Parkinson's disease pathology

The top hundred interacting partners among Aβ, Tau and α-Synuclein proteins further iterated with another top 100 interactors at high confidence revealed a set of 22 AβPP interacting proteins in AD while 6 in PD. Similarly, 12 Tau interacting proteins are repoted in AD and 8 in PD. Likewise, 6 α-Synuclein interacting proteins are found in AD and 10 in PD. In summary, the network identified 26 AD-related proteins, including ADAM10, APBB1, APH1A, APOE, APP, BACE1, BAD, CALM1, CAPN1, CAPN2, CASP3, CASSP8, CDK5, CDK5R1, GAPDH, GSK3B, MAPK1, MAPK3, MAPT, NAE1, NCSTN, PLCB2, PSEN1, PSEN2, PSENEN, and SNCA. However, it revealed 18 PD-related proteins, including PRKACB, PRKACG, PRKACA, GNAI3, TH, DRD2, GPR37, LRRK2, SNCA, SLC6A3, SNCAIP, PARK7, PARK2, CASP3, UBE2L6, UCHL1, PINK1, and UBB. The network maps of the functional partners are shown in **Figure 5.4,** and their corresponding functions are summarized in **Table 5.1.**

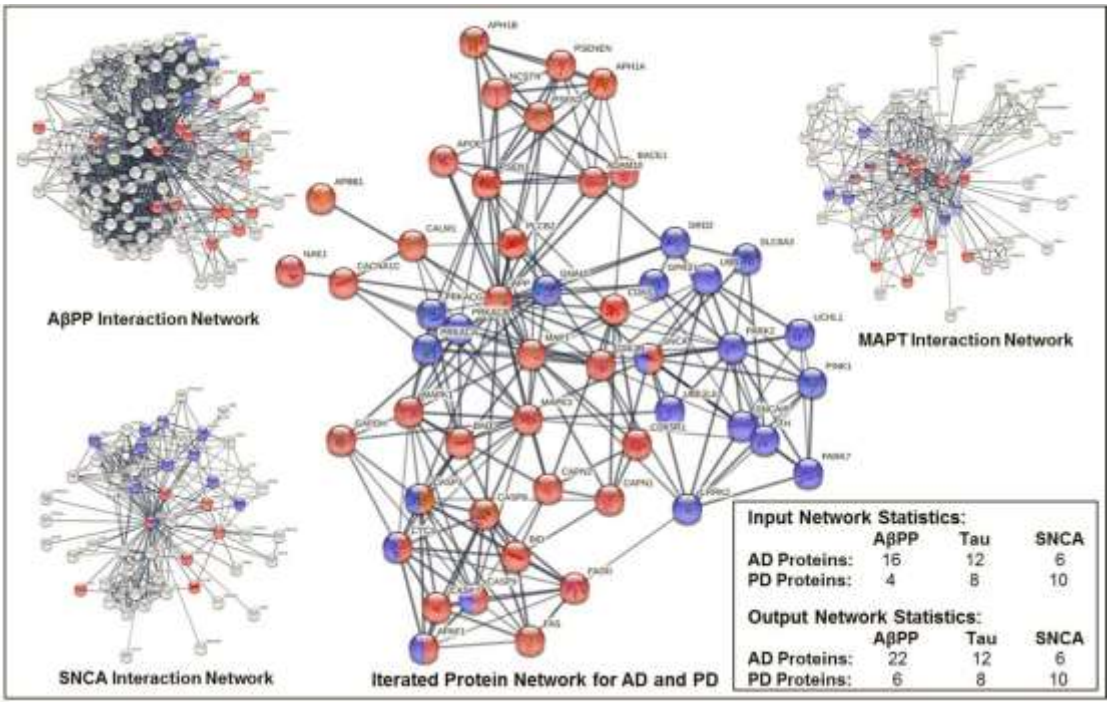


Figure 5.4: Functional association network of AβPP, Tau and α-Synuclein interacting-proteins in AD and PD- The top 100 amyloid beta precursor interactor input proteins involved 16 AD-related proteins- ADAM10, APBB1, APOE, BACE1, CAPN1, CAPN2, CASP8, GAPDH, MAPK1, MAPK3, MAPT, NAE1, NCSTN, PSEN1, PSEN2, SNCA and 4 PD-related proteins- DRD2, GNAI3, GPR37, SNCA. Similarly, top 100 tau interactor input proteins involved 12 AD-related proteins- APOE, APP, CALM1, CASP3, CDK5, CDK5R1, GSK3B, MAPK1, MAPK3, PSEN1, PSEN2, SNCA and 8 PD-related proteins- CASP3, LRRK2, PRKACA, PRKACB, PRKACG, SNCA, PARK2, UBB. Likewise, top 100 α-Synuclein interactor input proteins involved 6 AD-related proteins- APP, BAD, CAPN1, GSK3B, MAPT, PLCB2 and 10 PD-related proteins- LRRK2, PARK2, PARK7, PINK1, SLC6A3, SNCAIP, TH, UBB, UBE2L6, UCHL1. Moreover, the top 100 iterated interaction network of AβPP, Tau and α-Synuclein involved the following proteins. (**ADAM10-** Disintegrin and metalloproteinase domain-containing protein 10; **APBB1-** Amyloid-beta A4 precursor protein-binding family B member 1; **APH1A-** Gamma-secretase subunit APH-1A; **APOE-** Apolipoprotein E; **BACE1-** Beta-secretase 1; **BAD-** Bcl2-associated agonist of cell death; **CALM1-** Calmodulin-1; **CAPN1-** Calpain-1 catalytic subunit; **CAPN2-** Calpain-2 catalytic subunit; **CASP3-** Caspase-3; **CDK5-** Cyclin-dependent-like kinase 5; **CDK5R1-** Cyclin-dependent kinase 5 activator 1; **DRD2-** D(2) Dopamine receptor; **GAPDH-** Glyceraldehyde-3-phosphate dehydrogenase; **GNAI3-** Guanine nucleotide-binding protein G(k) subunit alpha; **GPR37-** Prosaposin receptor GPR37; **GSK3B-** Glycogen synthase kinase-3 beta; **LRRK2-** Leucine-rich repeat serine/threonine-protein kinase 2; **MAPK1-** Mitogen-activated protein kinase 1; **MAPK3-** Mitogen-activated protein kinase 3; **MAPT-** Microtubule-associated protein tau; **NAE1-** NEDD8-activating enzyme E1 regulatory subunit; **NCSTN-** Nicastrin; **PARK2-** E3 ubiquitin-protein ligase parkin; **PARK7-** Protein/nucleic acid deglycase DJ-1; **PINK1-** Serine/threonine-protein kinase PINK1; **PLCB2-** 1-

phosphatidylinositol 4;5-bisphosphate phosphodiesterase beta-2; **PRKACA-** cAMP-dependent protein kinase catalytic subunit alpha; **PRKACB-** cAMP-dependent protein kinase catalytic subunit beta; **PRKACG-** cAMP-dependent protein kinase catalytic subunit gamma; **PSEN1-** Presenilin-1; **PSEN2-** Presenilin-2; **PSENEN-** Gamma-secretase subunit PEN-2; **SLC6A3-** Sodium-dependent dopamine transporter; **SNCAIP-** Synphilin-1; **TH-** Tyrosine 3-monooxygenase; **UBB-** Polyubiquitin-B; **UBE2L6-** Ubiquitin/ISG15-conjugating enzyme E2 L6; **UCHL1-** Ubiquitin carboxyl-terminal hydrolase isozyme L1.)

Table 5.1: Interacting partners of AβPP, Tau and α-Synuclein proteins and their corresponding functions

Interacting Proteins		Molecular Functions
ADAM10	Disintegrin and metalloproteinase domain-containing protein 10	Responsible for the proteolytic release of TNF-alpha and several other cell-surface proteins, including heparin-binding epidermal growth-like factor, ephrin-A2, CD44, CDH2 and for constitutive and regulated alpha-secretase cleavage of amyloid precursor protein (APP)
APBB1	Amyloid-beta A4 precursor protein-binding family B member 1	Transcription coregulator with both coactivator and corepressor functions that forms a transcriptionally active complex with the gamma-secretase-derived amyloid precursor protein (APP) intracellular domain play a role in DNA damage response
APH1A	Gamma-secretase subunit APH-1A	Endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP
APOE	Apolipoprotein E	Mediates the binding, internalization, and catabolism of lipoprotein particles
BACE1	Beta-secretase 1	Responsible for the proteolytic processing of the amyloid precursor protein (APP)
BAD	Bcl2-associated agonist of cell death	Promotes cell death and appears to act as a link between growth factor receptor signaling and the apoptotic pathways
CALM1	Calmodulin-1	Mediates the control of a large number of enzymes, ion channels, aquaporins and other proteins through calcium-binding
CAPN1	Calpain-1 catalytic subunit	Catalyzes limited proteolysis of substrates involved in cytoskeletal remodeling and signal transduction
CAPN2	Calpain-2 catalytic subunit	Catalyzes limited proteolysis of substrates involved in cytoskeletal remodeling and signal transduction
CASP3	Caspase-3	Involved in the activation cascade of caspases responsible for apoptosis execution
CDK5	Cyclin-dependent-like kinase 5	Essential for neuronal cell cycle arrest and differentiation and may be involved in apoptotic cell death in neuronal diseases by triggering abortive cell cycle re-entry
CDK5R1	Cyclin-dependent kinase 5 activator 1	Neuron specific activator of CDK5 involved in dendritic spine morphogenesis and required for neurite outgrowth and cortical lamination
DRD2	D(2) dopamine receptor	Dopamine receptor whose activity is mediated by G proteins which inhibit adenylyl cyclase and promote cell proliferation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Play a role in glycolysis and nuclear functions via participating in transcription, RNA transport, DNA replication and apoptosis
GNAI3	Guanine nucleotide-binding protein G(k) subunit alpha	Function as transducers downstream of G protein-coupled receptors (GPCRs) in numerous signaling cascades

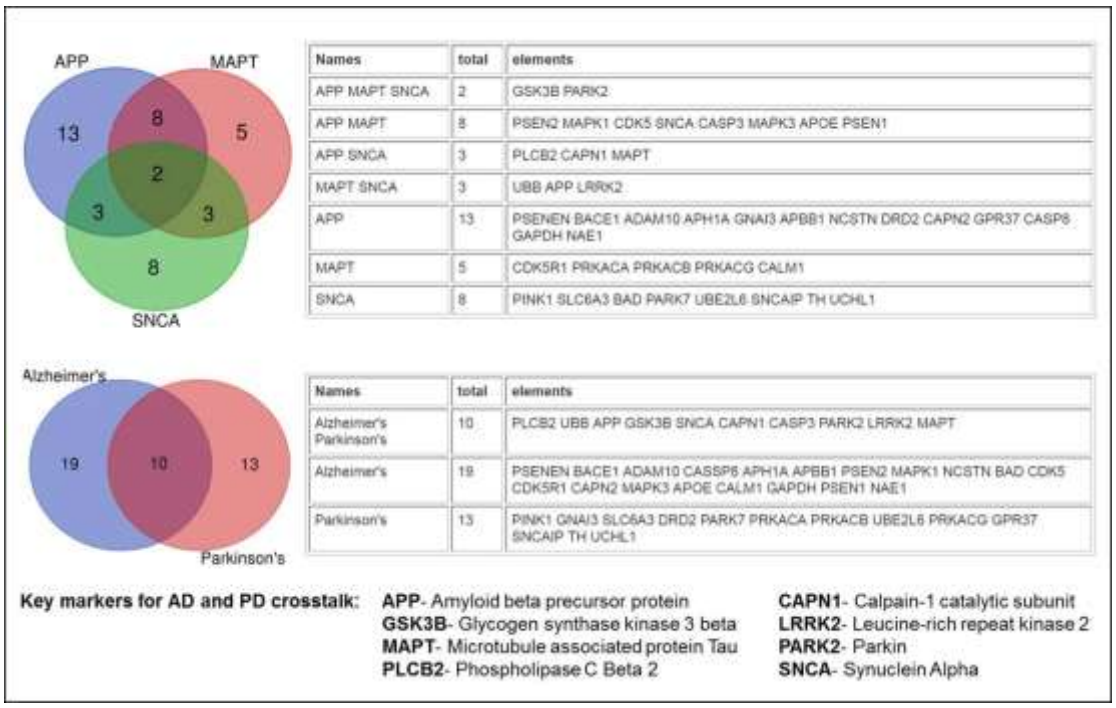
Interacting Proteins		Molecular Functions
GPR37	Prosaposin receptor GPR37	Receptor for the neuroprotective and glioprotective factor prosaposin where ligand binding induces endocytosis, followed by an ERK phosphorylation cascade
GSK3B	Glycogen synthase kinase-3 beta	Acts as a negative regulator in the hormonal control of glucose homeostasis, Wnt signaling and regulation of transcription factors and microtubules
LRRK2	Leucine-rich repeat serine/threonine-protein kinase 2	Positively regulates autophagy through a calcium- dependent activation of the CaMKK/AMPK signaling pathway
MAPK1	Mitogen-activated protein kinase 1	Acts as an essential component of the MAP kinase signal transduction pathway and mediates diverse biological functions such as cell growth, adhesion, survival and differentiation through the regulation of transcription, translation, cytoskeletal rearrangements
MAPK3	Mitogen-activated protein kinase 3	Acts as an essential component of the MAP kinase signal transduction pathway and mediates diverse biological functions such as cell growth, adhesion, survival and differentiation through the regulation of transcription, translation, cytoskeletal rearrangements
NAE1	NEDD8-activating enzyme E1 regulatory subunit	Regulatory subunit of the dimeric UBA3-NAE1 E1 enzyme
NCSTN	Nicastrin	Catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein)
PARK2	E3 ubiquitin-protein ligase parkin	Functions within a multiprotein E3 ubiquitin ligase complex, catalyzing the covalent attachment of ubiquitin moieties onto substrate proteins
PARK7	Protein/nucleic acid deglycase DJ-1	Catalyzes the deglycation of the Maillard adducts formed between amino groups of proteins or nucleotides and reactive carbonyl groups of glyoxals and functions as a protein deglycase that repairs methylglyoxal- and glyoxal-glycated proteins, and releases repaired proteins and lactate or glycolate, respectively
PINK1	Serine/threonine-protein kinase PINK1	Protects against mitochondrial dysfunction during cellular stress by phosphorylating mitochondrial proteins and triggering selective autophagy (mitophagy) by mediating activation and translocation of Parkin
PLCB2	1-phosphatidylinositol 4;5-bisphosphate phosphodiesterase beta-2	Involved in the production of the second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3)
PRKACA	cAMP-dependent protein kinase catalytic subunit alpha	Phosphorylates a large number of substrates in the cytoplasm and the nucleus and regulates the abundance of compartmentalized pools of its regulatory subunits
PRKACB	cAMP-dependent protein kinase catalytic subunit beta	Mediates cAMP-dependent signaling triggered by receptor binding to GPCRs that regulates diverse cellular processes such as cell proliferation, the cell cycle, differentiation and regulation of microtubule dynamics, chromatin condensation and decondensation, nuclear envelope disassembly and reassembly
PRKACG	cAMP-dependent protein kinase catalytic subunit gamma	Phosphorylates a large number of substrates in the cytoplasm and the nucleus

Interacting Proteins		Molecular Functions
PSEN1	Presenilin-1	Presenilin-2; Probable catalytic subunit of the gamma-secretase complex, an endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein) and may play a role in intracellular signaling and gene expression or in linking chromatin to the nuclear membrane
PSEN2	Presenilin-2	Presenilin-2; Probable catalytic subunit of the gamma-secretase complex, an endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein) and may play a role in intracellular signaling and gene expression or in linking chromatin to the nuclear membrane
PSENEN	Gamma-secretase subunit PEN-2	Catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein) and modulates both endoproteolysis of presenilin and gamma-secretase activity
SLC6A3	Sodium-dependent dopamine transporter	Terminates the action of dopamine by its high affinity sodium-dependent reuptake into presynaptic terminals
SNCAIP	Synphilin-1	Isoform 2 inhibits the ubiquitin ligase activity of SIAH1 and inhibits proteasomal degradation of target proteins
TH	Tyrosine 3-monooxygenase	Plays an important role in the physiology of adrenergic neurons
UBB	Polyubiquitin-B	Form polyubiquitin chains on target proteins and regulate different functions depending on the Lys residue of the ubiquitin that is linked
UBE2L6	Ubiquitin/ISG15-conjugating enzyme E2 L6	Catalyzes the covalent attachment of ubiquitin or ISG15 to other proteins
UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Ubiquitin-protein hydrolase involved both in the processing of ubiquitin precursors and of ubiquitinated proteins

5.3.3 GSK3B and PARK2 are key markers for AD-PD crosstalk commonly interacting with Aβ, Tau, and α-Synuclein

The Venn diagram analysis of the top interacting partners reported only two proteins, including GSK3B and PARK2 to be commonly interacting with Aβ, Tau and α-Synuclein. Instead other markers found to interact either any of the two or any one of the Aβ, Tau and α-Synuclein proteins. For instance, PSEN2, MAPK1, CDK5, SNCA, CASP3, MAPK3, APOE, and PSEN1 found to interact with AβPP and Tau, while PLCB2, CAPN1, and MAPT interacting with AβPP and α-Synuclein respectively. Likewise, UBB, APP, and LRRK2 observed to interact with Tau and α-Synuclein proteins. Moreover, their disease incidence analysis reported these eight markers, including APP, CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2, and SNCA involved in the crosstalk of Alzheimer's and Parkinson's disease at the molecular

level. While other markers specific for AD and PD interacting with any of their pathological partners Aβ, Tau and α-Synuclein is shown in **Figure 5.5.**



Names	total	elements
APP MAPT SNCA	2	GSK3B PARK2
APP MAPT	8	PSEN2 MAPK1 CDK5 SNCA CASP3 MAPK3 APOE PSEN1
APP SNCA	3	PLCB2 CAPN1 MAPT
MAPT SNCA	3	UBB APP LRRK2
APP	13	PSENEN BACE1 ADAM10 APH1A GNAI3 APBB1 NCSTN DRD2 CAPN2 GPR37 CASP8 GAPDH NAE1
MAPT	5	CDK5R1 PRKACA PRKACB PRKACG CALM1
SNCA	8	PINK1 SLC6A3 BAD PARK7 UBE2L6 SNCAIP TH UCHL1

Names	total	elements
Alzheimer's Parkinson's	10	PLCB2 UBB APP GSK3B SNCA CAPN1 CASP3 PARK2 LRRK2 MAPT
Alzheimer's	19	PSENEN BACE1 ADAM10 CASSP6 APH1A APBB1 PSEN2 MAPK1 NCSTN BAD CDK5 CDK5R1 CAPN2 MAPK3 APOE CALM1 GAPDH PSEN1 NAE1
Parkinson's	13	PINK1 GNAI3 SLC6A3 DRD2 PARK7 PRKACA PRKACB UBE2L6 PRKACG GPR37 SNCAIP TH UCHL1

Figure 5.5: Crosstalk markers involved in the pathology of Alzheimer's and Parkinson's disease- The first Venn diagram analysis highlighted the common interacting partners of Aβ, Tau and α-Synuclein while the second Venn diagram analysis showed the disease incidences of their interacting proteins into Alzheimer's and Parkinson's disease.

5.3.4 PARK2 and STUB1 are the key ubiquitin E3 ligases regulating the clearance of pathological markers in AD and PD

The ubiquitin E3 ligases were identified against all the AD-PD crosstalk markers and were classified as direct and indirect regulators depending on their potential interaction with Aβ, Tau and α-Synuclein. Those E3 ligases involved in the ubiquitination of Aβ, Tau and α-Synuclein was classified as direct regulators while those involved with other pathological markers were classified as indirect regulators. We reported only PARK2 and STUB1 to be commonly interacting with most of the AD-PD cross talk markers, including AβPP, MAPT, SNCA, and LRRK2. Instead other E3 ligases found to regulate AβPP, Tau, and α-Synuclein ubiquitination individually or in different combinations. Likewise, we reported indirect regulators that were involved with the ubiquitination of markers other than AβPP, Tau, and

α-Synuclein, such as CAPN1, GSK3B, LRRK2, PARK2, and PLCB2. Among them, TRAF2 found to commonly-regulate GSK3B, LRRK2, and PARK2 markers. Similarly, we defined a spectrum of E3 ligases involved in the ubiquitination of AD-PD crosstalk markers that have been summarized in **Table 5.2.** The comprehensive study reported 149 regulatory ubiquitin E3 ligases for the ubiquitination of PARK2 ubiquitin E3-ligase. It suggests the PARK2's involvement in both the pathology and clearance biology, i.e. negative and positive role in neurodegenerative disorders like AD and PD.

Table 5.2: The key ubiquitin E3 ligases regulating the clearance of AD-PD crosstalk markers

Direct regulators for AβPP, Tau, and α-Synuclein clearance		
Names	**Total**	**Elements**
APP, LRRK2, MAPT, PARK2, SNCA	1	STUB1
APP, LRRK2, MAPT, SNCA	1	PARK2
GSK3B, PARK2, SNCA	2	FBXW7, SIAH1
LRRK2, PARK2, SNCA	1	FBXO7
APP, CAPN1	1	WDTC1
APP, MAPT	1	SYVN1
GSK3B, SNCA	1	TRAF6
PARK2, SNCA	4	RNF19A, SIAH2, TRIM32, NEDD4
APP	1	FBXL2
MAPT	1	MARCH7
SNCA	1	FBXO45

Indirect regulators for AβPP, Tau, and α-Synuclein clearance		
Names	**Total**	**Elements**
GSK3B, LRRK2, PARK2	1	TRAF2
CAPN1, GSK3B	1	CDH1
CAPN1, PARK2	1	TRIM63
GSK3B, PARK2	5	CUL3, SKP1, RBX1, CUL1, UBE3A
LRRK2, PARK2	4	WSB1, RANBP2, HERC2, HACE1
PARK2, PLCB2	1	RNF41
GSK3B	9	UBR5, TRIM29, BIRC2, MAP3K1, PIAS1, XIAP, NHLRC1, MDM2, APC2
LRRK2	4	ERCC8, TRIM23, RHOBTB1, RHOBTB3

Indirect regulators for AβPP, Tau, and α-Synuclein clearance		
Names	**Total**	**Elements**
PARK2	149	TRAF7, MUL1, RNF114, SPSB2, RNF217, HEWE1, DET1, ASB7, HERC1, BTBD1, VPRBP, TRIM69, HECW2, FBXL18, RNF115, ASB14, HERC3, ASB11, LRRC41, FBXW4, RNF182, KLHL2, UBR1, SKP2, ASB17, RNF25, TCEB1, FBXL16, UBE3C, ASB4, RNF34, FBXL5, FBXO4, FBXL12, FBXO21, FBXO6, CDC20, MYLIP, TRIM9, TRIM11, FBXL22, KBTBD6, UBOX5, KBTBD8, ASB16, NEDD4L, ASB6, KLHL22, CDC23, RNF14, HERC4, RBCK1, ASB1, KLHL21, TCEB2, HECTD3, ASB9, FBXL3, SOCS3, ASB12, FBXW8, TRIP12, FBXL15, CUL2, ZBTB16, MGRN1, FBXL4, RNF7, CUL7, RNF19B, SPSB1, FBXO41, RNF123, RNF31, KLHL25, ITCH, HECTD1, RNF138, ARIH1, FBXO2, RFWD2, AMFR, TRIM36, UBR4, FBXL13, LRR1, MARCH5, TRIM39, KLHL20, CCNF, CUL5, FBXL19, TRIM71, SOCS1, WWP1, FBXL8, LNX1, ASB13, FBXO27, RNF111, RCHY1, TRIM21, PJA2, FBXO22, PJA1, TRIM4, FBXO17, LRSAM1, FBXO10, UBE3B, ASB8, FBXO30, SH3RF1, FBXO44, FBXW10, ASB18, RNF130, VHL, FBXO15, DZIP3, FBXW2, TRIM37, ASB5, ZNF645, FBXO9, FBXW9, ZNRF1, UBE4A, UBE3D, CDC26, ASB10, UBR2, ASB2, FBXW5, FBXL7, ASB15, FBXL14, TRIM50, ARIH2, HECTD2, FBXO11, FBXO31, FBXO40, KLHL9, KLHL11, RBBP6, TRIM41, RLIM, TRAIP

5.3.5 Ubiquitination biology of toxic Aβ, Tau, and α-synuclein protein clearance

The ubiquitination reaction of Aβ, Tau, and α-synuclein clearance is a complex biology of interactions among a series of E1-activating, E2-conjugating, E3-ligating and deubiquitinating enzymes. Here, we reported the important ubiquitination markers, including the E3 ligases- PARK2, STUB1, FBXW7 SIAH1, FBXO7, WDTC1, SYVN1, TRAF6, RNF19A, SIAH2, TRIM32, NEDD4, FBXL2, MARCH7, and FBXO45, and the deubiquitinases- ATXN3, USP8, PSMD14, and UCHL1 directly regulating the clearance of Aβ, Tau and α-synuclein. Furthermore, identified their corresponding ubiquitin E2-conjugating enzymes- UBE2A/B/C/F/G1/G2/H/J1/J2/K/L6/M/N/O/Q1/S/U/W/Z and ubiquitin E1-activating enzymes- ATG7, NAE1, SAE1, UBA1/3/5/6/7 involved in the ubiquitination biology of all these protein aggregates. In addition, we reported the majority with a similar set of E2s and E1s with crucial differences in their deubiquitinases. The important ubiquitination enzymes for the clearance of AD-PD cross talk markers are summarized in **Table 5.3.** Moreover, the enzymatic regulation of ubiquitination

reaction for Aβ, Tau, and α-synuclein with their specific ubiquitin E3-ligases are mapped in **Figure 5.6A.** These UPS enzymes along with their target AD-PD cross-talk markers were further investigated for their functional annotations, including the biological processes, reactome pathways, protein domains, and molecular functions **(Figure 5.6B).** The biological process analysis revealed only the UPS enzymes including **UbE1s-** *SAE1, UBA1/6;* **UbE2s-** *BIRC6, UBE2A/B/C/G1/G2/H/J2/K/L3/N/S/T/W/Z;* **UbE3s-** *FBXL2, FBXO7/45, FBXW7, NEDD4, PARK2, STUB1, SYVN1, TRIM32, WDTC1;* and **DUBs-** *USP7, VCPIP1* to be associated with protein ubiquitination, while **UbE1s-** *UBA6;* **UbE2s-** *BIRC6, UBE2A/B/C/G1/G2/H/I/K/L3/L6/N/S/Z;* **UbE3s-** *FBXL2, FBXO7/45, NEDD4, RNF19A, SIAH1/2, STUB1, SYVN1, TRIM32,* and **DUBs-** *ATXN3, BAP1, PSMD14, USP2/4/6/7/8/14/15/18/20/21/25/28/30/36/47* to be linked with the ubiquitin dependent protein catabolic process. On the other hand, AD-PD cross-talk markers were reported to be linked with both positive (APP, GSK3B, MAPT, SNCA) and negative regulation (APP, GSK3B, LRRK2, SNCA) of neuronal death. The analysis revealed the peculiarity of LRRK2 in the pathogenesis of disease. Furthermore, the reactome pathway analysis identified three pathways with most significant scores, including i) UPS associated antigen processing (**UbE1s-** *ATG7, UBA1/3/5/6/7;* **UbE2s-** *UBE2A/B/2C/F/G1/G2/H/J1/J2/K/L3/L6/M/N/O/Q1/S/U/W/Z;* **UbE3s-** *FBXO7, FBXW7, NEDD4, RNF19A, SIAH1/2, STUB1, TRIM32;* **DUBs-** *PSMD14*), ii) CDK5 linked neurodegeneration (AD-PD cross-talk markers- APP, CAPN1), and iii) synthesis of active ubiquitin protein (**UbE1s-** *UBA1/6;* **UbE2s-** *UBE2A/B/C/G1/G2/H/K/L3/S/T/W/Z;* **DUBs-** *USP7*). In addition, the Protein domain analysis revealed the majority of ubiquitin E1 activating enzymes with UBA_e1_C domain, while ubiquitin E3 ligases with FBOX domain. Likewise, it reported DUSP domain in most of the deubiquitinases, while S_TKc domain in the majority of the AD-PD crosstalk markers. Moreover, the molecular functions and biological processes analysis of AD-PD crosstalk markers identified their roles in microglial activation, synapse organization, mitochondrial fission, amyloid fibril formation and binding with various cellular proteins, including actin, dynactin, apolipoprotein, tau and microtubule. Altogether, these findings have provided new insights into the molecular mechanisms for neurotoxic protein clearance in AD and PD.

Table 5.3: Ubiquitinational markers for AD-PD crosstalk proteins

Pathological Targets	Key Ubiquitin E3 ligases	Ubiquitinating E2 Conjugating Enzymes	Ubiquitin E1 Activating Enzymes	Deubiquitinating Enzymes
AβPP, Tau, α-Synuclein, LRRK2, PARK2	**STUB1**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2L3, BIRC6, UBE2I, UBE2T	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	ATXN3, USP8, USP7, USP19, PSMD14, SENP3
AβPP, Tau, α-Synuclein, LRRK2	**PARK2**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2L3	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	ATXN3, USP8, USP30, UCHL1, BAP1, PSMD14, USP15
AβPP, α-Synuclein, LRRK2, PARK2	**FBXO7 (FBXL2, FBXO45)**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP47, UCHL1
α-Synuclein, GSK3B, PARK2	**FBXW7**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP47, USP28, USP7, USP36
α-Synuclein, GSK3B, PARK2	**SIAH1**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2I	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP19, USP4, USP6, USP15, USP20
AβPP, Tau	**SYVN1**	UBE2G2, UBE2J1, ATG3, UBE2J2, UBE2K, UBE2G1, UBE2S	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	ATXN3, PSMD14, USP19, VCPIP1

Pathological Targets	Key Ubiquitin E3 ligases	Ubiquitinating E2 Conjugating Enzymes	Ubiquitin E1 Activating Enzymes	Deubiquitinating Enzymes
α-Synuclein, PARK2	**NEDD4**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2I, UBE2J1, UBE2J2, UBE2K, UBE2L3, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2T, UBE2U, UBE2W, UBE2Z	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP2, USP7, USP8, USP14, USP18, USP20, USP25, USP28, STAMBP
-Synuclein, PARK2	**RNF19A**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2L3	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	–
α-Synuclein, PARK2	**SIAH2**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2I	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP19, USP4, USP6, USP15, USP20
α-Synuclein, PARK2	**TRIM32**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	–
AβPP, CAPN1	**WDTC1**	UBE2M	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	SENP8
α-Synuclein, GSK3β	**TRAF6**	UBE2N, UBE2I, UBE2K, UBE2L3, UBE2O, UBE2S	ATG7, UBA1, UBA3, UBA5, UBA6, UBA7	USP4, USP7, OTUB1, OTUB2, TNFAIP3, ZRANB1, OTUD5, OTUD7A, STAMBP, UCHL1, USP2, USP15, USP20, USP21, USP25
Tau	**MARCH7**	UBE2G2, UBE2K, UBE2N	ATG7, UBA1, UBA3, UBA5, UBA6, UBA7	USP7

Red- Highest Confidence; Blue- High Confidence; Green- Medium Confidence

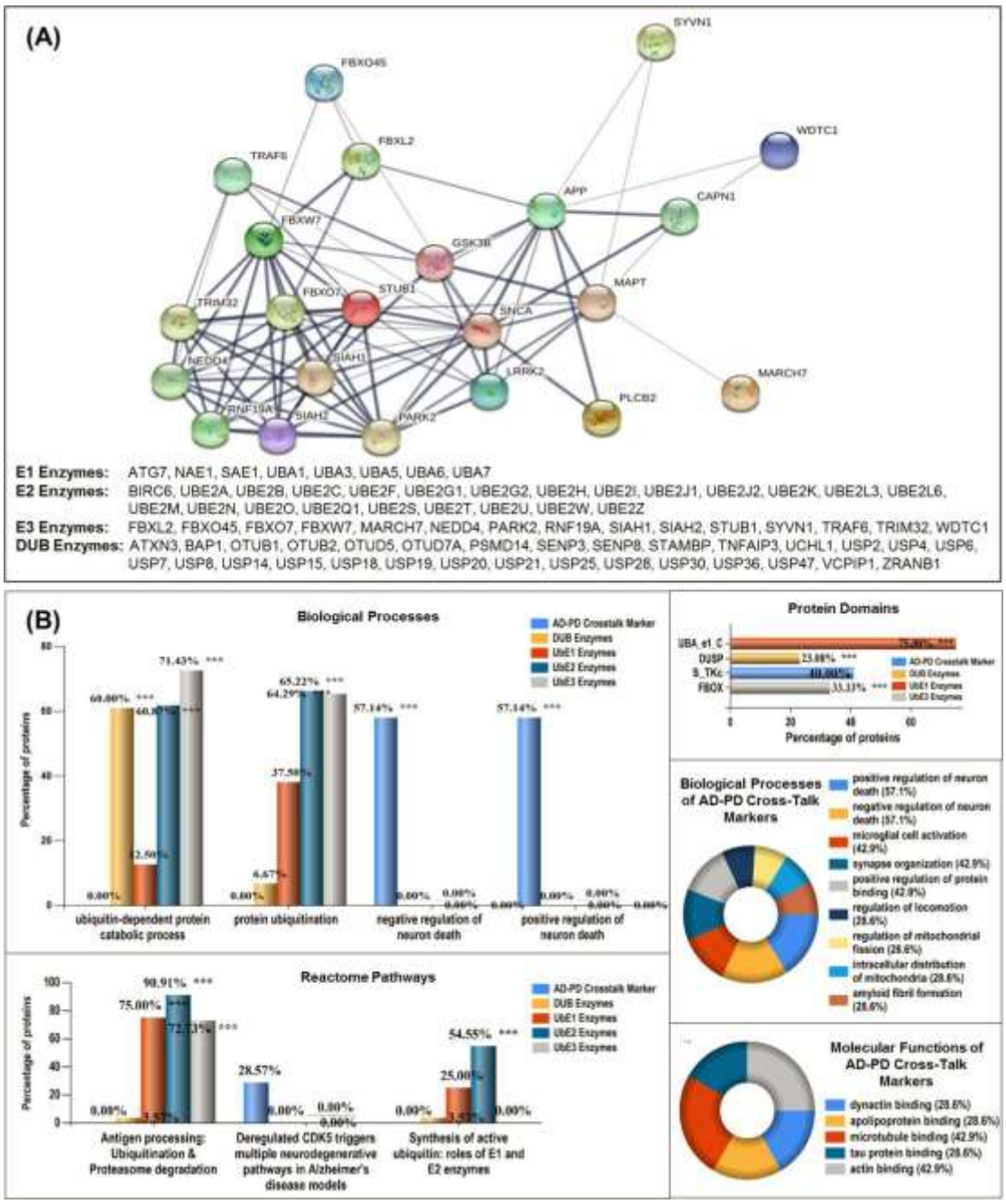


Figure 5.6: Ubiquitination enzyme network for the clearance of AD-PD crosstalk markers, including Aβ, Tau and α-synuclein- E1-activating enzymes: ATG7- Ubiquitin-like modifier-activating enzyme ATG7; **NAE1-** NEDD8-activating enzyme E1 regulatory subunit; **SAE1-** SUMO-activating enzyme subunit 1; **UBA1/3/5/6/7-** Ubiquitin-like modifier-activating enzyme 1/3/5/6/7. **E2 conjugating enzymes: BIRC6-** Baculoviral IAP repeat-containing protein 6; **UBE2A/B/C/F/G1/G2/H/I/J1/J2/K/L3/L6/M/N/O/Q1/S/T/U/W/Z-** Ubiquitin Conjugating Enzyme E2 A/B/C/F/G1/G2/H/I/J1/J2/K/L3/L6/M/N/O/Q1/S/T/U/W/Z. **E3 ligases: FBXL2-** F-box/LRR-repeat protein 2; **FBXO45-** F-box/SPRY domain-containing protein 1; **FBXO7-** F-box only protein 7; **FBXW7-** F-box/WD repeat-containing protein 7; **MARCH7-** Membrane Associated Ring-CH-Type Finger 7; **NEDD4-** Neural

precursor cell expressed developmentally down-regulated protein 4; **PARK2**- Parkin; **RNF19A-** Ring finger protein19A; **SIAH1/2-** Siah E3 Ubiquitin Protein Ligase 1/2; **STUB1-** Ubiquitin-protein ligase CHIP; **SYVN1-** Synoviolin; **TRAF6-** TNF receptor-associated factor 6; **TRIM32-** Tripartite Motif Containing 32. **Deubiquitinases: ATXN3-** Ataxin-3; **BAP1-** Ubiquitin carboxyl-terminal hydrolase BAP1; **OTUB1/2-** OTU Deubiquitinase 1/2; **OTUD5/7A-** OTU domain-containing protein 5/7A; **PSMD14-** 26S proteasome non-ATPase regulatory subunit 14; **SENP3/8-** Sentrin-specific protease 3/8; **STAMBP-** STAM-binding protein; **TNFAIP3-** Tumor necrosis factor alpha-induced protein 3; **UCHL1-** Ubiquitin carboxyl-terminal hydrolase isozyme L1; **USP2/4/6/7/8/14/15/18/19/20/21/25/28/30/36/47-** Ubiquitin carboxyl-terminal hydrolase 2/4/6/7/8/14/15/18/19/20/21/25/28/30/36/47; **VCPIP1-** Valosin Containing Protein Interacting Protein 1; **WDTC1-** WD and tetratricopeptide repeats protein 1; **ZRANB1-** Zinc Finger RANBP2-Type Containing 1. **(B) Functional enrichment analysis of AD-PD cross talk markers and the ubiquitination enzymes:** It has analyzed the most important biological processes, reactome pathways, molecular functions, and protein domains at high significance P-values, i.e. P<0.001. The bar graph compares the percentage of input proteins with their associated top scoring biological processes, reactome pathways and protein domains. **Biological processes:** Ubiquitin-dependent protein catalytic process (**UbE1s-** UBA6; **UbE2s-** BIRC6, UBE2A/B/C/G1/G2/H/I/K/L3/L6/N/S/Z; **UbE3s-** FBXL2, FBXO7/45, NEDD4, RNF19A, SIAH1/2, STUB1, SYVN1, TRIM32; **DUBs-** ATXN3, BAP1, PSMD14, USP2/4/6/7/8/14/15/18/20/21/25/28/30/36/47), Protein ubiquitination (**UbE1s-** SAE1, UBA1/6; **UbE2s-** BIRC6, UBE2A/B/C/G1/G2/H/J2/K/L3/N/S/T/W/Z; **UbE3s-** FBXL2, FBXO7/45, FBXW7, NEDD4, PARK2, STUB1, SYVN1, TRIM32, WDTC1; **DUBs-** USP7, VCPIP1), Negative regulation of neuron death (AD-PD cross-talk markers- APP, GSK3B, LRRK2, SNCA), Positive regulation of neuron death (AD-PD cross-talk markers- APP, GSK3B, MAPT, SNCA). **Reactome pathways:** Antigen processing: Ubiquitination and Proteasome degradation (**UbE1s-** ATG7, UBA1/3/5/6/7; **UbE2s-** UBE2A/B/2C/F/G1/G2/H/J1/J2/K/L3/L6/M/N/O/Q1/S/U/W/Z; **UbE3s-** FBXO7, FBXW7, NEDD4, RNF19A, SIAH1/2, STUB1, TRIM32; **DUBs-** PSMD14), Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models (**AD-PD cross-talk markers-** APP, CAPN1), Synthesis of active ubiquitin: roles of E1 and E2 enzymes (**UbE1s-** UBA1/6; **UbE2s-** UBE2A/B/C/G1/G2/H/K/L3/S/T/W/Z; **DUBs-** USP7). **Protein domains:** UBA_e1_C (**UbE1s-** UBA1/6/7), DUSP (**DUBs-** USP4/15/20), S_TKc (**AD-PD cross-talk markers-** GSK3B, LRRK2), FBOX (**UbE3s-** FBXL2, FBXO7/45, FBXW7). The doughnut chart depicted the biological processes and molecular functions associated with the AD-PD cross talk markers. **Biological processes:** Positive regulation of neuron death (APP, GSK3B, MAPT, SNCA), Negative regulation of neuron death (APP, GSK3B, LRRK2, SNCA), Microglial cell activation (APP, MAPT, SNCA), Synapse organization (APP, MAPT, SNCA), Positive regulation of protein binding (APP, GSK3B, LRRK2), Regulation of locomotion (LRRK2, SNCA), Regulation of mitochondrial fission (MAPT, LRRK2), Intracellular distribution of mitochondria (MAPT, LRRK2), Amyloid fibril formation (APP, MAPT). **Molecular functions:** Dynactin binding (MAPT, GSK3B), Apolipoprotein binding (APP, MAPT), Microtubule binding (LRRK2, MAPT, SNCA), Tau protein binding (GSK3B, SNCA), Actin binding (LRRK2, MAPT, SNCA).

5.3.6 Molecular cross talk among neurotoxic proteins and their clearance in Alzheimer's and Parkinson's disease

The functional enrichment analysis of the UPS enzymes has provided valuable insights for the choice of potential ubiquitination enzymes important for the proteasomal clearance of pathogenic proteins, while that of AD-PD cross talk markers revealed their role in protein aggregation. However, ubiquitination is a complex process of targeted protein degradation with the help of an array of ubiquitinational enzymes, especially the ubiquitin E3 ligases that are crucial for imparting the substrate specificity, but the obtained results have revealed the facts to a greater extent. Based on the evidence from our findings, we can hypothesize the molecular mechanism of the toxic protein aggregation and their clearance through the ubiquitination process for better understanding the medical state of Alzheimer's and Parkinson's disease **(Figure 5.7).** The clinical reports suggest that the identified AD-PD cross-talk markers have their pathogenic role in both the diseases, but CAPN1 and PLCB2 are two novel markers whose roles need to be investigated in both diseases, although they are known to play some role in Alzheimer's disease. Moreover, future research is required to translate these findings to devise better diagnostic and therapeutic avenues for life management and care for the patients suffering with Alzheimer's and Parkinson's disease.

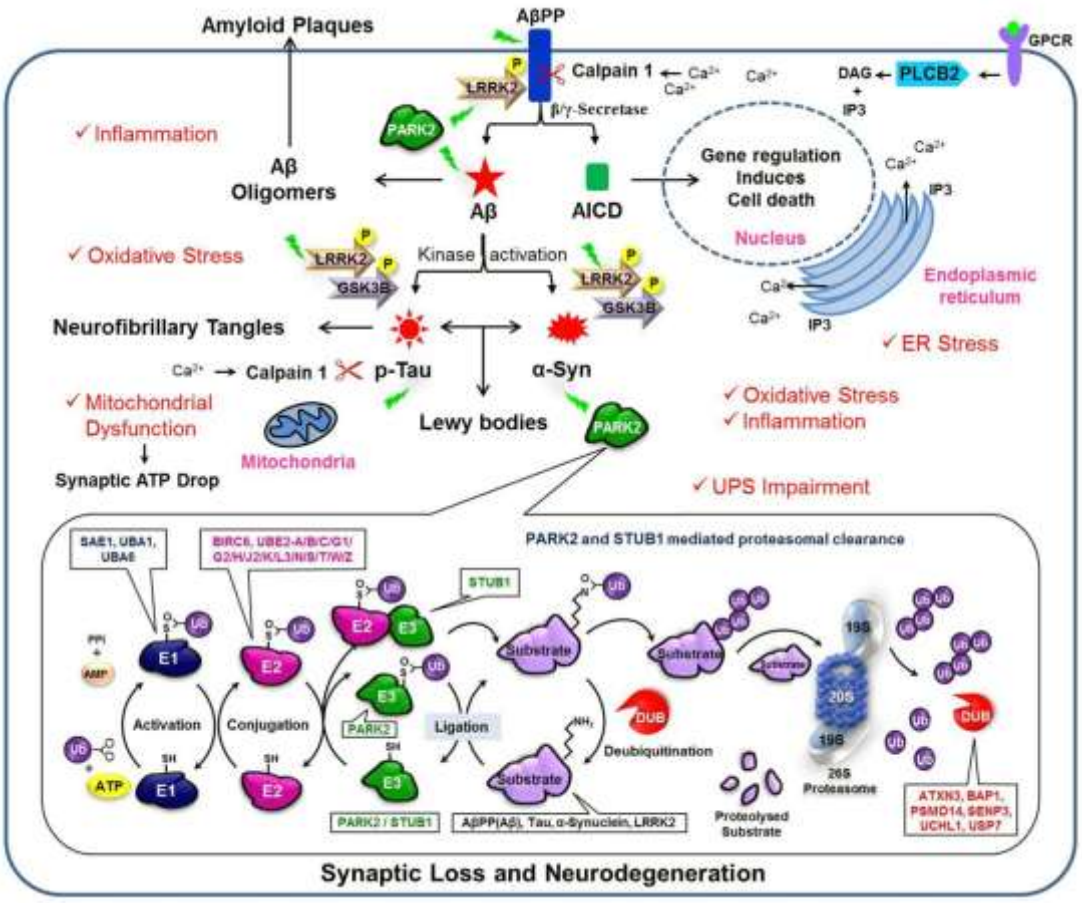


Figure 5.7: Molecular mechanism of AD-PD crosstalk marker's associated neurodegeneration and their proteasomal clearance: In diseased state, proteolytic processing of AβPP leads to the production of Aβ and amyloid intracellular domain. Here, amyloid production activates the kinases like LRRK2 and GSK3B, which in turn accelerate the phosphorylation of Tau and α-Synuclein protein thereby resulting neurofibrillary tangles and lewy bodies. On the other side, AICD triggers the apoptotic gene regulation and induces cell death. Furthermore, G-protein coupled receptor activates phospholipase B (PLCB2) that triggers the calcium release from ER and consequent action of calpain 1 on their substrates, including AβPP and Tau. Here, calapin 1 mediated tau cleavage products, induces mitochondrial dysfunction and thus ATP loss. On the other side, Parkin (PARK2) as an E3 ligase acts on the pathogenic proteins- AβPP(Aβ), Tau, α-Synuclein, and LRRK2 to mark them for proteasomal degradation with the help of diverse ubiquitin activating enzymes, conjugating enzymes, and deubiquitinases. Altogether, these toxic proteins are associated with the ER stress, oxidative stress, mitochondrial dysfunction, inflammation, and UPS impairment in neurons thereby leading to synaptic loss and neurodegeneration. **AICD-** Amyloid intracellular domain; **DAG-** Diacylglycerol; **PLCB2-** Phospholipase B.

5.4 DISCUSSION

The formation of misfolded protein aggregates is the key hallmark of many neurodegenerative diseases that trigger the neurotoxicity and consequent proteostatic collapse. In addition, active research is going on to unravel the mechanism of protein folding and aggregation. Here, the distortion of helical conformation into beta strands/bridges containing fibrils is the active principle for aggregation in amyloid beta, Tau and α-synuclein proteins. Recently, Balupuri *et al.*, has also shown the occurrence of α-strand in the monomer to drive sheet formation in the oligomers that initiates and promotes α-synuclein aggregation and fibrillation (Balupuri *et al.,* 2019). Moreover, the aggregation sites were highly populated with glycine and hydrophobic amino acid residues, including alanine, valine, isoleucine and phenylalanine involved in the intra-chain and inter-chain interactions. In support, Matsui *et al.,* has also demonstrated the α-helix rule, i.e. hydrophobicity of amino acids in the α-helix structure as a potential rationale for aggregation hotspot prediction (Matsui *et al.,* 2017). Moreover, the arrangement of monomer in antiparallel fashion leads to cooperative formation of β-sheet conformation (Lovas *et al.,* 2013), and they attain different topologies based on the diversity of their intra- and inter-chain interactions. Further, these pathological proteins (Aβ, Tau and α-synuclein) interact synergistically to accelerate the neuropathology (Clinton *et al.,* 2010); therefore, identifying their interaction with other proteins would help us to identify novel pathological markers in AD and PD. Here, the interactome analysis identified AβPP, CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2, and SNCA as key markers for AD-PD crosstalk with GSK3B and PARK2 as common interactor of amyloid beta, tau and alpha synuclein. However, these proteins were earlier known to involve in either of the disease, but recent findings have defined some of them in both diseases. For instance, LRRK2 mutations reported to promote AβPP phosphorylation and consequent AICD activity mediated neurotoxicity in PD (Chen *et al.,* 2017). Likewise, GSK3B dysregulations contributed towards Parkinson's like pathology with induced phosphorylation and aggregation of Tau and α-synuclein (Credle *et al.,* 2015). Similarly, PARK2 enhancement has compensated mitophagic alterations in AD pathology (Martín-Maestro *et al.,* 2016). Here, CAPN1 and PLCB2 are new markers, whose roles need to be investigated in both the diseases, however, they are known

to play some role in AD. Therefore, the elucidation of aggregation sites in these pathological proteins and identification of their interacting partners would enable us to identify novel therapies for multiple disease states. Further, the identification of ubiquitinational markers, including E3 ligases- PARK2, STUB1, FBXW7, SIAH1, FBXO7, WDTC1, SYVN1, TRAF6, RNF19A, SIAH2, TRIM32, NEDD4, FBXL2, MARCH7, and FBXO45 as direct regulators of Aβ, Tau and α-synuclein ubiquitination would help us to devise better therapeutic options for targeting misfolded proteins and large-scale rebalancing of proteostatic network. However, the ubiquitination reaction is a complex biology of interactions among a series of E1 activating, E2 conjugating, E3 ligating and deubiquitinating enzymes that are addressed here for the clearance of AD-PD crosstalk markers. These key findings can help the scientists to accelerate the identification of novel therapeutic modalities for such incurable neurodegenerative pathologies, including AD and PD.

Chapter VI

Summary, Discussion, and Future Perspective

CHAPTER VI

SUMMARY, DISCUSSION, AND FUTURE PERSPECTIVE

6.1 SUMMARY OF FINDINGS

Neurodegeneration is characterized by the progressive loss of neurons, which is attributed to the accumulation of misfolded or defective proteins in extra- or intra-cellular premises. However, different cellular mechanisms have been elucidated for such defective protein dynamics, including free radicals, oxidative stress, mitochondrial dysfunction, neuro-inflammation, compromised axonal transport and altered ubiquitin proteasome system (UPS). Here, UPS plays a cardinal role in regulating all these interrelated mechanisms through targeting pathological proteins for proteasomal degradation to maintain cellular homeostasis. Therefore, its perturbation is involved in the aggregation of amyloids, tau, and α-synuclein proteins in diverse neurodegenerative diseases such as Alzheimer's and Parkinson's disease. Moreover, one of the central mechanisms for their aggregation involves the role of amyloid-beta precursor protein that produces Aβ peptides ($A\beta_{40/42}$) and amyloid intracellular domain (AICD), which in turn facilitates the aggregation of other proteins and trigger neurotoxicity in AD and PD. Therefore, diagnosis of the UPS components is of great importance to devise the promising therapies for further managements. My studies have focused on the elucidation of the ubiquitination biology of the AβPP and the molecular biology of pathological protein aggregation and clearance in AD and PD. In this context, I answered the following questions regarding the potential ubiquitination sites in AβPP, lysine's impact on protein ubiquitination and other functions, lysine selection mechanism for ubiquitination, ubiquitination pattern for AβPP clearance, molecular basis of Aβ, Tau, and α-synuclein aggregation, molecular crosstalk between AD and PD pathology, and the ubiquitination markers for Aβ, Tau, and α-synuclein clearance. I reported K351, K377, K224, K363, K601, K751, and K763 as high-potential ubiquitination sites in AβPP. I also found that lysine is important for protein stability and non-covalent interactions for ubiquitin positioning and conjugation during ubiquitination. Moreover, I identified low disease susceptible lysine sites to be more favored for ubiquitination and found lysine to be crucial for other post-translational modifications for regulating different cellular functions. Besides, I observed that unique lysine site

sequences with glutamine and glutamic acid spanning hydrophobic residues and sterically allowed lysine sites are favored for ubiquitination reaction. Further, reported PARK2 and STUB1 E3 ligases, and USP25 and UCHL1 deubiquitinases as direct regulators of AβPP ubiquitination. Instead other E3 ligases- CDH1, FBXO2, TRIM13, TRIM55, and SART1 along with deubiquitinases- USP8, USP25, USP39, and UCHL1 to regulate ubiquitination of amyloid cascade enzymes- β-secretase (BACE1) and γ-subunit complex (PSEN1, PSNEN, NCSTN). Additionally, I reported the aggregation sequences in **$A\beta_{40/42}$:** HQKLVFFAEDV (14-24), GAIIGLMVGGVV (29-40) / HQKLVFFAEDVGS (14-26), GAIIGLMVGGVVI (29-41); **Tau:** GGG (650-652) and V630/G640 in paired and straight helical filaments, and VQI(I/V)(N/Y)K in amyloid spines, and **α-Synuclein:** GVVHGVATVAE (47-57) and GAVVTGVTAVA (68-78) to be rich in glycine and hydrophobic residues-alanine, valine, isoleucine and phenylalanine. Moreover, identified the key markers-AβPP, CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2 and SNCA for AD-PD crosstalk with GSK3B and PARK2 as common interactors of amyloid beta, tau and alpha synuclein protein. Furthermore, reported the E3 ligases- PARK2, STUB1, FBXW7 SIAH1, FBXO7, WDTC1, SYVN1, TRAF6, RNF19A, SIAH2, TRIM32, NEDD4, FBXL2, MARCH7, and FBXO45 and deubiquitinases- ATXN3, USP8, PSMD14, and UCHL1 as direct regulators of Aβ, Tau and α-synuclein ubiquitination. Moreover, I identified their corresponding E2-conjugating enzymes and the E1-activating enzymes regulating the ubiquitination biology of Aβ, Tau and α-synuclein clearance. These results collectively revealed the plausible mechanism for Aβ, Tau, and α-synuclein protein clearance through AβPP ubiquitination in Alzheimer's and Parkinson's disease **(Figure 6.1).**

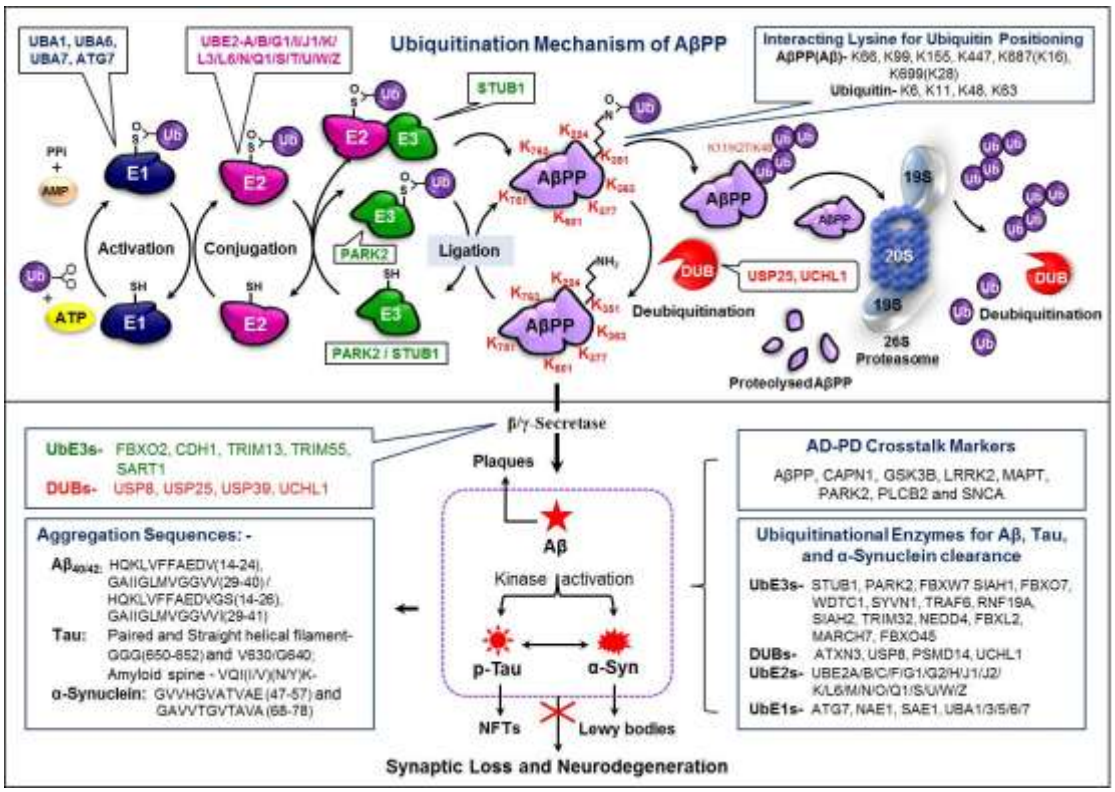


Figure 6.1: The pictorial representation of the plausible mechanism for Aβ, Tau, and α-Synuclein protein clearance through AβPP ubiquitination in Alzheimer's and Parkinson's disease.

6.2 THE IMPORTANCE OF AMYLOID BETA PRECURSOR PROTEIN UBIQUITINATION IN THE BRAIN

The amyloid beta synthesis is the consequence of AβPP processing by different proteolytic enzymes, including β- and γ-secretases in neurons. This pathway is known as amyloidogenic pathway, which is tightly regulated by the ubiquitination process. The levels of Aβ in the brain are properly regulated by the proteasomal degradation of their proteolytic enzymes and Aβ itself. However, any disruption in the ubiquitination of AβPP and Aβ, selectively amplify Aβ level that subsequently triggers AD pathogenesis. Moreover, recent studies have revealed that amyloid proteins further interact with other kinases and facilitate the phosphorylation of Tau and α-synuclein to trigger their consequent aggregation (Marsh and Blurton-Jones 2012; Tenreiro *et al.,* 2014; Spires-Jones *et al.,* 2017). Here, amyloid deposits form extracellular senile plaques, while hyperphosphorylated tau and α-synuclein deposits form neurofibrilliary tangles and lewy bodies in neurons respectively. They in turn, impede

the synaptic regulation and transmission, along with the nutritional transport across the neurons thereby affecting the normal functioning of the brain (Sheng *et al.*, 2012). Thus, they trigger different pathological events in neurons, including inflammation, oxidative stress, mitochondrial dysfunction, UPS and autophagy impairment (Ganguly *et al.*, 2017). Besides, the amyloid intracellular domain is also reported to regulate transcriptional activation of apoptotic markers and trigger the consequent neuronal death. Moreover, AβPP is proposed to be a cell-surface receptor (Thinakaran and Koo 2008); therefore, AβPP ubiquitination may also serve as a signaling event for some unknown cellular processes apart from merely a signal for degradation. Interestingly, Del Prete *et al.*, have hypothesized that AβPP may also facilitate the ubiquitination of other synaptic proteins with help of their interactions with ubiquitin E3 ligases (Del Prete *et al.*, 2016) that get dysregulated in the diseased state. Moreover, AβPP is overexpressed under diseased state that aggravates the pathological conditions in neurodegenerative disorders (Ting *et al.*, 2007). Importantly, AβPP processing and proteomic interactome are also evident to cause mitochondrial associated membranes (MAMs) deregulation in AD (Del Prete *et al.*, 2017). Together, these findings suggest that the ubiquitination mechanism of AβPP is crucial to prevent such a pathogenic event to overcome the proteinopathic insult in neurons. This study has revealed the dynamics of Aβ or AβPP ubiquitination, which is essential to devise the strategies to regulate the AβPP metabolism. This would enable us to avoid the complications arisen from the elimination of secretases mediated biological functions of AβPP fragments: ectodomain- sAβPPα, sAβPPβ, N-terminal- AβPP-NTFs, and intracellular- AICD in regulating gene transcriptions (Wang and Saunders 2014). Further investigations are required to develop the prospective therapeutic agents, which can address the clearance of such toxic proteins (Aβ) or their progenitors (AβPP) in a regulated way to ameliorate the neurodegenerative diseases like AD globally.

6.3 UBIQUITINATION BIOLOGY OF AMYLOID BETA PRECURSOR PROTEIN

6.3.1 Putative ubiquitination sites in Amyloid-beta precursor protein

Ubiquitination is a post-translational modification that controls the activity of various proteins and thus regulates different cellular processes and is implicated in many

diseases. Experimental identifications of the ubiquitination sites are challenging due to large set of ubiquitin modifier and rapid turnover of the ubiquitinated proteins. Moreover, it is the cost-effective and time-consuming process. Therefore, there is a need to develop computational approaches that can accurately and efficiently-determine the protein ubiquitination sites (He *et al.,* 2018). In this study, I utilized multiple approaches, including the *sequence identity analysis*, *sequence similarity analysis, protein-peptide docking approach* and *multiple machine-learning based computational tools* to predict the potential ubiquitination sites in AβPP. Therefore, this is an improvised algorithm, including their sequence and structure similarity information along with their physicochemical property comparison with the experimental data sets. Here, I reported twenty-seven lysine sites with different confidences for ubiquitination. Among them these seven lysines- K351, K377, K224, K363, K601, K751, and K763 are the potential ubiquitination sites in AβPP with high confidence, which is important for their trafficking and clearance in the neurons. My findings are well correlated with the findings of other scientists, including K751 in Crbn-KO mouse brains (Del Prete *et al.,* 2016) and K763 in PC12 cells (El Ayadi *et al.,* 2012). Likewise, other ubiquitination sites with low confidences were also supported by other results, including K699 in N2a cells, P0 mice brain (Williamson *et al.,* 2017) and K724 in Hela Cells, 2xTg Mice (FBL2/AD1), and 3xTg Mice (Watanabe *et al.,* 2012; Morel *et al.,* 2013). Ubiquitination at these sites is evident for their different functions, including AβPP metabolism, endosomal sorting and trafficking, and interactions with other proteins. Therefore, the identification of key lysine governing ubiquitination would be not only crucial for regulating Aβ level in neurons but also for determining the mechanism of AβPP's sub cellular trafficking and processing and consequent understanding of the pathophysiology of the disease.

6.3.2 The lysine residues are crucial for AβPP stability and functions

The covalent conjugation of ubiquitin at substrates determines a variety of biological processes ranging from proteolysis to DNA repair based on the site of lysine residues in ubiquitin and substrate protein. For instance, lysine selection on the substrate is important for the attachment of ubiquitin to it while lysine selection on ubiquitin is crucial for generating diverse substrate-polyubiquitin structures that determine their

cellular fate (Suryadinata *et al.,* 2013). Moreover, there are seven lysines in ubiquitin protein that form different structural topology of their polyubiquitin chains, including straight chain (K6, K63, M1), looped chain (K29, K33) and zigzag chain (K11, K27, K48). It triggers different cellular responses, including proteolysis (lysosomal or proteasomal), cell division, signal transduction, DNA transcription and repair, endocytosis, immune and inflammatory responses (Suryadinata *et al.,* 2014). Here, we reported that lysine is also important for AβPP stability. Since, lysines at high-potential ubiquitination sites were intolerant towards both positive and negative charged mutations, including lysine to arginine, histidine, aspartate and glutamate. However, only lysine to arginine mutations was reported to impart site-specific stability to AβPP. The finding that lysine and arginine are mostly exposed to the surface, and impart protein stability by forming electrostatic interactions, supports our result since, guanidium group in arginine allows interaction in three possible directions that enable it to form more electrostatic interaction in comparison to lysine residue (Sokalingam *et al.,* 2012). Therefore, Lysine to arginine mutation is being used to experimentally, determine the ubiquitination sites. Moreover, we have shown that low disease susceptible lysine sites to be more favored for ubiquitination in AβPP. Furthermore, lysine has also shown to undergo other post-translational modifications, including acetylation, glycosylation, phosphorylation, and SUMOylation to regulate the functionality of amyloid-beta precursor protein.

6.3.3 Lysine selection mechanism for AβPP ubiquitination

The ubiquitin attachment to the lysine residues on substrate or itself is a tightly regulated process with the help of ubiquitin E2 conjugating enzyme and ubiquitin E3 ligases. However, exact mechanism of lysine selection is still elusive, but the lysine positioning towards E2/E3's active site and residues proximal to the substrate lysine are crucial for their selection (Sadowski and Sarcevic 2010; Suryadinata *et al.,* 2013). In this regard, we have identified the lysines- K6, K11, K48, and K63 in ubiquitin and K66, K99, K155, K447, K687, and K699 in AβPP with non-covalent interactions between ubiquitin and AβPP, signifying their plausible role in ubiquitin positioning and conjugation during AβPP ubiquitination. Since, non-covalent interactions of ubiquitin are known with different proteins like DNA repair proteins, insulin-

degrading enzyme, SUMO protein for modulating their activity and correctly orienting ubiquitin for lysine specific conjugation (McKenna *et al.,* 2001; Saric *et al.,* 2003; Ouyang *et al.,* 2015). Moreover, we reported conserved glutamine and glutamate proximal to the ubiquitination sites in both ubiquitin and AβPP signifying their importance in lysine selection by E3 ligases and their interactions for ubiquitin positioning to govern lysine specific (K6, K11, K27, K29, K33, K48, K63) polyubiquitination. Interestingly, we also found that majority of putative ubiquitination sites were in α-helical region of protein in comparison with the non-ubiquitination sites, which were in mostly turn or loop region. This finding provides a clue that ubiquitin attachment might be a structural selective process, which requires further validations.

6.3.4 The key ubiquitination markers for AβPP regulation

The protein ubiquitination is a complex interplay of different classes of enzymes, including E1 activating enzymes (E1s), E2 conjugating enzymes (E2s ~40), E3 ligases (E3s ~800) and deubiquitinases (DUBs ~100). Primarily E1 activates ubiquitin by binding it to its own cysteine through ATP hydrolysis followed by its conjugation with help of E2s to its own cysteine. Then, ubiquitin is transferred to the amine group of the lysine's side chain on target protein with help of E3s either directly or through attachment on its own cysteine. The E3s are highly specific towards their targets. Therefore, it is quite intriguing to identify the ubiquitination enzymes involved in the ubiquitination of a particular protein. Here with help of protein interactome analysis, we identified PARK2 and STUB1 as the key E3 ligases, and USP25 and UCHL1 as key deubiquitinases directly regulating the ubiquitination and deubiquitination of AβPP respectively. These findings are supported by the insight that Parkin's over expression curtailed AβPP expression and Aβ burden in the AD mouse model (Sweeney *et al.,* 2017) while CHIP is also reported to restrict Aβ level (Gadhave *et al.,* 2016). Moreover, other E3 ligases- CDH1, FBXO2, TRIM13, TRIM55, and SART1 along with deubiquitinases- USP8, USP25, USP39, and UCHL1 are reported as the regulator of amyloid cascade enzymes- β-secretase (BACE1) and γ-subunit complex (PSEN1, PSNEN, NCSTN) thereby rescuing Aβ burden in neurodegenerative diseases like AD and PD. These ubiquitination markers may serve

as key therapeutic candidates for targeting neurodegenerative pathologies. Moreover, their identification is also crucial for developing proteolysis-targeting chimeric molecules (PROTACs) for achieving the target specific protein degradation in the diseased state, which is an interesting area of research for the scientist (Lai and Crews 2017; Gu *et al.,* 2018).

6.4 SURFACE HYDROPHOBICITY DETERMINES Aβ, TAU, AND α-SYNUCLEIN PROTEIN AGGREGATION

Altered proteostasis and inappropriate protein aggregations are the key pathological features of neurodegenerative disorders like AD and PD with Aβ, Tau and α-synuclein protein aggregates. However, the protein aggregation is a biological driven process that evades out the intrinsic hydrophobic regions due to mutations or protein misfolding events (Cox *et al.,* 2018). These hydrophobic residues rearrange their non-covalent interactions thereby disrupting their previous secondary structures. This results in a seed-nucleus that acts as surface for a series of misfolding events and interactions with other proteins to form oligomers and consequent protofibril (Marinko *et al.,* 2019). Here, we identified the aggregation sequences in Aβ, Tau and α-synuclein protein that was highly populated with glycine and hydrophobic residues predominating alanine, valine, isoleucine and phenylalanine. The aggregation sites in $A\beta_{40/42}$ included HQKLVFFAEDV (14-24), GAIIGLMVGGVV (29-40) and HQKLVFFAEDVGS (14-26), GAIIGLMVGGVVI (29-41) respectively, which were almost similar with only few residue differences. Interestingly, the presence of lysine in aggregation sequences was also reported by Sinha *et al.,* who have shown the role of lysine residues (K16) in Aβ folding, assembly and toxicity (Sinha *et al.,* 2012). Similarly, Tau proteins were reported with VQIVYK (623-628), GGG (650-652) and VQIVYK (623-628), V630, G640 to form paired and straight helical filament respectively while VQIINK (592-597) and VQIVYK (623-628) sequence segments to form amyloid spines. Likewise, α-synuclein have shown GVVHGVATVAE (47-57) and GAVVTGVTAVA (68-78) sequences to form fibril and amyloid structures respectively. Here, the presences of glycine residues were important for imparting flexibility to the protein structure, which can fit into both hydrophilic and hydrophobic environments due to its minimal side chain (Scott *et al.,* 2007).

Therefore, it sterically allows the protein aggregate to have different conformations. Besides, hydrophobic regions are responsible for the hydrophobic interactions among them that allow the protein to attain a variety of fibrillar forms with a common hydrophobic core (Kalinowska *et al.,* 2017). Together, these hydrophobic interactions reported to change the helical secondary structure of proteins to beta sheets and bridges. In fact, NMR studies have also shown the contribution of hydrophobic interaction and salt bridges in imparting the stability to beta sheets and turns in protein folding and assembly (Petkova *et al.,* 2002, 2006). Thus, surface hydrophobicity also guides neurotoxic protein aggregation in neurodegenerative disorders.

6.5 INTEGRATED ROLE OF PARK2 IN REGULATION AND CLEARANCE OF AD-PD CROSSTALK MARKERS

Alzheimer's and Parkinson's disease is two distinct neurodegenerative disorders with some pathological similarities. Therefore, numerous studies have investigated the links between Alzheimer's and Parkinson's disease at the protein level but there is hardly any evidence regarding genetic variants common to both (Moskvina *et al.,* 2013). However, latest research has come up demonstrating the plausible genetic links between them. For instance, LRRK2 mutant G2019S trigger phosphorylation of AβPP at T668 and promote APP intracellular domain's (AICD) nuclear translocation and consequent neurotoxicity in Parkinson's Disease (Zeng *et al.,* 2018). However, both diseases are associated with unique protein deposits, but they also displayed protein aggregates in common. For instance, more than 50 percent patients suffering from AD revealed amyloid-like alpha-synuclein peptide aggregates (Lippa *et al.,* 2005) while PD patients displayed frequent tau deposits (Lei *et al.,* 2010). Although, tau is predominantly aggregate in AD but researchers have also reported that in PD. Further, they demonstrated that the accumulation of tau proteins could take place with the help of specific strains of alpha-synuclein (Guo *et al.,* 2013). A recent study identified that Aβ seeding escalated the formation of big sized α-synuclein oligomers that efficiently hampered neuronal SNARE-mediated vesicle fusion thereby affecting synaptic transmission (Choi *et al.,* 2015). Likewise, the co-existence of numerous misfolded protein aggregates demonstrated *in-vivo,* which includes proteins like Aβ,

α-synuclein, Tau and prion protein (Giasson *et al.,* 2003; Busquets *et al.,* 2015). Here, we reported eight key markers, including AβPP (Aβ), CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2 and SNCA for AD-PD crosstalk; interacting with amyloid beta, Tau, and alpha-synuclein proteins individually or in combinations. However, only PARK2 and GSK3B found to interact commonly with all. Since, PARK2 is an ubiquitin E3 ligase, therefore, its interaction with Aβ, Tau and α-synuclein would certainly regulate their levels in our body. Moreover, these markers have shown their involvement in either of the disease previously, but recent findings have shown their role in both diseases. For instance, LRRK2 variant R1628P increased the risk of AD in the population, and *in-vitro* findings suggested its predisposition to apoptosis (Zhao *et al.,* 2011). Instead LRRK2 mutation G2019S reported to promote AβPP phosphorylation and consequent AICD activity mediated neurotoxicity in PD (Chen *et al.,* 2017). Likewise, apart from tau hyper-phosphorylation, increased β-amyloid production and inflammatory responses in AD (Hooper *et al.,* 2008); GSK3B dysregulations also contributed towards Parkinson's like pathology with induced phosphorylation and aggregation of Tau and α-synuclein (Credle *et al.,* 2015). Similarly, PARK2 has shown their role in AD, since its enhancement has compensated mitophagic alterations in their pathology (Martín-Maestro *et al.,* 2016). Here, CAPN1 and PLCB2 are novel markers for AD-PD crosstalk whose roles need to be investigated in both diseases; however, they are known to play some role in Alzheimer's disease. Moreover, we reported the important ubiquitination markers, including the E3 ligases- PARK2, STUB1, FBXW7 SIAH1, FBXO7, WDTC1, SYVN1, TRAF6, RNF19A, SIAH2, TRIM32, NEDD4, FBXL2, MARCH7, and FBXO45, and the deubiquitinases- ATXN3, USP8, PSMD14, and UCHL1 directly regulating the clearance of Aβ, Tau and α-synuclein. Furthermore, identified E2s- UBE2A/B/C/F/G1/G2/H/J1/J2/K/L6/M/N/O/Q1/S/U/W/Z and E1s- ATG7, NAE1, SAE1, UBA1/3/5/6/7 involved in the ubiquitination biology of all these protein aggregates. However, for the clearance of these protein aggregates HSPs, CHIP and Parkin known to play a critical role in refolding or targeting proteins to ubiquitin proteasome system for degradation (Yao 2010). In case of AD, Parkin's levels have been found to be elevated along with HSPs and CHIP while in case of PD loss of Parkin has been observed with high HSP and CHIP level (Kumar *et al.,* 2012).

Apart from neurotoxic protein deposition, their clearing mechanisms and some pathological symptoms there is hardly any other similarity. Together, these findings reinstate that Parkin has a dual role, i.e. itself a molecular marker for AD-PD crosstalk, and it is also involved in the ubiquitination biology of the toxic aggregates.

6.6 FUTURE DIRECTIONS

Although new insights into the molecular mechanisms underlying AβPP ubiquitination, neurotoxic protein (Aβ, Tau, and α-synuclein) aggregation and clearance have been achieved; several important questions remained to answer. For instance, dynamics of ubiquitination on Tau and α-synuclein protein remained elusive. However, the characteristic ubiquitination enzymes for their modification have identified here, but their exact ubiquitination site remained unmapped. Moreover, the algorithm presented in this study may be employed to develop the improvised ubiquitination prediction tools to reduce the time span for ubiquitination site discovery. Another important question to address is to identify the type of polyubiquitin chain they form at these potential lysine sites, which will help us to determine their fate in cellular milieu and thus their functional regulation in neurons. Here, in-depth studies pertaining to the lysine potential in AβPP processing, stability, interaction, ubiquitination, and identification of key pathological and ubiquitinational markers have narrowed down the search space for the experimentalist that can be translated into *in-vivo* studies to devise novel therapeutic modalities against Alzheimer's and Parkinson's disease. Ultimately, the identifications of potential ubiquitination enzymes, i.e. E3 ligases and deubiquitinases are also crucial for developing chimera products like PROTACs for achieving the target specific protein degradation in the diseased state as potential therapy against such incurable neurodegenerative diseases avoiding non-specific side effects.

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Appendices

APPENDIX-I

SUPPLEMENTARY TABLES

Table S3.1: The structure validation scores of Verify 3D and RAMPAGE for Modeled and Refined AβPP

S.No.	Protein	VERIFY 3D	RAMPAGE	
		% of residues had an averaged 3D-1D score >= 0.2	No. of residues in (Favored + Allowed region)	No. of residues in (Outlier region)
1	**Modeled AβPP (P)**	33.12	655	113
2	**Modeled AβPP (P1)**	46.75	718	50
3	**Modeled AβPP (P2)**	40	732	36
4	**Modeled AβPP (P3)**	49.22	745	23
5	**Modeled AβPP (P4)**	44.16	751	17
6	**Modeled AβPP (P5)**	48.57	756	12
7	**Modeled AβPP (P6)**	42.99	755	13
8	**Modeled AβPP (P7)**	48.31	757	11
9	**Modeled AβPP (P8)**	47.27	758	10
10	**Modeled AβPP (P9)**	40.26	758	10
11	**Modeled AβPP (P10)**	47.4	757	11
12	**Modeled AβPP (P11)**	51.69	755	13
13	**Modeled AβPP (P12)**	47.66	757	11
14	**Modeled AβPP (P13)**	49.35	758	10
15	**Modeled AβPP (P14)**	48.18	757	11
16	**Modeled AβPP (P15)**	46.1	758	10
17	**Modeled AβPP (P16)**	45.45	757	11
18	**Modeled AβPP (P17)**	46.49	759	9
19	**Modeled AβPP (P18)**	51.17	758	10
20	**Modeled AβPP (P19)**	51.04	758	10
21	**Modeled AβPP (P20)**	50.39	760	8
22	**Modeled AβPP (P21)**	53.51	761	7
23	**Modeled AβPP (P22)**	47.66	760	8
24	**Modeled AβPP (P23)**	48.44	762	6
25	**Modeled AβPP (P24)**	54.03	761	7
26	**Modeled AβPP (P25)**	55.71	760	8
27	**Modeled AβPP (P26)**	52.86	761	7
28	**Modeled AβPP (P27)**	47.79	762	6
29	**Modeled AβPP (P28)**	49.48	762	6
30	**Modeled AβPP (P29)**	51.56	763	5
31	**Modeled AβPP (P30)**	50.13	764	4
32	**Modeled AβPP (P31)**	50	763	5
33	**Modeled AβPP (P32)**	48.83	763	5
34	**Modeled AβPP (P33)**	44.94	762	6

S.No.	Protein	VERIFY 3D	RAMPAGE	
		% of residues had an averaged 3D-1D score >= 0.2	No. of residues in (Favored + Allowed region)	No. of residues in (Outlier region)
35	**Modeled AβPP (P34)**	46.49	763	5
36	**Modeled AβPP (P35)**	44.42	764	4
37	**Modeled AβPP (P36)**	45.32	764	4
38	**Modeled AβPP (P37)**	47.79	764	4
39	**Modeled AβPP (P38)**	48.31	764	4
40	**Modeled AβPP (P39)**	49.09	763	5
41	**Modeled AβPP (P40)**	46.36	762	6
42	**Modeled AβPP (P41)**	44.03	762	6
43	**Modeled AβPP (P42)**	46.88	763	5
44	**Modeled AβPP (P43)**	47.66	763	5
45	**Modeled AβPP (P44)**	47.01	763	5
46	**Modeled AβPP (P45)**	50.52	763	5
47	**Modeled AβPP (P46)**	48.31	764	4
48	**Modeled AβPP (P47)**	47.01	764	4
49	**Modeled AβPP (P48)**	51.69	764	4
50	**Modeled AβPP (P49)**	47.27	764	4
51	**Modeled AβPP (P50)**	48.7	764	4
52	**Modeled AβPP (P51)**	49.35	764	4
53	**Modeled AβPP (P52)**	49.35	764	4
54	**Modeled AβPP (P53)**	44.42	764	4
55	**Modeled AβPP (P54)**	46.36	764	4
56	**Modeled AβPP (P55)**	45.19	764	4
57	**Modeled AβPP (P56)**	43.51	764	4
58	**Modeled AβPP (P57)**	47	764	4
59	**Modeled AβPP (P58)**	49.35	764	4
60	**Modeled AβPP (P59)**	49.61	763	5
61	**Modeled AβPP (P60)**	48.83	762	6
62	**Modeled AβPP (P61)**	47.66	762	6
63	**Modeled AβPP (P62)**	50.91	762	6
64	**Modeled AβPP (P63)**	49.35	763	5
65	**Modeled AβPP (P64)**	53.12	763	5
66	**Modeled AβPP (P65)**	47.66	763	5
67	**Modeled AβPP (P66)**	47.01	764	4
68	**Modeled AβPP (P67)**	47.92	764	4
69	**Modeled AβPP (P68)**	46.62	764	4
70	**Modeled AβPP (P69)**	50.26	763	5
71	**Modeled AβPP (P70)**	47.01	764	4
72	**Modeled AβPP (P71)**	46.75	764	4
73	**Modeled AβPP (P72)**	43.9	764	4

Table S3.2: Pairwise alignment scores among AβPP and Ubiquitin lysine sites

S.No.	Lysine in AβPP	Sequence Identity Score						
		Lysine in Ubiquitin						
		K6	K11	K27	K29	K33	K48	K63
1	K51	0.1935484	0.1666667	0.1351351	0.105263	0.1351351	0.1666667	0.1351351
2	K60	0.2333333	0.2	0.3125	0.3125	0.2727273	0.1666667	0.2
3	K66	0.2333333	0.2352941	0.3125	0.3125	0.2727273	0.1666667	0.2
4	K99	0.1935484	0.2	0.2352941	0.235294	0.1666667	0.2	0.1666667
5	K103	0.1935484	0.1666667	0.2	0.2	0.1666667	0.1666667	0.2
6	K106	0.15625	0.1666667	0.2	0.2	0.2	0.1351351	0.2352941
7	K132	0.1935484	0.2	0.2352941	0.235294	0.1666667	0.2	0.2
8	K134	0.15625	0.2	0.2352941	0.235294	0.1666667	0.2	0.2
9	K155	0.2333333	0.2	0.1666667	0.135135	0.1666667	0.1666667	0.2
10	K161	0.1935484	0.2	0.2	0.166667	0.1666667	0.1666667	0.2352941
11	K178	0.15625	0.2	0.1666667	0.166667	0.1351351	0.1351351	0.1666667
12	K224	0.1212121	0.1666667	0.1666667	0.166667	0.1052632	0.1666667	0.1666667
13	K315	0.15625	0.1666667	0.2	0.2	0.1052632	0.1351351	0.0769231
14	K351	0.2333333	0.2727273	0.1666667	0.2	0.1666667	0.2	0.2352941
15	K363	0.2333333	0.2727273	0.1666667	0.235294	0.2	0.2352941	0.1666667
16	K377	0.1935484	0.2	0.2	0.235294	0.1666667	0.2352941	0.1666667
17	K393	0.15625	0.1351351	0.2	0.2	0.2352941	0.2	0.2352941
18	K395	0.15625	0.1666667	0.2	0.2	0.2352941	0.2	0.1666667
19	K401	0.15625	0.2	0.1351351	0.135135	0.2	0.2352941	0.2
20	K421	0.1212121	0.1351351	0.1666667	0.166667	0.1666667	0.1666667	0.1351351
21	K425	0.15625	0.1351351	0.2	0.166667	0.2	0.1351351	0.1666667
22	K428	0.1935484	0.1666667	0.2	0.2	0.2	0.1666667	0.1666667
23	K429	0.1935484	0.2	0.2	0.2	0.2	0.1666667	0.1666667
24	K438	0.15625	0.2	0.1666667	0.166667	0.2	0.1351351	0.1666667
25	K495	0.1212121	0.1666667	0.1666667	0.2	0.2	0.2	0.1666667
26	K496	0.1212121	0.1666667	0.1666667	0.2	0.2	0.2	0.1666667
27	K503	0.2333333	0.2	0.1666667	0.166667	0.2	0.2	0.2
28	K510	0.1935484	0.2352941	0.2	0.166667	0.1666667	0.2	0.2352941
29	K521	0.15625	0.1666667	0.1666667	0.166667	0.2	0.1351351	0.1351351
30	K522	0.1612903	0.1714286	0.1714286	0.171429	0.2058824	0.1388889	0.1388889
31	K568	0.15625	0.2	0.1666667	0.2	0.2352941	0.2	0.2
32	K601	0.2333333	0.2727273	0.2	0.2	0.1052632	0.1666667	0.2
33	K662	0.2333333	0.2727273	0.2352941	0.235294	0.1351351	0.2	0.2352941
34	K670	0.15625	0.2352941	0.2352941	0.235294	0.2	0.1666667	0.2
35	K687	0.1212121	0.1666667	0.2	0.2	0.1666667	0.2727273	0.1666667
36	K699	0.1935484	0.2	0.2352941	0.166667	0.2	0.1666667	0.1666667
37	K724	0.2333333	0.2	0.2	0.2	0.1666667	0.1351351	0.1666667
38	K725	0.2333333	0.2352941	0.2	0.166667	0.1666667	0.1351351	0.1666667
39	K726	0.2333333	0.2352941	0.2	0.166667	0.1666667	0.1351351	0.1666667
40	K751	0.15625	0.1666667	0.2	0.166667	0.1351351	0.2	0.1666667
41	K763	0.1333333	0.1142857	0.1470588	0.114286	0.1470588	0.1470588	0.1470588

Table S3: Internal potential energy of Modeled and Mutated AβPP protein

Modeled Protein			Energy (KJ/mol)						
			Bonds	**Angles**	**Torsion**	**Improper**	**Non-Bonded**	**Electrostatic**	**Total**
Refined AβPP (P30) Emin			790.612	5419.073	6695.087	1360.861	-14768.62	-18967.48	-19470.473
Confidence Level	**Mutational Site**	**Mutated Protein**	**Energy (KJ/mol)**						
			Bonds	**Angles**	**Torsion**	**Improper**	**Non-Bonded**	**Electrostatic**	**Total**
Very High	**K351**	**Lys(K)-Arg(R)**	802.431	5427.866	6694.257	1360.854	-8679.2	-19200.66	-13594.459
		Lys(K)-His(H)	815.851	5438.06	6695.279	1365.08	-14594.73	-18967.59	-19248.053
		Lys(K)-Asp(D)	794.221	5424.052	6696.3	1361.063	-14776.03	-18965.45	-19465.84
		Lys(K)-Glu(E)	795.899	5428.607	6696.188	1360.894	-14735.92	-18954.65	-19408.977
	K377	**Lys(K)-Arg(R)**	806.277	5425.048	6696.321	1360.712	-13342.04	-19222.43	-18276.109
		Lys(K)-His(H)	827.33	5432.853	6696.176	1366.362	-14779.36	-18951.61	-19408.256
		Lys(K)-Asp(D)	794.99	5421.954	6697.881	1360.883	-14772.59	-18958.12	-19455.002
		Lys(K)-Glu(E)	798.301	5425.638	6698.115	1360.731	-14778.98	-18959.72	-19455.916
High	**K224**	**Lys(K)-Arg(R)**	802.183	5425.641	6695.116	1360.184	-14769.02	-19223	-19708.891
		Lys(K)-His(H)	814.685	5433.525	6695.32	1365.42	-14776.11	-18968.28	-19435.436
		Lys(K)-Asp(D)	795.388	5422.884	6696.647	1359.784	-14762.41	-18965.37	-19453.078
		Lys(K)-Glu(E)	795.759	5426.603	6696.865	1360.066	-14766.68	-18963.09	-19449.477
	K363	**Lys(K)-Arg(R)**	807.077	5425.851	6696.442	1361.297	-13513.97	-19220.91	-18444.213
		Lys(K)-His(H)	829.593	5,436	6697.103	1366.678	-4986.73	-18975.3	-9632.712
		Lys(K)-Asp(D)	795.767	5421.912	6697.829	1360.917	-14761.31	-18952.02	-19436.908
		Lys(K)-Glu(E)	799.457	5426.508	6697.715	1361.207	-937.18	-18969.35	-5621.648
	K601	**Lys(K)-Arg(R)**	801.879	5425.684	6692.721	1361.378	2069043.24	-19174.4	2064150.5
		Lys(K)-His(H)	818.389	5433.463	6692.847	1365.85	1694.37	-18971.49	-2966.571
		Lys(K)-Asp(D)	793.886	5423.07	6693.462	1361.567	-14766.85	-18964.48	-19459.35
		Lys(K)-Glu(E)	796.41	5426.988	6694.113	1361.304	-14772.31	-18968.21	-19461.703
	K751	**Lys(K)-Arg(R)**	800.885	5425.582	6695.996	1361.291	-12077.12	-19203.91	-16997.271
		Lys(K)-His(H)	818.69	5434.352	6696.117	1366.893	-14658.24	-18962.36	-19304.551
		Lys(K)-Asp(D)	791.496	5422.378	6697.489	1360.563	-14781.76	-18961.27	-19471.102
		Lys(K)-Glu(E)	794.058	5426.416	6697.869	1360.924	-14798.68	-18960.28	19479.688
	K763	**Lys(K)-Arg(R)**	809.097	5433.8	6698.789	1359.286	4876117.16	-19224.98	4871193
		Lys(K)-His(H)	823.013	5440.111	6696.942	1363.613	-8764.7	-18966.96	-13407.986
		Lys(K)-Asp(D)	800.614	5431.836	6701.67	1359.471	-14780.25	-18977.19	-19463.852
		Lys(K)-Glu(E)	802.296	5434.789	6700.779	1359.307	141680.53	-18975.59	137002.109
Medium	**K60**	**Lys(K)-Arg(R)**	807.875	5426.131	6694.119	1361.002	-14773.41	-19222.37	-19706.652
		Lys(K)-His(H)	824.745	5434.341	6695.589	1363.859	-14727.41	-18955.08	-19363.947
		Lys(K)-Asp(D)	798.338	5423.702	6694.298	1361.17	-14734.46	-18964.56	-19421.512
		Lys(K)-Glu(E)	801.337	5427.718	6695.27	1360.905	-14757.42	-18963.8	-19435.982
	K161	**Lys(K)-Arg(R)**	803.024	5428.39	6695.768	1360.767	-14705.49	-19237.49	-19655.035
		Lys(K)-His(H)	819.898	5436.15	6695.521	1364.922	99999900	-18107.09	7.19899E+11
		Lys(K)-Asp(D)	794.113	5425.411	6696.486	1360.924	-14751.34	-18968.63	-19443.033
		Lys(K)-Glu(E)	796.357	5429.109	6697.021	1360.703	-14764.03	-18976.91	-19457.756
	K393	**Lys(K)-Arg(R)**	805.819	5424.226	6696.503	1362.218	-14767.45	-19226.37	-19705.053
		Lys(K)-His(H)	826.324	5433.08	6698.082	1367.856	-14754.27	-18967.41	-19396.338
		Lys(K)-Asp(D)	795.307	5420.752	6697.824	1361.659	-14763.43	-18958.76	-19446.648
		Lys(K)-Glu(E)	798.059	5424.88	6698.121	1362.047	-14766.01	-18967.17	-19450.074
	K401	**Lys(K)-Arg(R)**	821.647	5453.024	6691.961	1360.044	-14768.41	-19223.58	-19665.314
		Lys(K)-His(H)	832.929	5467.628	6693.324	1364.507	-14776.48	-18965.4	-19383.496
		Lys(K)-Asp(D)	813.279	5445.782	6692.49	1360.213	-14770.44	-18959.72	-19418.393
		Lys(K)-Glu(E)	814.202	5451.659	6693.34	1360.124	-14763.86	-18963.74	-19408.279
	K496	**Lys(K)-Arg(R)**	801.467	5427.565	6695.162	1360.041	-14772.4	-19221.94	-19710.109
		Lys(K)-His(H)	818.446	5436.725	6695.3	1365.885	-14763.6	-18963.86	-19411.105
		Lys(K)-Asp(D)	793.19	5425.26	6696.634	1360.161	-14757.77	-18967.53	-19450.057
		Lys(K)-Glu(E)	795.564	5429.406	6697.023	1360.046	-14768.75	-18971.65	-19458.359
	K510	**Lys(K)-Arg(R)**	801.601	5426.743	6695.284	1360.842	-14769.52	-19225.96	-19711.006
		Lys(K)-His(H)	819.57	5436.276	6695.73	1366.648	602234.47	-19065.8	597486.875
		Lys(K)-Asp(D)	792.995	5424.347	6696.662	1360.822	-14760.84	-18962.53	-19448.551

Confidence Level	Mutational Site	Mutated Protein	Energy (KJ/mol)						
			Bonds	**Angles**	**Torsion**	**Improper**	**Non-Bonded**	**Electrostatic**	**Total**
		Lys(K)-Glu(E)	795.402	5428.672	6697.048	1360.701	-14764.2	-18970.15	-19452.52
	K522	**Lys(K)-Arg(R)**	806.978	5435.505	6695.48	1358.961	-14759.06	-19239.56	-19701.695
		Lys(K)-His(H)	825.757	5446.636	6696.073	1363.499	-14725.92	-18986.39	-19380.352
		Lys(K)-Asp(D)	798.844	5431.752	6696.87	1358.517	-14758.78	-18963.61	-19436.402
		Lys(K)-Glu(E)	801.741	5436.82	6697.45	1358.917	-14758.87	-18977.04	-19440.977
	K662	**Lys(K)-Arg(R)**	801.327	5421.911	6694.192	1361.846	99999900	-19223.38	765408192
		Lys(K)-His(H)	816.73	5432.4	6694.53	1367.552	53591.42	-18963.11	48939.527
		Lys(K)-Asp(D)	792.766	5417.718	6695.584	1361.235	-14550.5	-18960.49	-19243.689
		Lys(K)-Glu(E)	794.47	5422.452	6695.924	1361.669	-14197.53	-18945.62	-18868.637
Low	**K503**	**Lys(K)-Arg(R)**	802.324	5426.054	6695.49	1360.559	-14031.68	-19235.98	-18983.23
		Lys(K)-His(H)	821.485	5435.13	6695.674	1366.46	-14749.88	-18969.19	-19400.318
		Lys(K)-Asp(D)	792.677	5424.024	6697.012	1360.629	-14754.62	-18976.95	-19457.229
		Lys(K)-Glu(E)	795.526	5428.115	6697.413	1360.556	-14592.22	-18986.58	-19297.186
	K568	**Lys(K)-Arg(R)**	801.921	5427.606	6696.514	1360.156	99999900	-19224.47	1.21228E+11
		Lys(K)-His(H)	817.592	5435.976	6697.213	1364.1	-14770.64	-18962.72	-19418.48
		Lys(K)-Asp(D)	794.554	5424.745	6698.018	1360.108	-14770.54	-18953.62	-19446.729
		Lys(K)-Glu(E)	796.59	5428.699	6697.896	1359.829	-14768.55	-18961.6	-19447.135
	K724	**Lys(K)-Arg(R)**	802.13	5426.248	6695.752	1361.157	-14779.53	-19222.01	-19716.252
		Lys(K)-His(H)	820.277	5435.344	6695.969	1366.748	8853.67	-18987.21	4184.802
		Lys(K)-Asp(D)	792.963	5422.939	6697.18	1360.572	-14753.78	-18954.48	-19434.609
		Lys(K)-Glu(E)	795.71	5427.048	6697.644	1360.947	-14763.08	-18969.29	-19451.016
Very Low	**K51**	**Lys(K)-Arg(R)**	807.802	5434.936	6693.311	1360.492	-14772.64	-19222.47	-19698.568
		Lys(K)-His(H)	790.612	5419.073	6695.087	1360.861	-14768.62	-18967.48	-19470.473
		Lys(K)-Asp(D)	799.388	5429.487	6695.032	1360.641	-14772.19	-18968.19	-19455.824
		Lys(K)-Glu(E)	801.531	5435.172	6695.086	1360.498	-14772.99	-18963.67	-19444.375
	K134	**Lys(K)-Arg(R)**	799.946	5425.159	6691.868	1360.926	-14631.64	-19247.86	-19601.6
		Lys(K)-His(H)	813.689	5434.433	6691.831	1366.012	-14775.71	-18983.46	-19453.203
		Lys(K)-Asp(D)	792.505	5421.616	6693.529	1360.54	-14759.3	-18972.21	-19463.322
		Lys(K)-Glu(E)	794.205	5426.14	6693.665	1360.773	-14760.55	-18974.84	-19460.613
	K155	**Lys(K)-Arg(R)**	802.016	5426.307	6696.181	1362.104	-12986.42	-19200.24	-17900.53
		Lys(K)-His(H)	819.993	5435.586	6696.08	1367.774	-14777.3	-18981.41	-19439.281
		Lys(K)-Asp(D)	792.944	5422.851	6697.673	1361.544	-14772.93	-18962.16	-19460.078
		Lys(K)-Glu(E)	795.229	5426.957	6697.972	1361.946	-14655.54	-18953.06	-19326.496
	K395	**Lys(K)-Arg(R)**	801.035	5425.859	6696.095	1361.561	3140.14	-19256.51	-1831.82
		Lys(K)-His(H)	817.702	5436.056	6695.903	1366.969	-14566.99	-18982.79	-19233.15
		Lys(K)-Asp(D)	792.329	5422.174	6697.629	1361.004	-14729.7	-18977.6	-19434.17
		Lys(K)-Glu(E)	794.242	5426.809	6697.042	1361.308	-14767.73	-18970.86	-19459.186
	K425	**Lys(K)-Arg(R)**	805.408	5425.781	6696.595	1362.304	-14765.96	-19225.77	-19701.641
		Lys(K)-His(H)	827.037	5435.978	6697.527	1367.379	-14767.81	-18966.12	-19406.004
		Lys(K)-Asp(D)	794.485	5421.756	6698.692	1362.062	-14765.87	-18957.41	-19446.281
		Lys(K)-Glu(E)	797.941	5426.331	6698.523	1362.222	-14766.53	-18959.11	-19440.623
	K495	**Lys(K)-Arg(R)**	802.884	5429.238	6695.895	1360.332	99999900	-19109.65	331698624
		Lys(K)-His(H)	817.489	5437.169	6696.177	1363.455	-14652.57	-18979.18	-19317.465
		Lys(K)-Asp(D)	796.316	5427.054	6697.379	1360.508	-14479.47	-18962.91	-19161.131
		Lys(K)-Glu(E)	798.16	5430.859	6697.877	1360.384	7624466.59	-18946.21	7619807.5
	K521	**Lys(K)-Arg(R)**	800.795	5426.545	6695.783	1360.228	-14766.17	-19219.69	-19702.514
		Lys(K)-His(H)	817.561	5434.005	6696.937	1364.709	-14769.44	-18964.37	-19420.6
		Lys(K)-Asp(D)	792.56	5424.429	6696.775	1360.262	-14765.79	-18951.12	-19442.887
		Lys(K)-Glu(E)	794.634	5427.922	6697.276	1360.157	-14766.61	-18959.32	-19445.941
	K670	**Lys(K)-Arg(R)**	800.78	5425.9	6696.322	1361.799	-11692.4	-19263.42	-16671.027
		Lys(K)-His(H)	817.275	5435.004	6696.06	1367.277	-14701.71	-18945.07	-19331.168
		Lys(K)-Asp(D)	792.739	5422.215	6697.894	1361.262	38990960.16	-19002	38986232
		Lys(K)-Glu(E)	794.501	5426.41	6698.111	1362.09	-13664.33	-19025.36	-18408.576
	K699	**Lys(K)-Arg(R)**	802.104	5426.108	6695.04	1361.694	-14766.29	-19224.83	-19706.18
		Lys(K)-His(H)	820.031	5436.419	6695.485	1367.188	99999900	-18975.16	4256345088
		Lys(K)-Asp(D)	793.081	5422.183	6696.508	1361.305	-14764.75	-18955.72	-19447.393
		Lys(K)-Glu(E)	795.318	5426.894	6697.127	1361.645	-14766.23	-18960.96	-19446.203

Table S3.4: Mutational effect of Lysine (K) to Arginine (R), Histidine (H), Aspartate (D), and Glutamate (E) on internal potential energy of AβPP Protein

Confidence Level	Mutational Site	Energy Total (KJ/mol)					ΔE Total (KJ/mol) ($E_{Mutation}$-$E_{No\ Mutation}$)			
		No Mutation	Mutation Type				Mutation Type			
			Lys(K)-Arg(R)	Lys(K)-His(H)	Lys(K)-Asp(D)	Lys(K)-Glu(E)	Lys(K)-Arg(R)	Lys(K)-His(H)	Lys(K)-Asp(D)	Lys(K)-Glu(E)
Very High	K351	-19470.473	-13594.459	-19248.053	-19465.84	-19408.977	5876.014	222.42	4.633	61.496
	K377	-19470.473	-18276.109	-19408.256	-19455.002	-19455.916	1194.364	62.217	15.471	14.557
High	K224	-19470.473	-19708.891	-19435.436	-19453.078	-19449.477	-238.418	35.037	17.395	20.996
	K363	-19470.473	-18444.213	-9632.712	-19436.908	-5621.648	1026.26	9837.761	33.565	13848.825
	K601	-19470.473	2064150.5	-2966.571	-19459.35	-19461.703	2083620.973	16503.902	11.123	8.77
	K751	-19470.473	-16997.271	-19304.551	-19471.102	19479.688	2473.202	165.922	-0.629	38950.161
	K763	-19470.473	4871193	-13407.986	-19463.852	137002.109	4890663.473	6062.487	6.621	156472.582
Medium	K60	-19470.473	-19706.652	-19363.947	-19421.512	-19435.982	-236.179	106.526	48.961	34.491
	K161	-19470.473	-19655.035	7.19899E+11	-19443.033	-19457.756	-184.562	7.19899E+11	27.44	12.717
	K393	-19470.473	-19705.053	-19396.338	-19446.648	-19450.074	-234.58	74.135	23.825	20.399
	K401	-19470.473	-19665.314	-19383.496	-19418.393	-19408.279	-194.841	86.977	52.08	62.194
	K496	-19470.473	-19710.109	-19411.105	-19450.057	-19458.359	-239.636	59.368	20.416	12.114
	K510	-19470.473	-19711.006	597486.875	-19448.551	-19452.52	-240.533	616957.348	21.922	17.953
	K522	-19470.473	-19701.695	-19380.352	-19436.402	-19440.977	-231.222	90.121	34.071	29.496
	K662	-19470.473	765408192	48939.527	-19243.689	-18868.637	765427662.5	68410	226.784	601.836
Low	K503	-19470.473	-18983.23	-19400.318	-19457.229	-19297.186	487.243	70.155	13.244	173.287
	K568	-19470.473	1.21228E+11	-19418.48	-19446.729	-19447.135	1.21228E+11	51.993	23.744	23.338
	K724	-19470.473	-19716.252	4184.802	-19434.609	-19451.016	-245.779	23655.275	35.864	19.457
Very Low	K51	-19470.473	-19698.568	-19470.473	-19455.824	-19444.375	-228.095	0	14.649	26.098
	K134	-19470.473	-19601.6	-19453.203	-19463.322	-19460.613	-131.127	17.27	7.151	9.86
	K155	-19470.473	-17900.53	-19439.281	-19460.078	-19326.496	1569.943	31.192	10.395	143.977
	K395	-19470.473	-1831.82	-19233.15	-19434.17	-19459.186	17638.653	237.323	36.303	11.287
	K425	-19470.473	-19701.641	-19406.004	-19446.281	-19440.623	-231.168	64.469	24.192	29.85
	K495	-19470.473	331698624	-19317.465	-19161.131	7619807.5	331718094.5	153.008	309.342	7639277.973
	K521	-19470.473	-19702.514	-19420.6	-19442.887	-19445.941	-232.041	49.873	27.586	24.532
	K670	-19470.473	-16671.027	-19331.168	38986232	-18408.576	2799.446	139.305	39005702.47	1061.897
	K699	-19470.473	-19706.18	4256345088	-19447.393	-19446.203	-235.707	4256364558	23.08	24.27

Table S3.5: Lysine mutations and their disease susceptibility

Confidence Level	Mutational Site	AβPP Mutation	Untransformed Results						Transformed Results					
			PANTHER	SNAP2	Polyphen2	Pmut	PhD-SNP	SIFT	PANTHER	SNAP2	Polyphen2	PMut	PhD-SNP	SIFT
Very High	K351	K351R	Probably Benign	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	0	0	1	0	0	0
		K351H	Probably Benign	Effect	Possibly Damaging	Neutral	Neutral	Not Tolerated	0	2	1	0	0	2
		K351D	Probably Benign	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	0	2	1	0	0	0
		K351E	Probably Benign	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	0	2	1	0	0	0
	K377	K377R	Probably Damaging	Neutral	Benign	Neutral	Neutral	Tolerated	2	0	0	0	0	0
		K377H	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	2	2	1	0	0	0
		K377D	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Tolerated	2	2	2	0	0	0
		K377E	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	2	2	1	0	0	0
High	K224	K224R	Possibly Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	1	0	1	0	0	0
		K224H	Possibly Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	1	2	1	0	0	0
		K224D	Possibly Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	1	2	1	0	0	0
		K224E	Possibly Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	1	0	1	0	0	0
	K363	K363R	Probably Benign	Neutral	Benign	Neutral	Neutral	Not Tolerated	0	0	0	0	0	2
		K363H	Probably Benign	Effect	Probably Damaging	Neutral	Neutral	Not Tolerated	0	2	2	0	0	2
		K363D	Probably Benign	Effect	Possibly Damaging	Neutral	Neutral	Not Tolerated	0	2	1	0	0	2
		K363E	Probably Benign	Effect	Possibly Damaging	Neutral	Neutral	Not Tolerated	0	2	1	0	0	2
	K601	K601R	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Tolerated	2	0	2	0	0	0

Confidence Level	Mutational Site	AβPP Mutation	Untransformed Results						Transformed Results					
			PANTHER	SNAP2	Polyphen2	Pmut	PhD-SNP	SIFT	PANTHER	SNAP2	Polyphen2	PMut	PhD-SNP	SIFT
		K601H	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Tolerated	2	0	2	0	0	0
		K601D	Probably Damaging	Neutral	Benign	Neutral	Neutral	Tolerated	2	0	0	0	0	0
		K601E	Probably Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	2	0	1	0	0	0
	K751	K751R	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	2	2	1	0	0	0
		K751H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K751D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K751E	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Tolerated	2	2	2	0	0	0
	K763	K763R	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Tolerated	2	2	2	0	0	0
		K763H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K763D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K763E	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
Medium	K60	K60R	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	2	2	1	0	0	0
		K60H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K60D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K60E	Probably Damaging	Effect	Possibly Damaging	Disease	Disease	Not Tolerated	2	2	1	2	2	2
	K161	K161R	Probably Damaging	Neutral	Benign	Neutral	Neutral	Tolerated	2	0	0	0	0	0
		K161H	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Not Tolerated	2	0	2	0	0	2
		K161D	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Not Tolerated	2	2	2	0	0	2

Confidence Level	Mutational Site	AβPP Mutation	Untransformed Results						Transformed Results					
			PANTHER	SNAP2	Polyphen2	Pmut	PhD-SNP	SIFT	PANTHER	SNAP2	Polyphen2	PMut	PhD-SNP	SIFT
		K161E	Probably Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	2	0	1	0	0	0
	K393	K393R	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	2	2	1	0	0	0
		K393H	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K393D	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K393E	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Not Tolerated	2	2	1	0	0	2
	K401	K401R	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Tolerated	2	0	2	0	0	0
		K401H	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K401D	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K401E	Probably Damaging	Effect	Possibly Damaging	Disease	Neutral	Not Tolerated	2	2	1	2	0	2
	K496	K496R	Probably Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	2	0	1	0	0	0
		K496H	Probably Damaging	Neutral	Probably Damaging	Neutral	Disease	Not Tolerated	2	0	2	0	2	2
		K496D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K496E	Probably Damaging	Effect	Probably Damaging	Neutral	Disease	Not Tolerated	2	2	2	0	2	2
	K510	K510R	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Tolerated	2	0	2	0	0	0
		K510H	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Not Tolerated	2	0	2	0	0	2
		K510D	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K510E	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
	K522	K522R	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2

Confidence Level	Mutational Site	AβPP Mutation	Untransformed Results						Transformed Results					
			PANTHER	SNAP2	Polyphen2	Pmut	PhD-SNP	SIFT	PANTHER	SNAP2	Polyphen2	PMut	PhD-SNP	SIFT
		K522H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K522D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K522E	Probably Damaging	Effect	Possibly Damaging	Disease	Disease	Tolerated	2	2	1	2	2	0
	K662	K662R	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Not Tolerated	2	0	2	0	0	2
		K662H	Probably Damaging	Neutral	Probably Damaging	Disease	Neutral	Not Tolerated	2	0	2	2	0	2
		K662D	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Tolerated	2	0	2	0	0	0
		K662E	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Tolerated	2	0	2	0	0	0
Low	K503	K503R	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K503H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K503D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K503E	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
	K568	K568R	Probably Damaging	Neutral	Benign	Neutral	Neutral	Tolerated	2	0	0	0	0	0
		K568H	Probably Damaging	Neutral	Probably Damaging	Neutral	Neutral	Tolerated	2	0	2	0	0	0
		K568D	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	2	2	1	0	0	0
		K568E	Probably Damaging	Neutral	Benign	Neutral	Neutral	Tolerated	2	0	0	0	0	0
	K724	K724R	Probably Damaging	Effect	Benign	Neutral	Neutral	Tolerated	2	2	0	0	0	0
		K724H	Probably Damaging	Effect	Probably Damaging	Neutral	Disease	Not Tolerated	2	2	2	0	2	2
		K724D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2

Confidence Level	Mutational Site	AβPP Mutation	Untransformed Results						Transformed Results					
			PANTHER	SNAP2	Polyphen2	Pmut	PhD-SNP	SIFT	PANTHER	SNAP2	Polyphen2	PMut	PhD-SNP	SIFT
		K724E	Probably Damaging	Effect	Possibly Damaging	Disease	Disease	Not Tolerated	2	2	1	2	2	2
Very Low	K51	K51R	Probably Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	2	0	1	0	0	0
		K51H	Probably Damaging	Neutral	Probably Damaging	Disease	Disease	Not Tolerated	2	0	2	2	2	2
		K51D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K51E	Probably Damaging	Effect	Probably Damaging	Neutral	Disease	Not Tolerated	2	2	2	0	2	2
	K134	K134R	Probably Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	2	0	1	0	0	0
		K134H	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Not Tolerated	2	2	2	0	0	2
		K134D	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K134E	Probably Damaging	Effect	Possibly Damaging	Disease	Neutral	Not Tolerated	2	2	1	2	0	2
	K155	K155R	Probably Damaging	Effect	Possibly Damaging	Neutral	Neutral	Tolerated	2	2	1	0	0	0
		K155H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K155D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K155E	Probably Damaging	Effect	Possibly Damaging	Disease	Neutral	Not Tolerated	2	2	1	2	0	2
	K395	K395R	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K395H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K395D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K395E	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Tolerated	2	2	2	2	2	0
	K425	K425R	Probably Damaging	Neutral	Benign	Neutral	Neutral	Tolerated	2	0	0	0	0	0
		K425H	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2

Confidence Level	Mutational Site	AβPP Mutation	Untransformed Results						Transformed Results					
			PANTHER	SNAP2	Polyphen2	Pmut	PhD-SNP	SIFT	PANTHER	SNAP2	Polyphen2	PMut	PhD-SNP	SIFT
		K425D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K425E	Probably Damaging	Effect	Possibly Damaging	Disease	Disease	Not Tolerated	2	2	1	2	2	2
	K495	K495R	Probably Damaging	Neutral	Possibly Damaging	Neutral	Neutral	Tolerated	2	0	1	0	0	0
		K495H	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K495D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K495E	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
	K521	K521R	Probably Damaging	Neutral	Possibly Damaging	Disease	Neutral	Not Tolerated	2	0	1	2	0	2
		K521H	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K521D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K521E	Probably Damaging	Neutral	Benign	Neutral	Neutral	Tolerated	2	0	0	0	0	0
	K670	K670R	Probably Damaging	Effect	Benign	Neutral	Neutral	Tolerated	2	2	0	0	0	0
		K670H	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Tolerated	2	2	2	0	0	0
		K670D	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Not Tolerated	2	2	2	2	0	2
		K670E	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Tolerated	2	2	2	0	0	0
	K699	K699R	Probably Damaging	Effect	Probably Damaging	Neutral	Neutral	Tolerated	2	2	2	0	0	0
		K699H	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Tolerated	2	2	2	2	0	0
		K699D	Probably Damaging	Effect	Probably Damaging	Disease	Disease	Not Tolerated	2	2	2	2	2	2
		K699E	Probably Damaging	Effect	Probably Damaging	Disease	Neutral	Tolerated	2	2	2	2	0	0

List of Publications

LIST OF PUBLICATIONS

[Cumulative Impact Factor: 49.85; Cumulative Citations: 170; h-Index: 7]

PUBLICATIONS FROM THESIS

I REFEREED JOURNALS

[1] **Dhiraj Kumar,** Pravir Kumar (2019), Integrated mechanism of Lysine-351, PARK2 and STUB1 in AβPP ubiquitination, *Journal of Alzheimer's Disease,* 68(3): 1125-1150. doi 10.3233/JAD-181219. PubMed ID: 30958363. **(Impact factor: 3.7)**

[2] **Dhiraj Kumar,** Pravir Kumar (2019), Aβ, Tau, and α-Synuclein aggregation and integrated role of PARK2 in the regulation and clearance of toxic peptides, *Neuropeptides,* [Epub ahead of print], doi:10.1016/j.npep.2019.101971. PubMed ID: 31540705. **(Impact factor: 2.41)**

[3] **Dhiraj Kumar,** Pravir Kumar (2018), An in-silico investigation of key lysine residues and their selection for clearing off Aβ and Holo-AβPP through ubiquitination, *Interdisciplinary Sciences: Computational Life Sciences,* [Epub ahead of print], doi:10.1007/s12539-018-0307-2. PubMed ID: 30194628. **(Impact factor: 1.4)**

[4] **Dhiraj Kumar,** Rashmi K Ambasta, Pravir Kumar (2019), Recent breakthroughs in the ubiquitination research of neurodegenerative pathologies: Molecular mechanisms to therapeutic applications, In Submission.

II PRESENTATIONS IN CONFERENCES

[1] **Dhiraj Kumar** and Pravir Kumar **(2015)**. Functional lysine residues in Aβ clearance, 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), 19-21 December, **North-Eastern Hill University, Shillong,** INDIA **[Oral presentation]**

[2] **Dhiraj Kumar**, Rashmi K. Ambasta and Pravir Kumar (**2014**). Anti-cancerous drugs as a neuroprotectant: a therapeutic intervention in neurodegenerative disorders, International Symposium on Translational Neuroscience and XXXII Annual Conference of the Indian Academy of Neurosciences, 01-03 November, **National Institute of Mental Health and Neurosciences, Bangalore,** INDIA **[Poster presentation]**

OTHER COLLABORATIVE WORKS IN THE LABORATORY

[1] Rashmi K Ambasta, Rohan Gupta, **Dhiraj Kumar**, Saurabh Bhattacharya, Aditi Sarkar, Pravir Kumar (2018), *Can luteolin be a therapeutic molecule for both colon cancer and diabetes?* **Briefings in Functional Genomics,** [Epub ahead of print] ***(Impact factor: 3.4)***

[2] Niraj Kumar Jha, Saurabh Kumar Jha, Renu Sharma, **Dhiraj Kumar**, Rashmi K Ambasta, Pravir Kumar (2018), *Hypoxia induced signaling activation in Neurodegenerative Diseases: Targets for new therapeutic strategies*, **Journal of Alzheimer's Disease,** 62(1):15-38. ***(Impact factor: 3.7)***

[3] Renu Sharma, **Dhiraj Kumar**, Niraj Kumar Jha, Saurabh Kumar Jha, Rashmi K. Ambasta, Pravir Kumar (2017), *Reexpression of cell cycle markers in aged neurons and muscles: whether cell should divide or die?* **(BBA) Molecular Basis of Disease,** 1863(1):324-336. ***(Impact factor: 5.9)***

[4] Saurabh Kumar Jha, Niraj Kumar Jha, **Dhiraj Kumar**, Renu Sharma, Abhishek Shrivastav, Rashmi K Ambasta, Pravir Kumar, (2017), *Stress-induced synaptic dysfunction and neurotransmitter release in Alzheimer's disease: Can neurotransmitters and neuromodulators be potential therapeutic targets?* **Journal of Alzheimer's Disease,** 57(4):1017-1039. ***(Impact factor: 3.7)***

[5] Saurabh Kumar Jha, Niraj Kumar Jha, **Dhiraj Kumar**, Rashmi K. Ambasta, Pravir Kumar (2017), *Linking mitochondrial dysfunction, metabolic syndrome and stress signaling in Neurodegeneration*, **(BBA) Molecular Basis of disease,** 1863(5):1132-1146. ***(Impact factor: 5.9)***

[6] Pravir Kumar, **Dhiraj Kumar**, Saurabh Kumar Jha, Niraj Kumar Jha, Rashmi K Ambasta (2016), *Ion channels in neurological disorders*, **Advances in Protein Chemistry and Structural Biology,** 103:97-136. ***(Impact factor: 3.01)***

[7] Rashmi K. Ambasta, **Dhiraj Kumar**, Piyush Sawhney, Rajat Gupta, Parul Yadav, Pooja Pabari and Pravir Kumar (2016) *Epigenesis in Colorectal Cancer: A lethal change in the cell*, In: Manoj K. Mishra, Kumar S. Bishnupuri (Eds.), **Epigenetic Advancements in Cancer (Springer)**, 123-144.

[8] Rashmi K. Ambasta, Saurabh Kumar Jha, **Dhiraj Kumar**, Renu Sharma, Niraj Kumar Jha, and Pravir Kumar (2015) *Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules*, **Journal of Translational Medicine,** 13:307. ***(Impact factor: 3.9)***

[9] Niraj Kumar Jha, Saurabh Kumar Jha, **Dhiraj Kumar**, Noopur Kejariwal, Renu Sharma, Rashmi K Ambasta and Pravir Kumar (2015), *Impact of IDE and Neprilysin in Alzheimer's Disease biology: Characterization of putative coagnates for therapeutic applications*, **Journal of Alzheimer's Disease,** 48(4):891-917. ***(Impact factor: 3.7)***

[10] **Dhiraj Kumar**, Sakshi Sharma, Sagar Verma, Pravir Kumar and Rashmi Kumar Ambasta (2015), *Role Of wnt-p53-Nox Signaling Pathway In Cancer*

Development And Progression, **British Journal of Medicine and Medical Research,** 8(8):651-676.

[11] **Dhiraj Kumar**, Sakshi Sharma, Sagar Verma, Pravir Kumar and Rashmi Kumar Ambasta (2015), *Molecular signaling saga in tumour biology*, **Journal of Tumor,** 3(2):309-313.

OTHER CONFERENCE PROCEEDINGS

[1] Alka Raina, Saurabh Kumar Jha, Niraj Kumar Jha, **Dhiraj kumar**, Rashmi K Ambasta and Pravir Kumar **(2015)**, *Putative transcription factor binding elements of ubiquitin E3 ligase in neurodegenerative disorders*, 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), 19-21 December, NEHU, Shillong [Poster presentation]

[2] Abhisekh Srivastava, Puspendramani Mishra, **Dhiraj kumar**, Saurabh Kumar Jha, Niraj Kumar Jha, Rashmi K Ambasta and Pravir Kumar **(2015)**, *Relevance of terpenoids and alkaloids in neuroprotection*, 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), 19-21 December, NEHU, Shillong [Poster presentation]

[3] Swati Sharan, Niraj Kumar Jha, Saurabh Kumar Jha, **Dhiraj kumar**, Rashmi K Ambasta and Pravir Kumar **(2015)**, *Post-translational modification mechanism in Parkinson's disease pathology*, 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), 19-21 December, NEHU, Shillong [Poster presentation]

[4] Minal Singh, Niraj Kumar Jha, Saurabh Kumar Jha, **Dhiraj kumar**, Rashmi K Ambasta and Pravir Kumar **(2015)**, *In-silico characterization of holo AβPP promoter and its transactivation modules*, 29th Annual Conference of Society for Neurochemistry India and Advancement in computation Neurochemistry and Neurobiology (SNCI-ACNN), 19-21 December, NEHU, Shillong [Poster presentation]

[5] Pushpendra Mishra, Abhishek Srivastava, **Dhiraj Kumar**, Rashmi K Ambasta and Pravir Kumar **(2015)**, *Genetic Aberrations in Neurodegenerative disorders: A molecular link between Parkinson's and Huntington's disease*, International Congress on Friedreich's ataxia and DNA structure in Health and Disease, 11-13 April, AllMS, New Delhi, INDIA

[6] Abhishek Srivastava, Pushpendra Mishra, **Dhiraj Kumar**, Rashmi K Ambasta and Pravir Kumar **(2015)**, *Role of DNA damage and repair defects in Neurodegenerative disorders*, International Congress on Friedreich's ataxia and DNA structure in Health and Disease, 11-13 April, AllMS, New Delhi, INDIA

Curriculum Vitae

CURRICULUM VITAE

Dhiraj

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✉ **dhiraj1188@gmail.com**

Personal Information

Father's Name	: Dharmdev Ram
Sex	: Male
Nationality	: Indian
Marital Status	: Married
Languages	: English, Hindi

Permanent Address:

House No	: A1-391
Street No	: 04
Area	: Madhu Vihar
P.O.	: Uttam Nagar
City	: New Delhi
PIN	: 110059
State	: Delhi
Country	: India

Academic Profile

- **Ph.D** in **Neuroscience** (2014-2019)
  Delhi Technological University
  Delhi, India.

- **M.Tech.** in **Bioinformatics** (2012-2014)
  Delhi Technological University
  Delhi, India. *(First Division)*

- **B.E.** in **Biotechnology** (2008-2012)
  Netaji Subhas Institute of Technology
  Delhi, India. *(First Division)*

Career Objectives

- To enhance and develop research competencies via active participation in personal and teamwork for planning, designing and conducting highly technical and innovative research projects based on the available resources.

Research Interests

- Molecular Neuroscience, Computational Biology, Mammalian cell culture, Drug discovery and Structural Biology

Presently Engaged in the Project

- Implication of computational approaches for ubiquitination profiling of Amyloid beta precursor protein as a therapeutic intervention for Alzheimer's and Parkinson's disease

Professional Training and Research Experiences

(A) **Graduate Researcher** *(August 2014 – October 2019)*
Research project on *"Characterization, Investigation and Clearance Mechanisms of Neurotoxic Proteins in Alzheimer's Disease and Parkinson's Disease",* under the supervision of Prof. Pravir Kumar, **Delhi Technological University**, Delhi, INDIA

(B) **Research Training** *(14^{th}-18^{th} December 2016)*
Short Term Training on *"Research Methodology"* at **Delhi Technological University**, Delhi, INDIA

(C) **M.Tech. Project** *(December 2012 – July 2014)*
Dissertation on *"Neurological Channelopathic Knowledge Base (NCKB): An application software for Ion channels and Neurological channelopathies",* under the supervision of Prof. Pravir Kumar, **Delhi Technological University**, Delhi, INDIA

(D) **B.E. Project** *(January 2012- May 2012)*
Dissertation on *"Characterization of Basic 7S globulin, A protein from Soy seed"* under the supervision of Dr. Imtaiyaz Hassan and Dr. Ashok Kumar Dubey, **Netaji Subhas Institute of Technology**, Delhi INDIA

(E) **Research Intern** *(1^{st}-30^{th} July 2011)*
Training on "Next Generation DNA Sequencing" under the supervision of Tony Jose (Scientist, Sandor Proteomics Private Limited), **Indian Biosciences**, Haryana, INDIA

(F) **Research Intern** *(1^{st}-30^{th} June 2011)*
Training on "*Advanced Molecular Biology Techniques and DNA Fingerprinting*", under the supervision of Dr. Kulmohan S. Mehta, (MD)**, Indian Biosciences Institute of Technology**, Gurgaon, Haryana, INDIA

Future Professions

I am trained as a molecular and structural biologist with broad interests and expertise in dissecting molecular mechanisms and employing in-silico methodologies to devise therapeutic interventions in diseases. Highly efficient in literature review, scientific writing and scientific editing, with neat and sharp presentation skills. I would love to be a true research expert with successful end-to-end project management experiences in the future years.

List of Publications

(Cumulative Impact Factor: 49.85)
(Cumulative Citations: 170)

[1] **Dhiraj Kumar**, Pravir Kumar (2019), *Aβ, Tau, and α-Synuclein aggregation and integrated role of PARK2 in the regulation and clearance of toxic peptides*, **Neuropeptides,** [Epub ahead of print]. doi: 10.1016/j.npep.2019.101971. PubMed ID: **31540705 (Impact factor: 2.41)**

[2] **Dhiraj Kumar**, Pravir Kumar (2019), *Integrated mechanism of Lysine 351, PARK2 and STUB1 in AβPP ubiquitination,* **Journal of Alzheimer's Disease,** 68(3):1125-1150. doi: 10.3233/JAD-181219. PubMed ID: 30958363. ***(Impact factor: 3.7)***

[3] Rashmi K Ambasta, Rohan Gupta, **Dhiraj Kumar**, Saurabh Bhattacharya, Aditi Sarkar, Pravir Kumar (2018), *Can luteolin be a therapeutic molecule for both colon cancer and diabetes?* **Briefings in Functional Genomics,** 18(4):230-239. doi:10.1093/bfgp/ely036. PubMed ID: 30462152. ***(Impact factor: 3.4)***

[4] **Dhiraj Kumar**, Pravir Kumar (2018), *An in-silico investigation of key lysine residues and their selection for clearing off Aβ and Holo-AβPP through ubiquitination*, **Interdisciplinary Sciences: Computational Life Sciences,** [Epub ahead of print], doi:10.1007/s12539-018-0307-2. PubMed PMID: 30194628. ***(Impact factor: 1.4)***

[5] Niraj Kumar Jha, Saurabh Kumar Jha, Renu Sharma, **Dhiraj Kumar**, Rashmi K Ambasta, Pravir Kumar (2018), *Hypoxia induced signaling activation in Neurodegenerative Diseases: Targets for new therapeutic strategies*, **Journal of Alzheimer's Disease,** 62(1):15-38. doi:10.3233/JAD-170589. PubMed PMID: 29439330. ***(Impact factor: 3.7)***

[6] Renu Sharma, **Dhiraj Kumar**, Niraj Kumar Jha, Saurabh Kumar Jha, Rashmi K. Ambasta, Pravir Kumar (2017), *Reexpression of cell cycle markers in aged neurons and muscles: whether cell should divide or die?* **Biochimica et Biophysica Acta, (BBA) Molecular Basis of Disease,** 1863(1):324-336. doi:10.1016/j.bbadis.2016.09.010. PubMed PMID: 27639832. ***(Impact factor: 5.9)***

[7] Saurabh Kumar Jha, Niraj Kumar Jha, **Dhiraj Kumar**, Renu Sharma, Abhishek Shrivastav, Rashmi K Ambasta, Pravir Kumar, (2017), *Stress-induced synaptic dysfunction and neurotransmitter release in Alzheimer's disease: Can neurotransmitters and neuromodulators be potential therapeutic targets?* **Journal of Alzheimer's Disease,** 57(4):1017-1039. doi:10.3233/JAD-160623. PubMed PMID: 27662312. ***(Impact factor: 3.7)***

[8] Saurabh Kumar Jha, Niraj Kumar Jha, **Dhiraj Kumar**, Rashmi K. Ambasta, Pravir Kumar (2017), *Linking mitochondrial dysfunction, metabolic syndrome and stress signaling in Neurodegeneration*, **(BBA) Molecular Basis of disease,** 1863(5):1132-1146. doi:10.1016/j.bbadis.2016.06.015. PubMed PMID: 27345267. ***(Impact factor: 5.9)***

[9] Pravir Kumar, **Dhiraj Kumar**, Saurabh Kumar Jha, Niraj Kumar Jha, Rashmi K Ambasta (2016), *Ion channels in neurological disorders*, **Advances in Protein Chemistry and Structural Biology,** 103:97-136. doi:10.1016/bs.apcsb.2015.10.006. PubMed PMID: 26920688. ***(Impact factor: 3.01)***

[10] Rashmi K. Ambasta, **Dhiraj Kumar**, Piyush Sawhney, Rajat Gupta, Parul Yadav, Pooja Pabari and Pravir Kumar (2016) *Epigenesis in Colorectal Cancer: A lethal change in the cell*, In: Manoj K. Mishra, Kumar S. Bishnupuri (Eds.), **Epigenetic Advancements in Cancer (Springer)**, 123-144. doi:10.1007/978-3-319-24951-3_6.

[11] Rashmi K. Ambasta, Saurabh Kumar Jha, **Dhiraj Kumar**, Renu Sharma, Niraj Kumar Jha, and Pravir Kumar (2015) *Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules*, **Journal of Translational Medicine,** 13:307. doi: 10.1186/s12967-015-0665-z. PubMed PMID: 26385094. ***(Impact factor: 3.9)***

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- ✓ ***Molecular Biology:*** DNA / RNA Isolation; Plasmid isolation, cloning and expression in bacterial systems; Transformation; PCR, Agarose gel electrophoresis
- ✓ ***Animal Cell Culture Basics:*** Media preparation; Cell disaggregation; Subculturing; Cell viability and Cytotoxicity assays
- ✓ ***Protein Purification:*** Isolation and purification of proteins; Dialysis; Ultra-filtration; Chromatography: Ion exchange, Gel-filtration and Affinity
- ✓ ***Bioinformatics:*** Sequence analysis tools; Secondary and tertiary structure prediction; Homology modeling using various online servers for protein structure and dynamics
- ✓ ***Structure Biology:*** Protein structure determination and refinement; Model building; Structural analysis and use of related software
- ✓ ***Microbiology:*** Media preparation; Plating; Pouring; CFU count; Infection; Antimicrobial action of different compounds
- ✓ ***Enzymology:*** Enzyme purification assay and Immobilization on different support
- ✓ ***Crystallization Techniques:*** Micro dialysis; Vapor diffusion (Hanging drop and Sitting drop); Co crystallization and soaking
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DECLARATION

I hereby declare that the given above information are true to the best of my knowledge and belief and can be supported with reliable documents when needed.

Place: New Delhi

Date*: 10/10/2019* ***(Dhiraj)***

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Integrated Mechanism of Lysine 351, PARK2, and STUB1 in AβPP Ubiquitination

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Abstract. Intracellular accumulation of aggregated amyloid-β, misfolded and non-functional proteinopathy, is the hallmark feature in Alzheimer's disease (AD). There are several mechanisms to clear the amyloid burden in a cell, including transcytosis across the blood-brain barrier, immune mediated, lysosomal pathway associated autophagy, enzymatic degradation by insulin degrading enzyme/neprilysin, and the proteasomal pathway. Among them, the ubiquitin proteasome system (UPS) is playing a critical role to prevent the intracellular amyloid-β deposition and to clear off the cellular burden in association with ubiquitin E3 ligase enzymes in AD. For ubiquitination, lysine moiety in a protein acts like a docking site for the attachment of ubiquitin molecule and different lysine residues act differently in this reaction. Therefore, it is pertinent to understand and link the role of various lysine residues along with their effector molecules, for instance, E3 ligases PARK2 and STUB1 in the ubiquitination cascade. Herein, we 1) modeled the structure of AβPP and determined its topologies and studied the impact of lysine residues in AβPP stability, 2) reported K351 as the most promising target for AβPP ubiquitination, 3) investigated the plausible role of lysine residues in non-covalent interactions mediated ubiquitin positioning in the ubiquitination, 4) detected conserved amino acids that is crucial for AβPP ubiquitination, and 5) identified the key ubiquitination enzymes and their interaction network playing major role in the ubiquitination of AβPP.

Keywords: Amyloid-β, amyloid-β protein precursor, lysine, neurodegeneration, therapeutics, ubiquitination

INTRODUCTION

Alzheimer's disease (AD) is characterized by the aberrant protein accumulation and aggregates such as amyloid-β (Aβ), leading to the development of senile plaques in the brain [1]. In this regard, the amyloid cascade hypothesis affirms the Aβ deposition as an early pathogenic event in the progression of AD [2]. The intra- and extracellular $A\beta_{40/42}$ deposits perturb the synaptic transmission between neurons and trigger the memory and cognitive decline in AD patients [3]. Here, Aβ and its precursor, AβPP, are the central players in the pathology of AD whose levels are regulated by the protein quality control: ubiquitin proteasome system (UPS). There are other mechanisms that regulate Aβ clearance including enzymatic pathways utilizing insulin degrading enzyme and neprilysin [4], immune-mediated transcytosis across the blood-brain barrier [5], and the lysosomal pathway through autophagy [6]. However, the ubiquitination pathway is central to regulate the level of proteins involved in all these pathways; therefore, it has a great importance in the cellular biology for the clearance of Aβ. The ubiquitination process involves the selection of key lysine residues of target protein by E3 ligases for ubiquitin attachment. This ubiquitin ligation at key lysine and their type of poly-ubiquitin chain determines the cellular fate of the target protein [7]. For instance, studies have identified K6 poly-Ub-chain to trigger DNA repair

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Table 1
Experimental studies reporting the ubiquitination of AβPP and Aβ to rescue the amyloid burden in AD

S. No.	Experimental Models	UPS Enzymes	Ubiquitination Sites	Ubiquitination Effects	Reported Studies
1	APP/PS1 Transgenic mice	Parkin	_	AβPP degradation	[11]
2	Human Neuroblastoma M17 Cells, Rat Brains		_	Aβ degradation	[12]
3	SHSY5Y Cells, Human and Transgenic Mouse (Parkin K/O) Brain Samples		_	Aβ/AβPP degradation	[13, 14]
4	APP23/PS45 Mice	UCHL1	_	Regulate AβPP degradation	[15]
5	Crbn-KO Mouse Brains	CRL4	K676(AβPP695)/ K751(AβPP770)	AβPP interactions with other proteins	[16]
6	HeLa Cells, 2 × Tg Mice (FBL2/AD1)/3 × Tg Mice	FBL2	K649–651(AβPP695)/ K724–726(AβPP770)	AβPP metabolism, Endocytic trafficking	[17, 18]
7	Hippocampal Neurons, Fbxo2 KO Mice	Fbxo2	_	AβPP processing and degradation	[19]
8	N2a Cells, P0 Mice Brain	_	K612(AβPP695)/ K687(AβPP770) K624(AβPP695)/ K699(AβPP770)	Endosomal sorting of AβPP	[20]
9	SHSY5Y Cells, ddY Mice	HRD1	_	AβPP degradation	[21]
10	CHO Cells	HRD1	_	AβPP degradation	[22]
11	SHSY5Y Cells, Human Brain Sample	CHIP	_	AβPP degradation	[23]
12	Primary Neuron Culture	_	_	Aβ degradation	[24]
13	Rat Cortical Neurons	_	_	Aβ/AβPP degradation	[25]
14	PC12 Cells	Ubiquilin-1	K688(AβPP695)/ K763(AβPP770)	AβPP biosynthesis, trafficking, and degradation	[26]
15	SHSY5Y Cells	_	_	AβPP degradation	[27]

responses and K33 poly-Ub-chain to initiate stress responses. Moreover, K63 poly-Ub-chain to govern DNA repair, endocytosis and inflammatory responses while K11, K27, K29, and K48 poly-Ub-chain to elicit proteasomal degradation [8, 9].

In this manner, UPS greatly influences Aβ production through AβPP ubiquitination and through proteasomal degradation of their regulatory enzymes (β- and γ-secretases). Moreover, there is increasing evidence of non-functional UPS, i.e., ubiquitinating and deubiquitinating enzymes and the proteasomal subunits that are responsible for the altered Aβ clearance in AD patients [10]. However, there are numerous reports depicting the ubiquitination of Aβ or AβPP *in vitro*, *ex vivo*, and *in vivo* but their exact site for ubiquitination remained unknown for most cases (Table 1). Moreover, the AβPP ubiquitination research is at the preliminary stage, where much needs to be investigated to unravel the mystery of amyloid burden mitigation. Therefore, identification of the key lysine residues and the ubiquitination components are crucial for determining the mechanism behind Aβ clearance. In this regard, we have identified the key lysine residues having great potential for AβPP ubiquitination. Moreover, the importance of these key lysine residues in AβPP processing, non-covalent interactions with ubiquitin and other functions have also studied. Further, we modeled the 3D structure of holo-AβPP to investigate the impact of key lysine residues on AβPP stability and their mutational disease susceptibility. Nevertheless, we have also identified the potential ubiquitination enzymes, E1s, E2s, E3s, and DUBs, and their complex interplay in the ubiquitination process of AβPP. In summary, this work demonstrates the AβPP ubiquitination mechanism and Aβ clearance to provide novel therapeutic targets against AD.

MATERIALS AND METHODS

AβPP structure modeling

The AβPP structure was modeled with a protein homology/analogy recognition tool Phyre[2]. It included the assembly of homologous sequences by multiple sequence alignments of query sequence with their sequence homologs and their consequent secondary structure prediction by PSI-pred pooled query hidden Markov model. Further, fold library was scanned for crude backbone construction based on top scoring alignments. Further, loop modeling for correcting insertions and deletions in the models followed by side chain placement to backbone to obtain a final protein structure model [28]. Here, six templates (c3ktmB, c3dxeB, c2yszA, d1rw6a, c1amlA, c2lp1A) were employed to model the AβPP protein

structure based on heuristics method to maximize the confidence, percentage identity, and alignment coverage to the queried sequence.

AβPP domain analysis

The protein domain analysis was done to identify the functional sites in the AβPP with MOTIF Search tool. It extracted domain results on the basis of matched bit score for AβPP sequence and each domain found in Pfam library with an E-value cut-off of 0.001 [29].

Structure refinement simulations

The predicted 3D-structure of AβPP was refined with an atomic-level, high-resolution protein structure refinement tool-ModRefiner [30]. We performed the conformational search for the backbone and side chain atoms to simulate the predicted model to their native state in terms of backbone topology, side chain positioning and hydrogen bonds directed by combined physics- and knowledge-based force field energy calculations.

AβPP structural validations through PROVE, ERRAT, VERIFY 3D, and RAMPAGE

The reliability of 3D-atomic models of predicted AβPP validated through the stereo chemical quality measurements of modeled proteins with different structural validation programs. ***PROVE*** analyzed the volume-based quality of protein crystal structure by computing the statistical Z-score deviations of the atomic volumes from their standard values [31]. The buried atoms less than 1% passed the structural quality test while greater than 5% implied the structural irregularities. ***ERRAT*** determined overall quality factor by identifying the statistical differences between the patterns of non-bonded atomic interactions (ordered versus randomized distribution) and the error functions of the predicted model with the statistics of highly refined structures [32]. ***VERIFY 3D*** compared 3D atomic model compatibility with its own primary sequence (1D) associated structural class assigned based on their environment and location [33]. ***RAMPAGE*** geometrically validated C-alpha neighboring residues of modeled AβPP [34], which allowed the detection of C-beta bond angle distortions. Moreover, it defined favored and allowed phi-psi regions for glycine, proline, and pre-proline residues to validate the accuracy of the model.

Identification of potential ubiquitination sites in AβPP

The important lysine residues for AβPP ubiquitination was identified with four different approaches, including 1) the sequence identity between AβPP and ubiquitin lysine sites, 2) sequence similarity between AβPP and ubiquitin lysine sites, 3) ubiquitin-ubiquitin and ubiquitin-AβPP lysine peptides docking, and 4) machine learning based ubiquitination site predictions.

Sequence identity analysis

Pairwise sequence alignment was performed using BioEdit Sequence Alignment Editor Software [35] using BLOSUM 62 substitution matrix. Further, it calculated the identity scores among the 21 window-size central lysine's neighboring residues of ubiquitin and AβPP sequences.

Sequence similarity analysis

Conserved sequence analysis of amino acid residues neighboring lysine sites was performed by aligning the 21 window-size multiple lysine site sequences in AβPP by the BioEdit Sequence Alignment Editor Software [35] at different thresholds to identify the important residues common to both ubiquitin and AβPP critical for the ubiquitination.

Ubiquitin-AβPP lysine peptides docking

The protein-peptide interactions of ubiquitin protein and AβPP lysine peptides were modeled by flexible docking approach using CABS-dock web server. It executed simulation searching for the binding sites in the receptor protein allowing full flexibility to the peptides being docked [36]. For ubiquitin-AβPP peptide docking, 21 window-size peptide sequences were prepared by taking 10 amino acid residues on either side of the lysine sites from AβPP protein as test set and from ubiquitin protein as control set, respectively. Further, ubiquitin protein (PDB ID: 1UBQ) is flexibly docked with both the test set peptides, and the control set peptides to identify the potential lysine for ubiquitination.

Machine learning techniques: UbiSite, BDM-PUB, hCKSAAP_UBSITE, UbPred, UbiPred

The potential ubiquitination sites for AβPP was predicted by diverse machine learning tools, including UbiSite, BDM-PUB, CKSAAP, UbPred, and UbiPred on the basis of the experimentally verified

ubiquitination site datasets. ***UbiSite*** employed maximal dependence decomposition method (specificity level High at 95%) and support vector machine based on the hybrid features for the large-scale ubiquitin conjugation sites data to predict ubiquitination sites [37]. ***BDM-PUB*** employed Bayesian Discriminant analysis (at balanced cut off) between high specificity and high sensitivity to apply a probabilistic approach for ubiquitination pattern recognition [38]. ***hCKSAAP_UBSITE*** utilized SVM classifiers based diverse amino acid pattern and propensities trained by logistic regression to predict the ubiquitination sites in AβPP [39]. ***UbPred*** relied upon a random forest-based prediction model trained on experimentally verified ubiquitination sites to predict the ubiquitination pattern in AβPP [40]. ***UbiPred*** employed SVM to predict the ubiquitination sites based on the principle of the informative physicochemical property mining algorithm [41].

Functional studies of lysine's impact on AβPP ubiquitination

The refined AβPP structure was incorporated with both positive (Arginine (R), Histidine (H)) and negative (Aspartic acid (D), Glutamic acid (E)) charged mutations at their lysine sites by Pymol software [42]. The above mutations were studied for their impact on AβPP stability, ubiquitination, modifications, and disease susceptibility.

Investigation of AβPP stability upon lysine mutations

The refined AβPP model and mutated AβPP (Lysine (K)$\longrightarrow$Arginine(R)/Histidine(H)/Aspartic acid(D)/Glutamic acid(E)) analyzed for their total force field energies by Swiss PDB viewer 4.0.2 software [43]. The variations in the total force field energies were estimated to observe the impact of lysine mutations on the amyloid beta precursor protein's stability.

Physico-chemical property analysis for AβPP ubiquitination

The physico-chemical properties of Aβ, AβPP, and ubiquitin proteins were computationally determined from their peptide sequences by ExPASy server tool ProtParam [44]. It computed various physico-chemical properties, including the atomic and amino acid compositions along with the count of positively and negatively charged residues, the instability index, aliphatic index, and the grand average of hydropathicity of the desired proteins. The physico-chemical properties of AβPP was compared with ubiquitin to investigate the crucial parameters important for the ubiquitination.

Examination of lysine sites crucial for AβPP modifications

The molecular mechanisms associated with the pathogenic amino acid substitutions in AβPP were identified by a machine-learning tool MutPred [45]. It quantified the pathogenicity of mutations based on the probabilistic modeling of a large repertoire of structural-functional alterations, including the disruptions in structure, stability, macromolecular binding, and post-translational modification sites in amino acid sequences.

Mutational analysis of lysine residues for disease susceptibility

The functional impacts of the introduced mutations were studied with different mutation analysis tools, including PANTHER [46], SNAP2 [47], Polyphen2 [48], Pmut [49], PhD-SNP [50], and SIFT [51]. The obtained results were transformed into numerical values to analyze them on the stacked bar graph. For instance, results with "Probably Benign", "Neutral", and "Tolerated" were assigned with '0' numerical value and "Possibly Damaging" with '1' numerical value. Similarly, results with "Probably Damaging", "Effect", and Not tolerated" were assigned with "2" numerical value and the threshold was taken '6', i.e., more than half prediction tools to predict high confidence lysine having disease susceptibility.

Characterization of ubiquitination pattern of AβPP

The ubiquitination pattern of AβPP was investigated through the combination of multiple studies, including 1) the AβPP-ubiquitin interaction studies, and 2) the prediction of ubiquitination enzyme's interactional network for AβPP or Aβ clearances in AD biology.

AβPP-ubiquitin interaction prediction and interface residue analysis

The interactions among the different domains of AβPP and ubiquitin proteins were predicted by the protein-protein interaction prediction server-PSOPIA [52]. It compared the sequence similarities to a known interacting protein pair, statistical propensities of the domain-domain interactions, and the

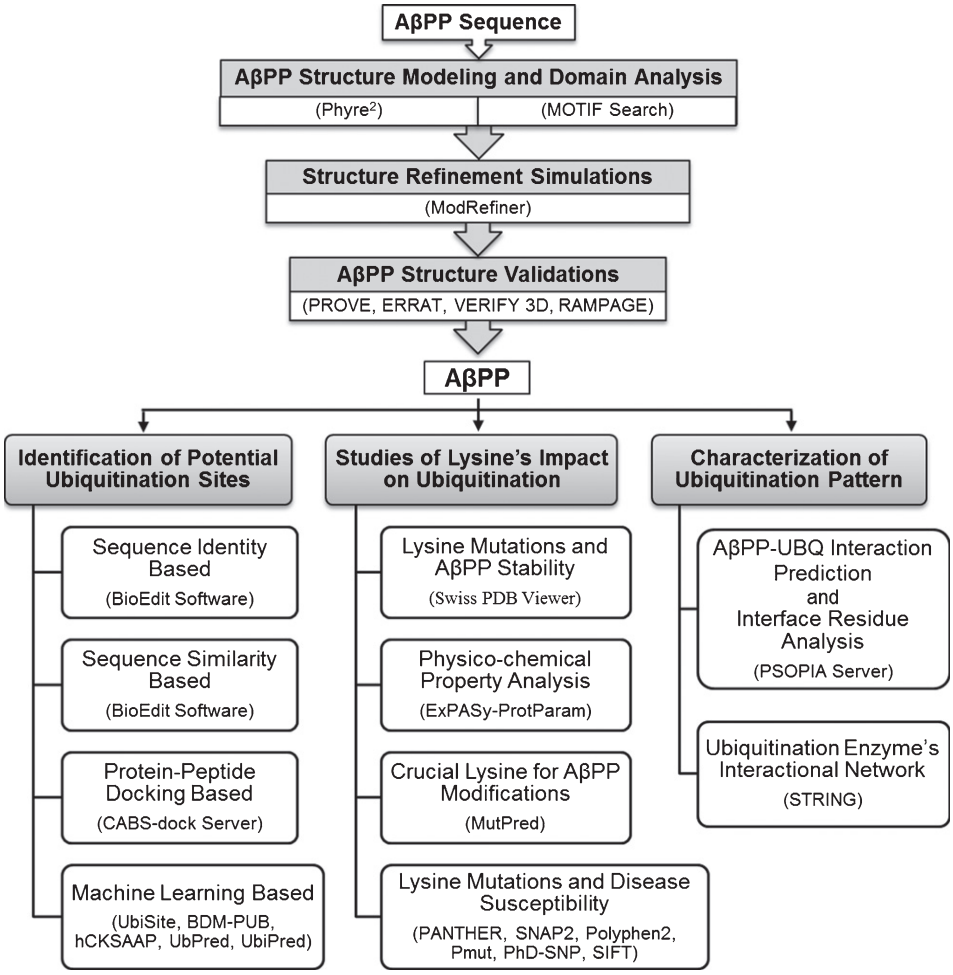


Fig. 1. Flow chart depicting the tools and techniques employed for the study of AβPP ubiquitination.

sum of edge weights along the shortest path between the homologous proteins in a PPI network. Moreover, the potential ubiquitin interacting domains of AβPP (PDB IDs: 4JFN_A, 2FKL_A, 1AAP_A, 3UMK_A, 1IYT_A, 2LP1_A) were docked with ubiquitin (PDB ID: 1UBQ) by rigid body docking approach using Fast Fourier Transformation methodology of GRAMM-X software [53]. Furthermore, their docked interface residues having distance <4.5A were identified by Pymol software [42] to identify the key lysine residues important for ubiquitin and AβPP interactions.

Prediction of ubiquitination enzymes interactional network for AβPP

The potential ubiquitination enzymes regulating the AD biology of Aβ and AβPP proteasomal clearance were identified by determining the interaction among all the ubiquitin E1-activating enzymes, E2-conjugating enzymes, E3-ligating enzymes, and deubiquitinating enzymes with amyloid forming proteins: AβPP, β-secretases and γ-secretases. Further, the protein-protein interactional network among the identified proteins was designed by functional protein association network prediction STRING tool [54]. Lastly, the overall methodology along with the tools and techniques employed in this study has shown in Fig. 1.

RESULTS

3D-model of AβPP

The three-dimensional structure of AβPP is predicted to analyze the potential lysine residues important for their ubiquitination, since its full

experimental structure was lacking. The structural topology of the modeled AβPP has identified an N-terminal signal peptide MLPGLALLLLAAWTARALEVPT (1–22), an extracellular region (1–698), a trans-membrane helix S1 (699–721), and a cytoplasmic region (722–770). Further, domain analysis of full length AβPP (770aa) revealed six functional domains, namely: 1) N-terminal domain, 2) Copper binding domain, 3) Protease inhibitor domain, 4) E2 domain, 5) Aβ peptide domain, and 6) C-terminal domain. The combined results suggest that the first four domains parses in the extracellular region and the Aβ peptide in the trans-membrane region while the C-terminal domain in the cytoplasmic region. Moreover, the modeled structure was spanned by 22 alpha-helices (37%), 7 beta-strands (4%), and one trans-membrane alpha-helix (3%). In addition, the tertiary structure of the AβPP model is enriched with two disulfide linkages one at cysteine144-cystein174 and another at cysteine158-cysteine186 that are very important for their stability. Further, the structural validation of the refined model by PROVE analysis passed their structural quality test with less than 1% buried outlier atoms. Similarly, the sequence information based tertiary structure, i.e., 3D–1D score $\geq$0.2 was found to be more than 50% for our refined model as per the verify-3D tool. Moreover, the geometrical conformation of the refined model analyzed by RAMPAGE server was found to be greater than 99.5% for the favored and allowed region residues except four amino acids, which included Alanine 35, Aspartic acid 360, Proline 365, and Arginine 653. The refined model of AβPP along with their molecular descriptors and structural refinement simulations is shown in Fig. 2. Further, the structure validation scores of modeled and refined models obtained by verify 3D and RAMPAGE can be accessed from Supplementary Table 1.

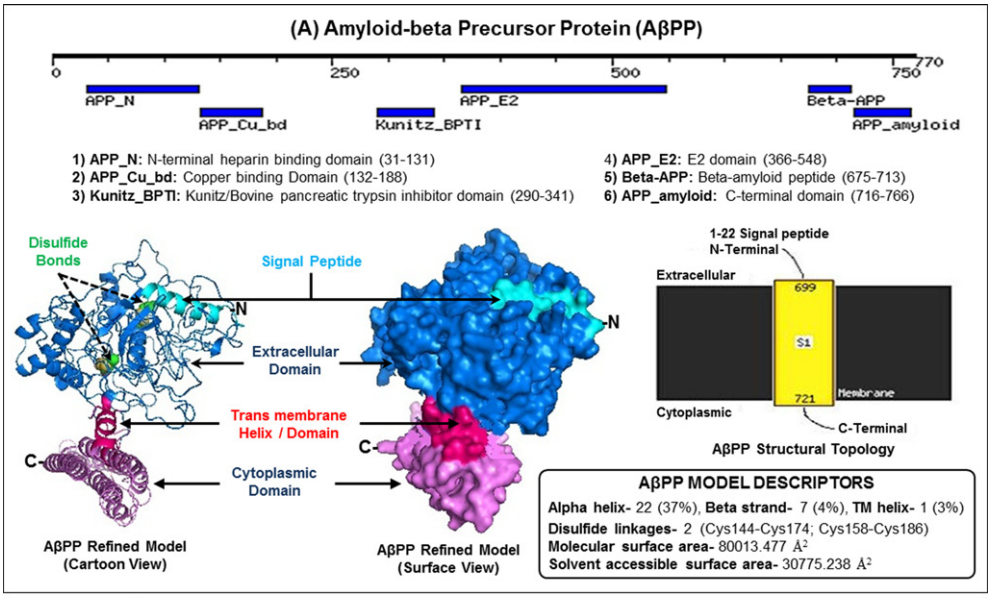
K351 is the most promising target for AβPP ubiquitination

The potential lysine sites which are favorable for the ubiquitination of AβPP are identified by four different methodologies, including sequence identity, sequence similarity, protein-peptide docking, and machine learning techniques. Since, ubiquitin is the pre-eminent protein that is ubiquitinated the most in any cellular processes due to the poly-ubiquitination phenomenon; therefore, its lysine site features are of great importance to unravel the ubiquitination mystery. These informative sites of ubiquitin are utilized by above mentioned methodologies to deduce the potential lysine sites in AβPP.

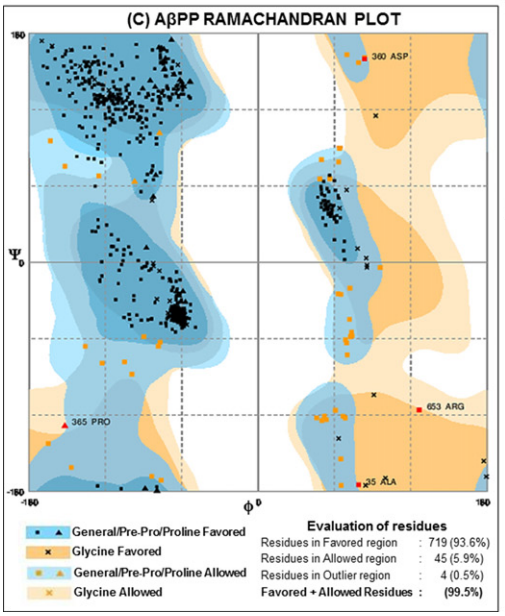
Sequence identity based potential lysine in AβPP

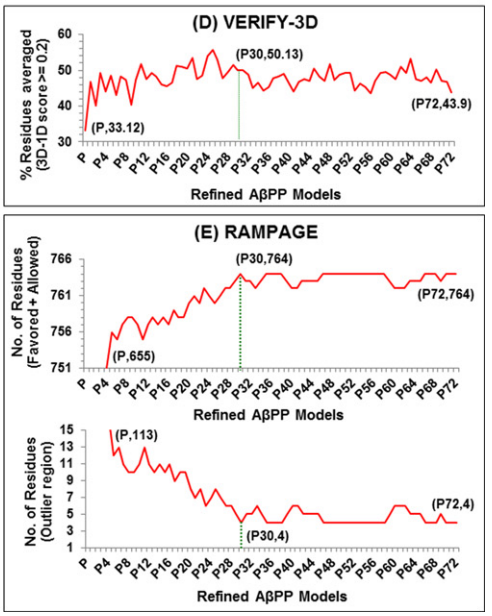
The sequence identity between the 21 window-size peptide sequence with central lysine at seven lysine sites of ubiquitin and forty-one lysine sites of AβPP is determined to infer the promising ubiquitination sites in AβPP. The sequence-identity of at least three amino acids, i.e., identity score >0.2 was taken as the threshold, which identified 26 key lysine sites in AβPP including K60, K66, K99, K106, K132, K134, K155, K161, K351, K363, K377, K393, K395, K401, K503, K510, K522, K568, K601, K662, K670, K687, K699, K724, K725, and K726 as favorable sites for the ubiquitination (Fig. 3A). Among them these seven K60, K66, K351, K363, K601, K662, and K687 lysine sites displayed the higher identity scores. For detailed sequence identity scores obtained for

Fig. 2. AβPP Modeling and Structural Refinement. A) *AβPP Model:* The modeled AβPP (shown in cartoon and surface view) represented an N-terminal signal peptide (Cyan in color), Trans-membrane helix/domain (Hot pink in color), and a C-terminal region (Purple in color) along with the disulfide bonds (Green/Yellow in color). The domain analysis identified six domains including N-terminal, copper binding, protease inhibitor, E2, Aβ, and C-terminal domain spanned across the full length 770aa protein. The structural topology of the predicted model has identified a membrane spanning trans-membrane helix S1 at 699 to 721 amino acid residues with an extracellular N-terminal (1–699) carrying signal peptide (1–22) and a cytoplasmic C-terminal (721–770). The structural analysis of the predicted model revealed 22 alpha-helices, 7 Beta-sheets, and 1 Trans-membrane helix and 2 disulfide linkages (Cys144–Cys174 and Cys158–Cys186). Moreover, the solvent accessible surface area (30775.238 angstrom$\wedge$2) was less than the molecular surface area (80013.477 angstrom$\wedge$2) of the model due to inaccessibility of the solvent in trans-membrane region. B) *Structural Refinement of AβPP:* The modeled AβPP was refined with structural refinement simulations up to 72 cycles (designated P1,P2,... P72) and validated for the refinements by different structural validation programs- PROVE, ERRAT, Verify-3D, and RAMPAGE. C) *AβPP Ramachandran Plot:* Evaluated 99.5% residues in geometrical (Φ–ψ) favored (719 residues (93.6%)) + allowed residues (45 (5.9%)) regions while only four residues Ala35, Asp360, Pro365, and Arg653 ~0.5% in outlier region; other structural validation programs including PROVE and ERRAT identified to PASS the modeled structure for their overall quality. D) *Verify-3D:* The 3D atomic model compatibility assessment revealed the refinement of modeled AβPP from zero cycle (P)– 33.12% to 72 cycle (P72)– 43.9% residues, with 50.13% residues of the best obtained model P30 at 30th cycle showing 2D–3D structural compatibility. E) *RAMPAGE:* The geometrical validation of C-alpha neighboring residues revealed the refinement from zero cycle (P)– 655 (favored + allowed residues) and 113 (outlier residues) to 72 cycle (P72)– 764 (favored + allowed residues) and 4 (outlier residues). The best geometrical configuration with minimum simulations was achieved at 30th cycle (P30) signifying it as a best-predicted model.



Protein	(B) Structural Refinement of AβPP			
	PROVE	ERRAT	VERIFY 3D	RAMPAGE
Modeled AβPP (P)	The total number of buried outlier protein atoms was 344 (22.5 %) of scored atoms	Overall quality factor 25.658	33.12% of the residues had an averaged 3D-1D score >= 0.2	No. of residues in Favored region : 525 (68.4%) Allowed region : 130 (16.9%) Outlier region : 113 (14.7%)
Refined AβPP (P30)	PASS	–	50.13% of the residues had an averaged 3D-1D score >= 0.2	No. of residues in Favored region : 719 (93.6%) Allowed region : 45 (5.9%) Outlier region : 4 (0.5%)





each pairwise sequence alignment, Supplementary Table 2 can be explored.

Sequence similarity based potential lysine in AβPP

The sequence conservation analysis among lysine neighboring residues in ubiquitin revealed the conservation of hydrophobic leucine (L), isoleucine (I), valine (V) and polar negatively charged aspartic acid (D), glutamic acid (E) at 57% similarity threshold. Further, the conservation in ubiquitin at 42% similarity threshold provided the majority of informative amino acid residues in common with AβPP that are crucial for the ubiquitination process. These conserved residues included the hydrophobic methionine (M), leucine (L), isoleucine (I), and valine (V), polar negatively charged glutamic acid (E), and polar uncharged glutamine (Q). Moreover, in AβPP only glutamic acid (E) and glutamine (Q) is conserved at the higher threshold of 34% sequence similarity, which depicted K224, K351, K377, K393, K401, K503, K510, K522, K662, K724, K751, and K763 as potential lysine sites (Fig. 3B).

Ubiquitin-AβPP lysine site peptide docking based potential lysine in AβPP

The affinity of lysine specific ubiquitin-ubiquitin interaction in the poly-ubiquitination process is taken as the principal to investigate the potential lysine sites in AβPP important for ubiquitination. The flexible docking of ubiquitin with the best conformations of ubiquitin's seven lysine peptides, K6, K11, K27, K29, K33, K48, and K63, resulted in the average root mean square deviation (Avg. RMSD) of 2.92, 5.31, 4.92, 3.88, 4.49, 1.96, and 4.63 respectively. Further, the obtained docking results of ubiquitin with ubiquitin and AβPP lysine peptides are summarized in Table 2. Interestingly, the Avg. RMSD of K48 was minimal, i.e., 1.96 showing the best affinity for K48 linked poly-ubiquitination than the K11 site, which had highest Avg. RMSD of 5.31 implying the least propensity of K11 linked poly-ubiquitination. Here, the minimal Avg. RMSD, i.e., best binding affinity than the K11 (5.31) was taken as a threshold to find the best propensity ubiquitination sites in AβPP. The 21 potential sites were identified with good propensities for ubiquitination, including K51, K60, K99, K132, K161, K178, K351, K393, K401, K425, K495, K496, K503, K510, K521, K522, K568, K687, K724, K725, and K726 (Fig. 4A). Among them, the lowest Avg. RMSD of 0.906538 was obtained for the K687 site in AβPP or corresponding K16 site in Aβ showing best potential for ubiquitination.

Machine learning ubiquitination tools based potential lysine in AβPP

The different machine learning approaches, including maximal dependence decomposition, Bayesian discriminant analysis, random forest models, and support vector machine classifier-based tools (UbiSite, BDM-PUB, CKSAAP, UbPred and UbiPred) have been employed to determine the potential lysine sites in AβPP for ubiquitination on the basis of experimentally verified ubiquitination site datasets. The predicted ubiquitination sites and scores obtained by UbiSite, BDM-PUB, CKSAAP, UbPred, and UbiPred tools are summarized in Table 3. Moreover, the stacked bar graph of the ubiquitination prediction scores versus AβPP lysine sites are plotted to identify the potential lysine in AβPP as shown in Fig. 4B. The best ubiquitination aggregate scores were obtained for K351 and K377, i.e., 3.19 and 2.47, respectively, with maximal predictions by four tools: UbiSite, BDM-PUB, UbPred, and UbiPred.

Fig. 3. Potential lysine prediction for ubiquitination in AβPP based on: A) *Sequence Identity:* The sequence identity scores among 21 window size polypeptides carrying central lysine (K6-Blue, K11-Pink, K27-Brown, K29-Green, K33-Yellow, K48-Red, K63-Purple) in ubiquitin and (K51, K60, K66, K99, K103, K106, K132, K134, K155, K161, K178, K224, K315, K351, K363, K377, K393, K395, K401, K421, K425, K428, K429, K438, K495, K496, K503, K510, K521, K522, K568, K601, K662, K670, K687, K699, K724, K725, K726, K751, K763) in AβPP are plotted on a bar graph. The identity scores greater than 0.2 represented the conservation of more than two amino acid residues in the aligned sequences signifying the presence of potential lysine for ubiquitination based on sequence identity (shown in Red)- K60, K66, K99, K106, K132, K134, K155, K161, K351, K363, K377, K393, K395, K401, K503, K510, K522, K568, K601, K662, K670, K687, K699, K724, K725, K726. B) *Sequence Similarity:* The lysine neighboring amino acid conservation analysis among 21 window size polypeptides carrying central lysine have identified the conservation of Asp(D), Glu(E), Ile(I), Leu(L), and Val(V) at 57% similarity threshold and Asp(D), Gln(Q), Glu(E), Ile(I), Leu(L), Met(M), Thr(T), and Val(V) at 42% similarity threshold in ubiquitin while the conservation of Gln(Q) and Glu(E) at 34% similarity threshold; Gln(Q), Glu(E), Ile(I), Leu(L), Met(M), and Val(V) at 31% similarity threshold and Asn(N), Gln(Q), Glu(E), Ile(I), Leu(L), Met(M), and Val(V) at 29% similarity threshold in AβPP. The amino acid residues conserved at the common sites of both ubiquitin and AβPP are enclosed by red rounded rectangles signifying the critical amino acids for ubiquitination including Glu (E), Gln (Q), Val (V), Leu (L), Met (M), and Ile (I). Further, on the basis of best conserved amino acids, i.e., glutamic acid and glutamine common to both ubiquitin and AβPP (marked by yellow arrow), the potential lysine for ubiquitination are identified and encircled in pink rectangles including K224, K351, K377, K393, K401, K503, K510, K522, K662, K724, K751, and K763.

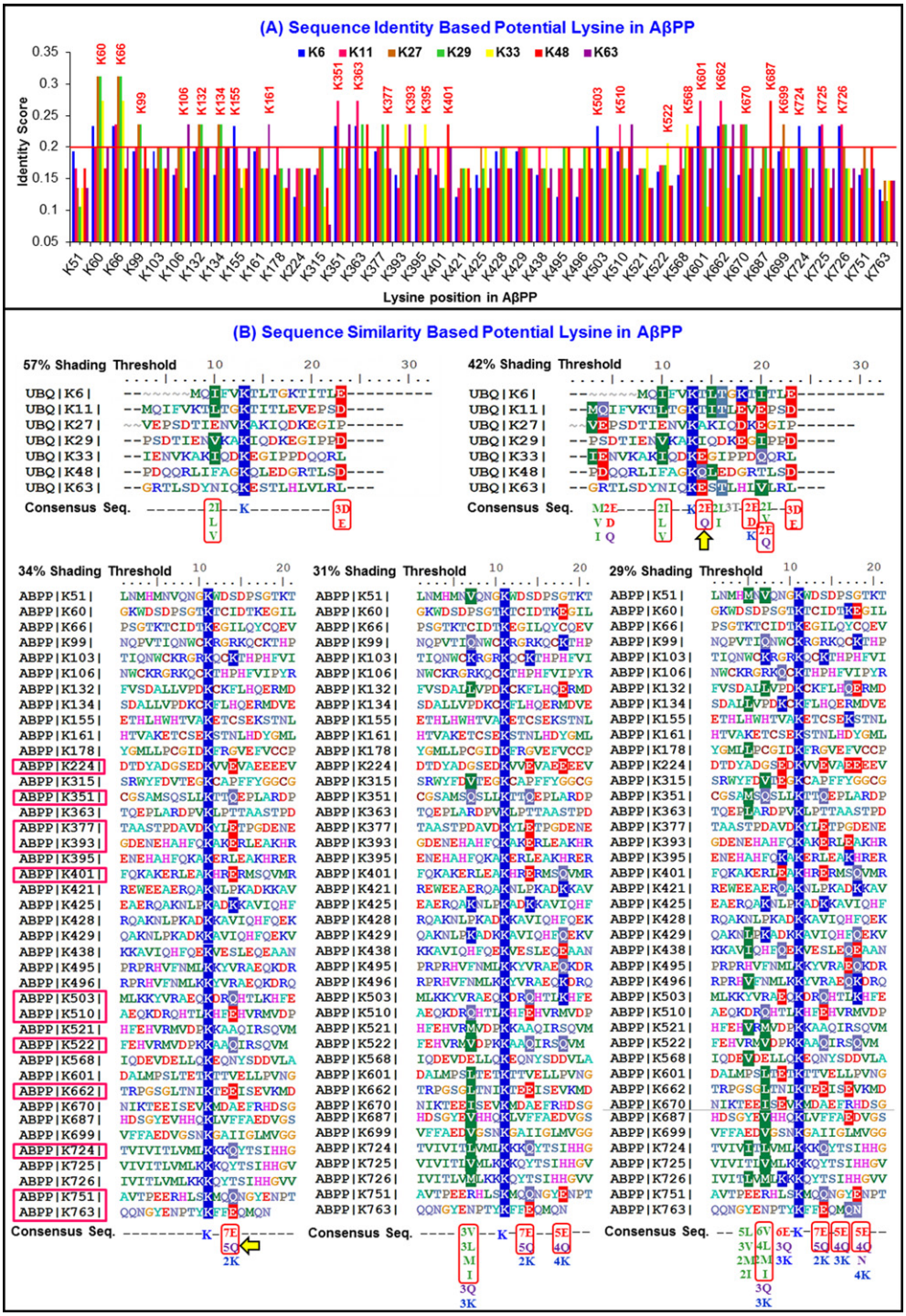

(A) Sequence Identity Based Potential Lysine in AβPP
K6 K11 K27 K29 K33 K48 K63
Identity Score
Lysine position in AβPP
(B) Sequence Similarity Based Potential Lysine in AβPP
57% Shading Threshold
42% Shading Threshold
34% Shading Threshold
31% Shading Threshold
29% Shading Threshold
Consensus Seq.

Table 2
Ubiquitin and AβPP lysine peptides docking scores with ubiquitin protein using CABS-dock

Ubiquitin						
S.No.	Ubiquitin Lysine Site	21 residues key lysine sequence	Cluster Density	Average RMSD	Max RMSD	No of elements
1	K6	MQIFV**K**TLTG**K**TITLE	26.338	2.92353	19.4007	77
2	K11	MQIFV**K**TLTG**K**TITLEVEPSD	23.1489	5.31343	23.9976	123
3	K27	VEPSDTIENV**K**A**K**IQD**K**EGIP	27.6251	4.92306	25.651	136
4	K29	PSDTIENV**K**A**K**IQD**K**EGIPPD	38.8683	3.88491	23.829	151
5	K33	IENV**K**A**K**IQD**K**EGIPPDQQRL	27.5913	4.49417	17.6504	124
6	K48	PDQQRLIFAG**K**QLEDGRTLSD	24.0229	1.95647	7.09756	47
7	K63	GRTLSDYNIQ**K**ESTLHLVLRL	25.5963	4.64911	26.4507	119

AβPP						
S.No.	AβPP Lysine Site	21 residues key lysine sequence	Cluster Density	Average RMSD	Max RMSD	No of elements
1	K51	LNMHMNVQNG**K**WDSDPSGT**K**T	21.5806	5.09718	27.0102	110
2	K60	G**K**WDSDPSGT**K**TCIDT**K**EGIL	26.0199	4.3044	24.3425	112
3	K66	PSGT**K**TCIDT**K**EGILQYCQEV	19.0914	9.00931	24.3529	172
4	K99	NQPVTIQNWC**K**RGR**K**QC**K**THP	41.1493	3.57236	14.4164	147
5	K103	TIQNWC**K**RGR**K**QC**K**THPHFVI	24.9614	6.08941	22.8975	152
6	K106	NWC**K**RGR**K**QC**K**THPHFVIPYR	19.9487	9.17352	24.6746	183
7	K132	FVSDALLVPD**K**C**K**FLHQERMD	34.6382	3.0602	18.6577	106
8	K134	SDALLVPD**K**C**K**FLHQERMDVE	17.7294	7.38886	30.4831	131
9	K155	ETHLHWHTVA**K**ETCSE**K**STNL	18.8584	9.22667	27.4895	174
10	K161	HTVA**K**ETCSE**K**STNLHDYGML	25.5239	3.44775	14.9316	88
11	K178	YGMLLPCGID**K**FRGVEFVCCP	31.9959	4.53183	19.5177	145
12	K224	DTDYADGSED**K**VVEVAEEEEV	21.3651	7.58246	19.827	162
13	K315	SRWYFDVTEG**K**CAPFFYGGCG	28.418	5.38391	18.2749	153
14	K351	CGSAMSQSLL**K**TTQEPLARDP	26.6421	4.42907	21.7612	118
15	K363	CGSAMSQSLL**K**TTQEPLARDP	22.2974	9.59752	30.1062	214
16	K377	TAASTPDAVD**K**YLETPGDENE	30.3162	5.50861	30.4716	167
17	K393	GDENEHAHFQ**K**A**K**ERLEA**K**HR	34.6566	4.09735	12.2452	142
18	K395	ENEHAHFQ**K**A**K**ERLEA**K**HRER	26.0746	6.36634	25.9803	166
19	K401	FQ**K**A**K**ERLEA**K**HRERMSQVMR	23.396	4.2315	18.9197	99
20	K421	REWEEAERQA**K**NLP**K**AD**KK**AV	21.1402	10.4067	31.0374	220
21	K425	EAERQA**K**NLP**K**AD**KK**AVIQHF	32.8454	3.83616	27.7128	126
22	K428	RQA**K**NLP**K**AD**KK**AVIQHFQE**K**	24.0608	5.94329	22.5957	143
23	K429	QA**K**NLP**K**AD**KK**AVIQHFQE**K**V	24.0077	6.70617	28.9284	161
24	K438	**KK**AVIQHFQE**K**VESLEQEAAN	37.1462	6.38019	28.0285	237
25	K495	PRPRHVFNML**KK**YVRAEQ**K**DR	22.7752	4.1712	14.4324	95
26	K496	RPRHVFNML**KK**YVRAEQ**K**DRQ	39.035	4.73934	17.5878	185
27	K503	ML**KK**YVRAEQ**K**DRQHTL**K**HFE	26.2182	4.61512	15.6449	121
28	K510	AEQ**K**DRQHTL**K**HFEHVRMVDP	26.9482	5.19515	20.7974	140
29	K521	HFEHVRMVDP**KK**AAQIRSQVM	20.0593	5.23448	19.223	105
30	K522	FEHVRMVDP**KK**AAQIRSQVM	29.0115	4.10182	18.1209	119
31	K568	IQDEVDELLQ**K**EQNYSDDVLA	36.6961	2.9431	28.6506	108
32	K601	DALMPSLTET**K**TTVELLPVNG	19.4168	11.5879	26.6436	225
33	K662	TRPGSGLTNI**K**TEEISEV**K**MD	22.9797	8.39872	29.6782	193
34	K670	NI**K**TEEISEV**K**MDAEFRHDSG	27.64	8.53834	32.0268	236
35	K687	HDSGYEVHHQ**K**LVFFAEDVGS	40.8146	0.906538	1.81447	37
36	K699	VFFAEDVGSN**K**GAIIGLMVGG	19.2171	9.62683	22.5839	185
37	K724	TVIVITLVML**KKK**QYTSIHHG	20.3169	5.1189	28.5382	104
38	K725	VIVITLVML**KKK**QYTSIHHGV	29.4156	2.00574	19.6385	59
39	K726	IVITLVML**KKK**QYTSIHHGVV	31.7439	4.75682	19.5129	151
40	K751	AVTPEERHLS**K**MQQNGYENPT	21.713	11.0533	30.6823	240
41	K763	QQNGYENPTY**K**FFEQMQN	23.4377	5.54662	28.2897	130

Further, these potential lysine residues were classified into different confidence levels: very high, high, medium, low, and very low based on the evidence from ubiquitination prediction tools and their verification by other methods, including sequence identity, sequence similarity, and flexible protein-peptide docking. For instance, the potential ubiquitination sites predicted by at least three ubiquitination pre-

diction tools and is verified by at least any two other methods are classified as "Very High" confidence, while those verified by any one of the other methods are classified as "High" confidence. In a similar way, the potential ubiquitination sites predicted by any two ubiquitination prediction tools and are verified by at least any one of the other methods are categorized into "Medium" confidence. Additionally, the potential ubiquitination sites predicted by any one of the ubiquitination prediction tools, which is verified by at least, any two of the other methods, are assigned as "Low" confidence whereas if verified by any one of the other methods then classified as "Very Low" confidence. The key lysine residues important for the ubiquitination of AβPP with predicted confidence are listed in Table 4. Moreover, the Venn diagrams comprehensively reviewed the predicted ubiquitination sites and illustrated the logical relations among the key ubiquitination sites in AβPP (Fig. 4C). The Venn diagram analysis clearly depicted K351 as the most promising ubiquitination site at high confidence; K393, K401, K510, and K522 at medium confidence; and K503 and K724 at low confidence.

Lysine residues are crucial for AβPP stability, ubiquitination, and other functions

The mutational studies on AβPP revealed the importance of lysine residues in AβPP processing and ubiquitination. When we mutated the key lysine (Polar; positively charged; basic amino acid; pK = 10.5) with other polar; positively charged; basic amino acids- Arginine (pK = 12.5), Histidine (pK = 6.0) and polar; negatively charged; acidic amino acids- Aspartate (pK = 3.9) and Glutamate (pK = 4.2), we observed some interesting effects on the internal potential energy of mutated AβPP summarized in Table 5. We found that only arginine had imparted site-specific stability to even more than half of the predicted ubiquitination sites, including K51, K60, K134, K161, K224, K393, K401, K425, K496, K510, K521, K522, K699, and K724, and instability at the rest site of AβPP (Fig. 5A). Moreover, the lysine residues at high confidence ubiquitination sites were found intolerant towards all mutations against the stability of AβPP. Apart from arginine, histidine was observed to greatly impact the stability of AβPP followed by glutamate and aspartate. However, the effect of glutamate on AβPP stability was less than the arginine and histidine, but it affected nearly all the ubiquitination sites (Fig. 5A). Further, the detailed internal potential energies obtained for the modeled and mutated AβPP, and the mutational impact of lysine mutations on their total potential energies can be inferred from Supplementary Tables 3 and 4, respectively.

Further, the disease susceptibility of lysine mutations were checked through the mutational analysis tools, PANTHER, SNAP2, Polyphen2, PMut, PhdSNP, and SIFT. It revealed that mutations had an effect on all the sites, but they had very least effect on the high confidence ubiquitination sites in comparison with the medium and low confidence sites. The highly intolerant mutations that were most susceptible to the diseases are shown in Fig. 5B. In addition, the detailed results for the mutation associated disease susceptibility predicted by PANTHER, SNAP2, Polyphen2, PMut, PhD-SNP, and SIFT can be accessed through Supplementary Table 5. Further, we analyzed the physico-chemical and amino acid compositional analysis of ubiquitin, $A\beta_{42}$ peptide, and AβPP to understand the crucial factors that determine the ubiquitination of a protein. Interestingly we found that total number of positively and negatively charged residues were in same proportion in ubiquitin along with the aliphatic index-100 and GRAVY- –0.489 which could be the decisive factor for ubiquitin to be the top ubiquitination protein. Moreover, some amino acid compositions, including arginine, asparagine, aspartate, glutamate, phenyl-alanine, proline, and serine were found in the comparable range $\pm$ 1% in ubiquitin, $A\beta_{42}$ peptide, and AβPP (Fig. 5C). Further, lysine sites in AβPP were investigated for their role in the other cellular processes and functions by MutPred, which has been outlined in Table 6. The collective results depicted that apart from ubiquitination; lysine residues are also crucial for protein stability, modifications, and other functions.

Can lysine mediated non-covalent interactions prevail ubiquitin positioning and ubiquitin-AβPP conjugation during ubiquitination?

The non-covalent interactions of ubiquitin with different proteins, such as human DNA repair proteins, insulin-degrading enzyme, and SUMO protein, are well known for modulating their activity or correctly orienting ubiquitin for lysine specific conjugation [55–57]. These interactions can also impart stability to the ubiquitin-protein conjugates in the ubiquitination process [58]. In this regard, we predicted the interaction among all the domains of AβPP and ubiquitin and examined them for the lysine mediated polar

interactions among their interacting residues. The ubiquitin-AβPP interaction predictions reported that ubiquitin has nearly same interactive affinity toward all the domains of AβPP except Aβ peptide. The prediction of ubiquitin-AβPP interactions was highest on the basis of homologous protein interactional network (S_{Net}), rather than by the statistical domain-domain interactions (S_{Dom}) and sequence similarity based interacting protein pairs (S_{Seq}) shown in Fig. 6A. Further, lysine investigation in non-covalent electrostatic, polar interactions revealed the presence of lysine interactions with N-terminal domain (K66, K99), Copper binding domain (K155), E2 domain (K447), Aβ peptide (K28), and C-terminal domain of AβPP (K687). Apart from AβPP, ubiquitin's lysine residues, including K6, K11, K33, K48, and K63 were also involved in the polar interactions (Fig. 6B). The presence of lysine as interacting residues provided a clue for their role in ubiquitin positioning or ubiquitin-AβPP conjugation but further researches are required for clear understanding. These informative residues are the foundations for future avenues of AβPP ubiquitination mechanistic research.

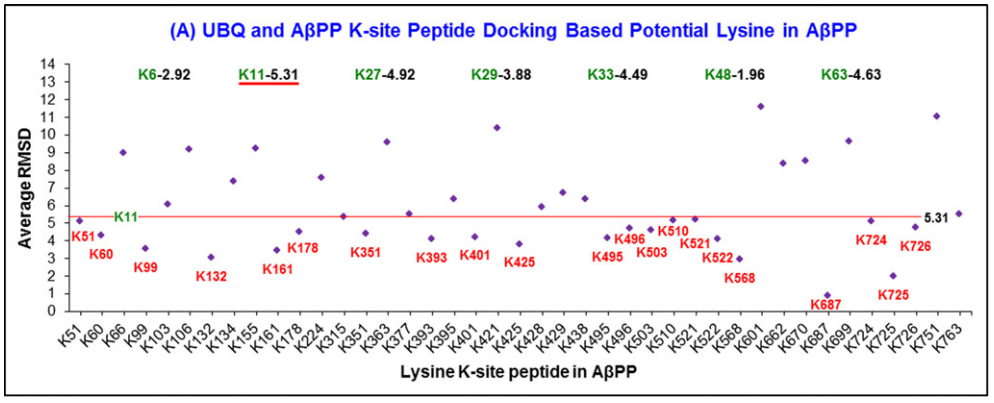
Interaction network of ubiquitination proteins for AβPP clearance

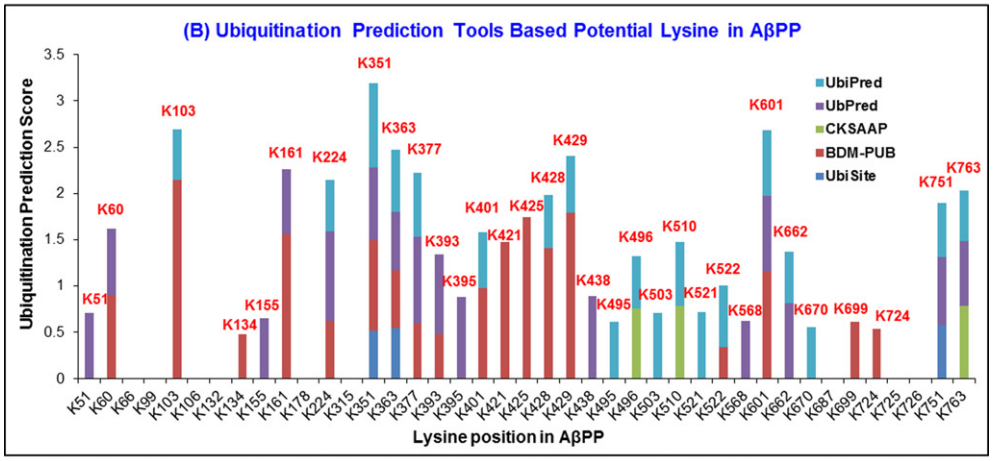
The accumulation of well-known pathogenic Aβ can be regulated by the clearance of its precursor, AβPP, through ubiquitination. However, ubiquitination is a well-defined process, but the association of different ubiquitination E1s, E2s, E3s, and deubiquitinating enzymes (DUBs) always remained an interesting field of research for target specific protein clearance. Here, we investigated the interaction network of E1s, E2s, E3s, and DUBs enzymes for the clearance of AβPP in AD biology. The interaction network identified ubiquitin E3 ligases, Park2 and STUB1, to be associated with AβPP ubiquitination while deubiquitination was by USP25 and UCHL1 (Fig. 7A). The ubiquitination processing of other enzymes that govern the synthesis of Aβ peptide are also crucial to restrict them. Here, certain E3 ligases like FBXO2 is reported to regulate the ubiquitination of BACE1; TRIM13 to regulate PSENEN; CDH1 to regulate PSEN1 and NCSTN; while TRIM55 and SART1 to regulate the ubiquitination of NCSTN. Likewise, specific deubiquitinase enzymes, including USP25 and UCHL, are found to regulate AβPP; USP8 to BACE1; and USP39 to NCSTN, respectively (Fig. 7A). Further, examinations on the potential E1 activating and E2 conjugating enzymes were done to identify the pre-processors of AβPP ubiquitin ligation by Park2 and STUB1 E3 ligases. We reported an array of ubiquitin E1 activating enzymes UBA1, UBA6, UBA7, and ATG7 that can possibly activate specific ubiquitin E2 conjugating enzymes including UBE2A, UBE2B, UBE2G1, UBE2J1, UBE2K, UBE2L3, UBE2L6, UBE2N, UBE2Q1, UBE2S, UBE2U, and UBE2Z to conjugate lysine to Park2 (Fig. 7B); while additional UBE2I, UBE2T, and UBE2W enzymes to conjugate lysine to STUB1 (Fig. 7C). The PPI network provided us the substantial information about the ubiquitination enzymes associated with the clearance of AβPP in the AD biology that can be further explored for the therapeutic avenues.

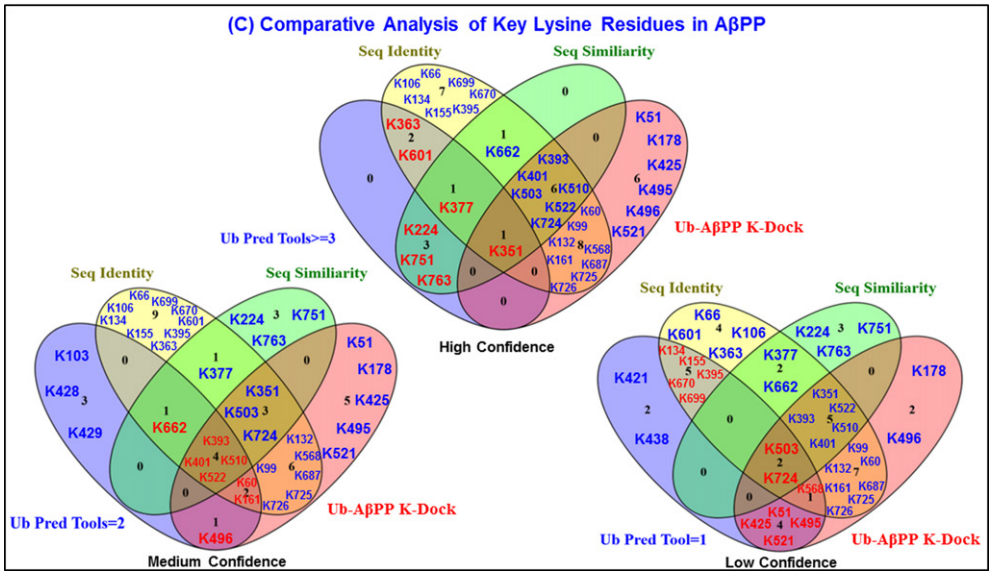
DISCUSSION

The structural topology of the modeled AβPP has identified 1) an N-terminal signal peptide of 22AA

Fig. 4. Potential lysine prediction in AβPP and their comparative analysis: A) *Ubiquitin and K-AβPP site Peptide Docking:* The average root mean square deviation (Avg. RMSD) graph of the docked 21 window size K-site AβPP peptide with ubiquitin is dot plotted and is compared with the Avg. RMSD of the docked 21 window size K-site Ubiquitin peptide with the ubiquitin protein. The Avg. RMSD of K-11 ubiquitin, i.e., 5.31, is taken as threshold to identify potential ubiquitination site in AβPP (K51, K60, K99, K132, K161, K178, K351, K393, K401, K425, K495, K496, K503, K510, K521, K522, K568, K687, K724, K725, and K726) with lower Avg. RMSD values. B) *Ubiquitination Prediction Tools:* The machine learning based ubiquitination prediction tools-UbiPred (Light Blue), UbPred (Purple), CKSAAP (Green), BDM-PUB (Dark Red), and UbiSite (Blue) has predicted potential ubiquitination sites in AβPP including K51, K60, K103, K134, K155, K161, K224, K351, K363, K377, K393, K395, K401, K421, K425, K428, K429, K438, K495, K496, K503, K510, K521, K522, K568, K601, K662, K670, K699, K724, K751, and K763. C) *Comparative Analysis of Ubiquitination Sites:* The comparison of the potential ubiquitination sites predicted by all the methods have revealed the most potential ubiquitination sites which is predicted by at least three ubiquitination prediction tools and verified by at least any two other methods are K351 and K377 (marked Red) with very high confidence, while verified by any one of the other methods are K224, K363, K601, K751, and K763 (marked Red) with high confidence. Similarly, the potential ubiquitination sites predicted by any two ubiquitination prediction tools and verified by at least any one of the other methods are K60, K161, K393, K401, K496, K510, K522, and K662 (marked Red) with medium confidence. Likewise, the potential ubiquitination sites predicted by any one of the ubiquitination prediction tool and is verified by at least any two of the other methods are K503, K568, and K724 (marked Red) with low confidence while verified by any one of the other methods are K51, K134, K155, K395, K425, K495, K521, K670, and K699 (marked Red) with very low confidence.


(A) UBQ and AβPP K-site Peptide Docking Based Potential Lysine in AβPP
Average RMSD
K6-2.92
K11-5.31
K27-4.92
K29-3.88
K33-4.49
K48-1.96
K63-4.63
5.31
Lysine K-site peptide in AβPP


(B) Ubiquitination Prediction Tools Based Potential Lysine in AβPP
Ubiquitination Prediction Score
UbiPred
UbPred
CKSAAP
BDM-PUB
UbiSite
Lysine position in AβPP


(C) Comparative Analysis of Key Lysine Residues in AβPP
Seq Identity
Seq Similiarity
Ub Pred Tools>=3
Ub-AβPP K-Dock
High Confidence
Ub Pred Tools=2
Medium Confidence
Ub Pred Tool=1
Low Confidence

.

Table 3
Ubiquitination Prediction Scores of Ubiquitination-site Prediction Tools

AβPP Key Lysine Site	Ubiquitination Site Prediction Tools					Aggregate Scores
	UbiSite	BDM-PUB	CKSAAP	UbPred	UbiPred	
K51	_		_	0.71	_	0.71
K60	_	0.9	_	0.72	_	1.62
K103	_	2.15	_	_	0.54	2.69
K134	_	0.48	_	_	_	0.48
K155	_	_	_	0.65	_	0.65
K161	_	1.56	_	0.7	_	2.26
K224	_	0.62	_	0.97	0.56	2.15
K351	0.510765	0.98	_	0.79	0.91	3.190765
K363	0.544687	0.62	_	0.64	0.67	2.474687
K377	_	0.59	_	0.94	0.69	2.22
K393	_	0.48	_	0.86	_	1.34
K395	_	_	_	0.88	_	0.88
K401	_	0.98	_	_	0.6	1.58
K421	_	1.47	_	_	_	1.47
K425	_	1.74	_	_	_	1.74
K428	_	1.41	_	_	0.57	1.98
K429	_	1.79	_	_	0.61	2.4
K438	_	_	_	0.89	_	0.89
K495	_	_	_	_	0.61	0.61
K496	_	_	0.751	_	0.57	1.321
K503	_	_	_	_	0.71	0.71
K510	_	_	0.7805	_	0.69	1.4705
K521	_	_	_	_	0.72	0.72
K522	_	0.34	_	_	0.66	1
K568	_	_	_	0.62	_	0.62
K601	_	1.16	_	0.81	0.71	2.68
K662	_	_	_	0.81	0.56	1.37
K670	_	_	_	_	0.55	0.55
K699	_	0.61	_	_	_	0.61
K724	_	0.53	_	_	_	0.53
K751	0.569523	_	_	0.74	0.59	1.899523
K763	_	_	0.7883	0.7	0.54	2.0283

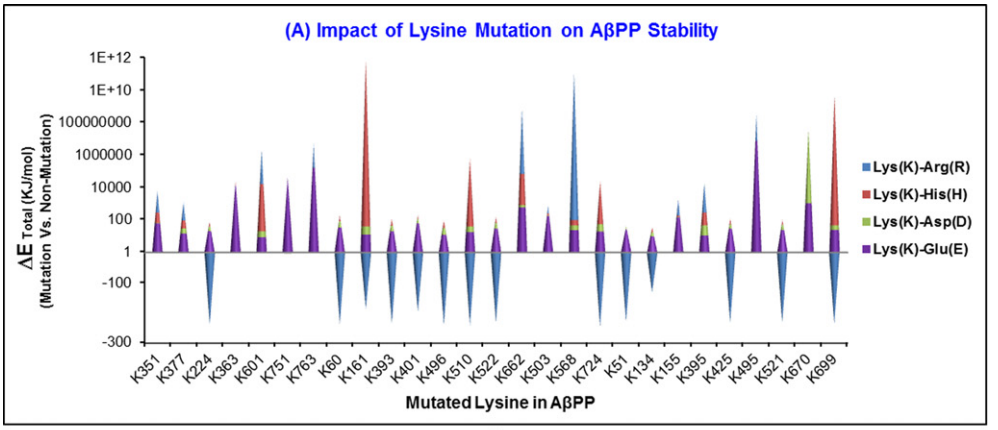
residues (MLPGLALLLLAAWTARALEVPT) and a trans-membrane segment S1 (699–721). Here, positively charged arginine (Arg16) and the N-terminal hydrophobic amino acids are crucial for AβPP's post-translational translocation across the ER membrane. Since, the removal of positively charged amino acid from signal peptide selectively impairs the translocation, while the presence of hydrophobic residues favors it through lipid bilayer of ER membrane toward the secretory pathway [59]. 2) The domain analysis revealed six domains that are contributing toward the functionality of AβPP. Among them four domains, including N-terminal, copper binding, protease inhibitor (Kunitz_BPTI), and E2 domain spans over the extracellular surface region and are responsible for copper binding, protease inhibition, platelet aggregation, and hemostasis. While the proteolytic product of cytoplasmic c-terminal domain acts as a transcriptional regulator in neurons. In addition, the cleavage of their trans-membrane domain is responsible for Aβ peptide formation [60]. 3) The secondary structure analysis identified a higher alpha-helical content ∼37% in AβPP indicating their potent ability to tolerate mutations, since helices can accrue more mutations than beta strands without structural distortion due to their higher numbers of inter-residue contacts [61]. 4) Their tertiary structure revealed two disulfide linkages (cysteine144-cystein174 and cysteine158-cysteine186) that imparted stability to the AβPP. Further, the potential ubiquitination sites in AβPP has identified by adopting the sequence identity, sequence similarity and protein-peptide docking

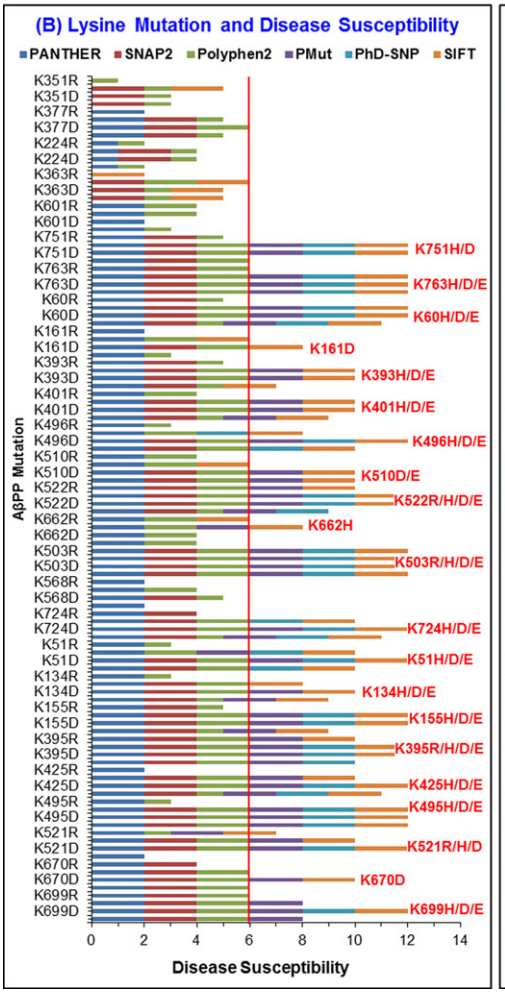
Table 4
Predicted Confidence of the Key Ubiquitination sites in AβPP

Key Lysine Sites	Potential lysine (K) in AβPP for ubiquitination based upon						Predicted Confidence
	Ubiquitination prediction tools			Amino Acid Sequence Identity Matrix	Conserved Similar Amino Acid Residues	Ubiquitin and AβPP K-site peptide docking	
	1 Tool	2 Tools	≥ 3 Tools	Sites >0.2 Sequence Identity Score	@34% Similarity Threshold	Sites < 5.31 Average RMSD Scores	
K51							Very Low
K60							Medium
K134							Very Low
K155							Very Low
K161							Medium
K224							High
K351							Very High
K363							High
K377							Very High
K393							Medium
K395							Very Low
K401							Medium
K425							Very Low
K495							Very Low
K496							Medium
K503							Low
K510							Medium
K521							Very Low
K522							Medium
K568							Low
K601							High
K662							Medium
K670							Very Low
K699							Very Low
K724							Low
K751							High
K763							High

approach using lysine site features of ubiquitin, for being the eminent protein for poly-ubiquitination [62, 63]. The comparative analysis of thus predicted ubiquitination sites with the potential sites identified by the machine learning approaches revealed K351 as most promising ubiquitination site at high confidence; K393, K401, K510, and K522 at medium confidence; and K503 and K724 at low confidence. Additionally, the site specific conserved amino acids, glutamic acid (E), glutamine (Q), valine (V), leucine (L), isoleucine (I), and methionine (M), were identified neighboring the lysine residue in AβPP. These conserved polar residues Glu(E) and Gln(Q) at ubiquitination site would display their propensities for salt-bridges while conservation of hydrophobic residues Val(V), Leu(L), Ile(I), and Met(M) indicate their propensities for buried contacts signifying their role in the ubiquitination reaction [64]. Interestingly, we can comprehend the presence of polar negatively charged Glu(E) amino acids neighboring lysine for their electrostatic interactions among each other in their native folded state [65] while ubiquitination directs lysine's covalent attachment with c-terminal glycine of ubiquitin molecule that opened the avenues for further validations.

Moreover, the mutational analysis of lysine's impact on AβPP stability revealed only arginine for imparting site-specific stability to the AβPP while rest mutations were detrimental for the structural conformation of AβPP. The stability associated with arginine mutations can be attributed to its highly basic nature and resonating states than lysine that can stabilize the positive charges in protein. The arginine has also shown protein stability results with other proteins [66] thereby also indicating their role in structural stability of AβPP. Interestingly, lysine residues at





(C) Physico-chemical Properties and Amino acid Composition

Physico-chemical Properties	Ubiquitin	Aβ42	AβPP
Number of amino acids	76	42	770
Molecular weight (KDa)	8.56484	4.5141	86.94325
Theoretical pI	6.56	5.31	4.73
Total number of negatively charged residues (Asp + Glu)	11	6	142
Total number of positively charged residues (Arg + Lys)	11	3	78
Instability index	36.06 (Stable)	18.17 (Stable)	40.69 (Unstable)
Aliphatic index	100	97.38	73.18
Grand average of hydropathicity (GRAVY)	-0.489	0.205	-0.584

Amino acid residues	Amino acid % Composition		
	Ubiquitin	Aβ42	AβPP
Ala (A)	2.60%	9.50%	8.20%
Arg (R)	5.30%	2.40%	4.80%
Asn (N)	2.60%	2.40%	4.00%
Asp (D)	6.60%	7.10%	6.50%
Cys (C)	0.00%	0.00%	2.30%
Gln (Q)	7.90%	2.40%	4.70%
Glu (E)	7.90%	7.10%	11.90%
Gly (G)	7.90%	14.30%	4.90%
His (H)	1.30%	7.10%	3.20%
Ile (I)	9.20%	7.10%	3.10%
Leu (L)	11.80%	4.80%	7.30%
Lys (K)	9.20%	4.80%	5.30%
Met (M)	1.30%	2.40%	3.10%
Phe (F)	2.60%	7.10%	2.70%
Pro (P)	3.90%	0.00%	4.50%
Ser (S)	3.90%	4.80%	4.50%
Thr (T)	9.20%	0.00%	6.50%
Trp (W)	0.00%	0.00%	1.20%
Tyr (Y)	1.30%	2.40%	2.60%
Val (V)	5.30%	14.30%	8.40%

Table 5
Effect of Lysine Mutation on Total Potential Energy of AβPP

Confidence Level	Mutational Site	ΔE Total (KJ/mol) ($E_{Mutation}$–$E_{NoMutation}$) Mutation Type			
		Lys(K)–Arg(R)	Lys(K)–His(H)	Lys(K)–Asp(D)	Lys(K)–Glu(E)
Very High	K351	5876.014	222.42	4.633	61.496
	K377	1194.364	62.217	15.471	14.557
High	K224	–238.418	35.037	17.395	20.996
	K363	1026.26	9837.761	33.565	13848.825
	K601	2083620.973	16503.902	11.123	8.77
	K751	2473.202	165.922	–0.629	38950.161
	K763	4890663.473	6062.487	6.621	156472.582
Medium	K60	–236.179	106.526	48.961	34.491
	K161	–184.562	7.19899E+11	27.44	12.717
	K393	–234.58	74.135	23.825	20.399
	K401	–194.841	86.977	52.08	62.194
	K496	–239.636	59.368	20.416	12.114
	K510	–240.533	616957.348	21.922	17.953
	K522	–231.222	90.121	34.071	29.496
	K662	765427662.5	68410	226.784	601.836
Low	K503	487.243	70.155	13.244	173.287
	K568	1.21228E + 11	51.993	23.744	23.338
	K724	–245.779	23655.275	35.864	19.457
Very Low	K51	–228.095	0	14.649	26.098
	K134	–131.127	17.27	7.151	9.86
	K155	1569.943	31.192	10.395	143.977
	K395	17638.653	237.323	36.303	11.287
	K425	–231.168	64.469	24.192	29.85
	K495	331718094.5	153.008	309.342	7639277.973
	K521	–232.041	49.873	27.586	24.532
	K670	2799.446	139.305	39005702.47	1061.897
	K699	–235.707	4256364558	23.08	24.27

high confidence ubiquitination sites were intolerant against all mutations thereby affecting AβPP stability and signifying their importance in protein's structural and functional regulation. Further, the evaluation of lysine mutations for disease susceptibility indicated their least effect on the high confidence ubiquitination sites than the other sites. This situation can be extrapolated that ubiquitination may be more selective towards highly conserved or stable lysine sites than the least stable sites as observed by Kim and Hahn, who reported the gain of ubiquitination sites in the highly conserved region of proteins [67]. Further analysis by Mutpred identified lysine residues to play a role in AβPP acetylation, glycosylation, phosphorylation, and SUMOylation apart from the ubiquitination. Additionally, investigations on AβPP-ubiquitin non covalent electrostatic, polar interactions reported K66, K99, K155, K447, K687, and K699 lysine residues having their plausible role in ubiquitin positioning or ubiquitin-AβPP conjugation that are the foundations for further research. Last but not the least, we investigated the interaction

Fig. 5. Mutational Analysis of Lysine residues for AβPP Ubiquitination. A) *Impact of Lysine Mutation on AβPP Stability:* The total energy change in AβPP upon Lysine mutations have identified the prominent effect of Lysine-Arginine and Lysine-Histidine mutations as the most detrimental to AβPP stability in comparison with Glutamic and Aspartic acid. Here, nearly 14 Lysine-Arginine mutations including K51, K60, K134, K161, K224, K393, K401, K425, K496, K510, K521, K522, K699, and K724 are reported to increase the stability of AβPP by reducing their total energy in KJ/mol. B) *Lysine Mutation and Disease Susceptibility:* The investigation by various mutation analysis tools including PANTHER, SNAP2, Polyphen2, Pmut, PhD-SNP, and SIFT have identified the key lysine sites where mutations are susceptible to disease pathogenesis and are marked with red color taking the threshold of predictions by more than three tools for best accuracy. The highly disease susceptible mutations include K51H/D/E, K60H/D/E, K134H/D/E, K155H/D/E, K161D, K393H/D/E, K395R/H/D/E, K401H/D/E, K425H/D/E, K495H/D/E, K496H/D/E, K503R/H/D/E, K510D/E, K521R/H/D, K522R/H/D/E, K662H, K670D, K699H/D/E, K724H/D/E, K751H/D, and K763H/D/E. C) *Physico-chemical Properties and Amino acid Composition:* The physico-chemical properties of Ubiquitin showed equal no of positive and negative charged residues, with low hydrophobicity and high stability, while $A\beta_{42}$ showed high hydrophobicity and stability in comparison to AβPP low hydrophobicity and instability. Amino acid compositional analysis revealed the comparable percentage within 1% range of Ubiquitin with $A\beta_{42}$ and AβPP including Arginine, Asparagine, Aspartic acid, Glutamic acid, Phenyl alanine, Proline, and Serine.

Table 6
Importance of Lysine residues in AβPP Ubiquitination, Processing and other Functions

AβPP Mutational Site (Ubiquitination Confidence)		Affected molecular mechanisms (p-values ≤ 0.05)		Affected Motifs		Pathogenic Score
K351 (Very High)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of O-linked glycosylation (T353)	Loss of Proteolytic cleavage (Q347) Gain of Proteolytic cleavage (Q347)	GSK3, CK2, PIKK Phosphorylation site and FHA Phosphopeptide ligand		0.6470.658 0.629
K377 (Very High)	Lys(K)–Asp(D)	Gain of Phosphorylation and Sulfation (Y378); Loss of Acetylation, SUMOylation, and Ubiquitylation (K377); Gain of Proteolytic cleavage (D376); Altered Coiled coil		–		0.517
K224 (High)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of SUMOylation and Ubiquitylation (K224); Loss of Proteolytic cleavage (D219); Altered Coiled coil and Trans membrane protein		– CK2 Phosphorylation site, SUMO Interaction site		0.7 0.7550.722
K601 (High)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of SUMOylation (K601); Altered Trans membrane protein		NEK2 Phosphorylation site and FHA Phosphopeptide ligand	– SUMO Interaction site	0.611 0.6010.567
K751 (High)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of Allosteric site (R747); Altered Metal binding; Altered Trans membrane protein	Altered Ordered and Disordered interface –	–		0.737 0.713 0.705
K763 (High)	Lys(K)–Arg(R) Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of N-terminal acetylation (M768); Altered Disordered interface Loss of Methylation (K763); Gain of N-terminal acetylation (M768)	Altered Metal binding; Altered Ordered and Disordered interface Gain of Sulfation (Y762)	Tyrosine based sorting signal, Phosphotyrosine binding ligand, PKC Phosphorylation site, Amyloidogenic glycoprotein intracellular domain		0.6010.8290.8390.832
K60 (Medium)	Lys(K)–Arg(R) Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of Relative solvent accessibility; Altered Trans membrane protein; Loss of Disulfide linkage (C62) Loss of Relative solvent accessibility; Altered Trans membrane protein; Loss of Loop	Gain of ADP-ribosylation (K60); – Altered Metal binding; Loss of Loop	GSK3, CK1 Phosphorylation site, FHA Phosphopeptide ligand, N-myristoylation site	– CK2 Phospho-rylation site	0.5090.8120.7970.79
K161 (Medium)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Gain of Loop; Loss of Disulfide linkage (C158); Altered Trans membrane protein	Altered Metal binding; Loss of Relative solvent accessibility –	GSK3, CK1, PKC Phosphorylation site, FHA Phosphopeptide ligand		0.6190.6350.629

K393 (Medium)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Altered Coiled coil; Loss of Acetylation (K393); Loss of Helix	– Gain of SUMOylation (K395)		–	0.783 0.782 0.737
K401 (Medium)	Lys(K)–Arg(R) Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of SUMOylation and Acetylation (K401); Altered Disordered interface	– Loss of Helix		–	0.525 0.798 0.774 0.782
K496 (Medium)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of Acetylation (K496); Altered Disordered interface			–	0.613 0.544 0.516
K510 (Medium)	Lys(K)–Arg(R) Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Altered Metal binding; Altered DNA binding; Loss of Allosteric site (R505); Loss of Acetylation (K510); Altered Disordered interface	– Loss of Helix –	PKC Phosphorylation site		0.524 0.814 0.801 0.806
K522 (Medium)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of Helix; Altered Disordered interface; Altered Coiled coil		–		0.773 0.745 0.754
K662 (Medium)	Lys(K)–Arg(R) Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Gain of ADP-ribosylation (K662) Loss of Phosphorylation (S667) Gain of Phosphorylation (S667) Loss of Phosphorylation (S667)	Loss of SUMOylation, Acetylation, Ubiquitylation (K662); Altered Trans membrane protein	GSK3, CK1 Phosphorylation site, SUMOylation site, FHA Phosphopeptide ligand	– CK2 Phospho-rylation site	0.541 0.808 0.813 0.803
K503 (Low)	Lys(K)–Arg(R) Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Altered Disordered interface; Loss of Acetylation (K503); Altered DNA binding; Loss of Allosteric site (R505)	– Altered Metal binding; Loss of Helix Loss of Helix	PKB Phosphorylation site LATS Kinase Phosphorylation site – TRAF2 Binding site		0.571 0.805 0.815 0.786

(*Continued*)

Table 6
(*Continued*)

AβPP Mutational Site (Ubiquitination Confidence)		Affected molecular mechanisms (p-values ≤ 0.05)		Affected Motifs	Pathogenic Score
K568 (Low)	Lys(K)–His(H)	Altered Coiled coil; Loss of Helix; Loss of Phosphorylation (Y572); Altered Trans membrane protein; Loss of N-linked glycosylation (N571)	Loss of Sulfation (Y572)	_	0.608
	Lys(K)–Asp(D)		Gain of Sulfation (Y572)		0.629
	Lys(K)–Glu(E)				0.621
K724 (Low)	Lys(K)–His(H)	Altered Trans membrane protein; Altered DNA binding; Altered Metal binding; Gain of Pyrrolidone carboxylic acid (Q727)	Altered Disordered interface	LATS Kinase Phosphorylation site	0.641
	Lys(K)–Asp(D)		_	Clathrin box, SUMO Interaction site	0.648
	Lys(K)–Glu(E)				0.638
K51 (Very Low)	Lys(K)–Arg(R)	Altered Trans membrane protein; Loss of Relative solvent accessibility; Loss of Ubiquitylation (K51)	_	Integrin Binding site	0.511
	Lys(K)–His(H)		Altered Ordered interface	_	0.773
	Lys(K)–Asp(D)		Altered Ordered interface; Altered Metal binding		0.778
	Lys(K)–Glu(E)		_		0.736
K134 (Very Low)	Lys(K)–His(H)	Altered Metal binding; Altered Trans membrane protein	Loss of Relative solvent accessibility	_	0.744
	Lys(K)–Asp(D)		_		0.769
	Lys(K)–Glu(E)		Gain of SUMOylation (K132)		0.726
K155 (Very Low)	Lys(K)–His(H)	Altered Metal binding; Altered Trans membrane protein	Loss of Helix; Loss of Disulfide linkage (C158)	CK2 Phosphorylation site, FHA Phosphopeptide ligand	0.815
	Lys(K)–Asp(D)		Gain of Disulfide linkage (C158)		0.801
	Lys(K)–Glu(E)		Loss of Disulfide linkage (C158)		0.811
K395 (Very Low)	Lys(K)–His(H)	Loss of Helix; Loss of Acetylation(K393); Altered Disordered interface; Loss of SUMOylation(K395)		_	0.751
	Lys(K)–Asp(D)				0.709
	Lys(K)–Glu(E)	Altered Coiled coil; Loss of Helix; Loss of Acetylation and SUMOylation (K393); Altered Disordered interface			0.73
K425 (Very Low)	Lys(K)–His(H)	Altered Coiled coil; Loss of Helix; Altered Disordered interface; Loss of Acetylation (K425); Gain of Ubiquitylation (K428)		_	0.506
	Lys(K)–Asp(D)				0.527

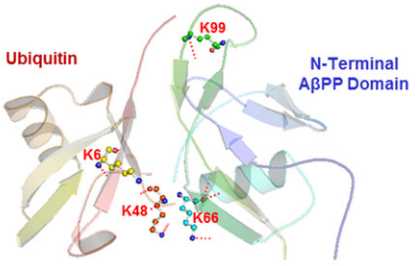
K495 (Very Low)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Altered Disordered interface; Loss of Acetylation (K495); Altered Coiled coil	–	–	0.779 0.781 0.731
		Altered Disordered interface; Gain of Acetylation (K496)	– Altered Coiled coil		
K521 (Very Low)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Loss of Helix; Altered Coiled coil	–	–	0.729 0.72 0.731
			Altered Disordered interface		
K670 (Very Low)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Altered Trans membrane protein; Loss of Acetylation (K670)	Loss of Phosphorylation (S667)	–	0.655 0.642 0.634
			Gain of Phosphorylation (S667)	CK2 Phosphorylation site	
K699 (Very Low)	Lys(K)–His(H) Lys(K)–Asp(D) Lys(K)–Glu(E)	Altered Trans membrane protein; Loss of Ubiquitylation (K699);	Loss of GPI-anchor amidation (N698)	PKC Phosphorylation site, N-myristoylation site	0.621 0.661 0.641
			Gain of GPI-anchor amidation (N698); Altered Metal binding		
			Gain of GPI-anchor amidation (N698)		

GSK3, Glycogen synthase kinase-3; CK1, Casein kinase-1; CK2, Casein kinase-2; PIKK, Phosphatidylinositol 3-kinase-related kinase; FHA, Forkhead-associated domain; SUMO, Small Ubiquitin-like Modifier; NEK2, NIMA Related Kinase 2; PKB, Protein Kinase B; PKC, Protein Kinase C; LATS, Large tumor suppressor kinase 1; TRAF2, TNF receptor-associated factor 2.

(A) Prediction of AβPP – Ubiquitin Interaction

Ubiquitin (1UBQ) interactions with	S_{Seq}	S_{Dom}	S_{Net}	S_{All}
APP Full Length	0.3537	0.5146	0.6359	0.5577
N-Terminal APP (4JFN_A)	0.3613	0.5146	0.6359	0.5555
Cu-binding APP (2FKL_A)	0.3537	0.5146	0.6359	0.5577
Protease inhibitor APP (1AAP_A)	0.3613	0.5146	0.6359	0.5555
E2 Domain APP (3UMK_A)	0.3537	0.5146	0.6359	0.5577
Aβ-42 of APP (1IYT_A)	0	0	0	0
C-Terminal APP (2LP1_A)	0.3537	0.5146	0.6359	0.5577

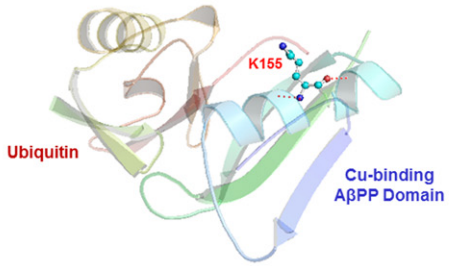
(B) Important Lysine for AβPP – Ubiquitin Interactions



Interacting Residues

N-AβPP: M36, F37, C38, R40, C62, I63, D64, T65, **K66**, I69, V85, V86, E87, A88, N89, P91, **K99**, Q104, T107, H108, H110, F111, V112, I113, Y115

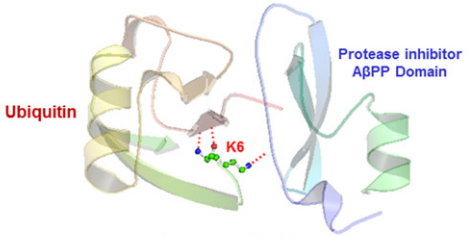
Ubiquitin: **K6**, L8, T9, R42, I44, A46, G47, **K48**, Q49, E51, H68, V70, L71, R72, L73, G75, G76



Interacting Residues

Cu-AβPP: A126, L127, L128, H147, H151, **K155**, S159, T163, N164, L165, H166, D167, Y168, G169, M170, P173, G175, D177, F179, E183

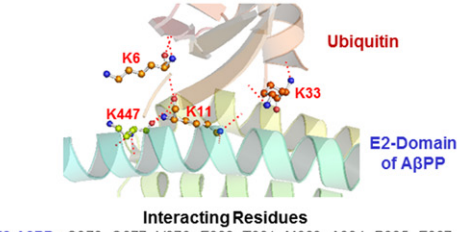
Ubiquitin: L8, T9, G10, R42, I44, G47, Q49, V70, L71, R72, L73, G75, G76



Interacting Residues

Protease inhibitor AβPP: S6, E7, Q8, A9, E10, T11, G12, P13, M17, Y22, F23, D24, V25, T26, E27, F33, F34, G39, G40

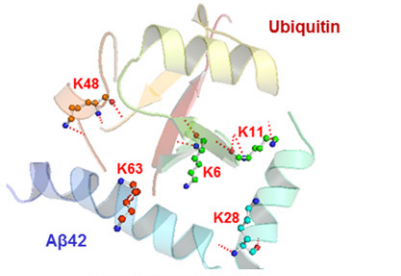
Ubiquitin: **K6**, T7, L8, T9, G10, T12, R42, G47, Q49, H68, L69, V70, L71, R72, L73, R74, G75, G76



Interacting Residues

E2-AβPP: Q376, Q377, V379, E380, T381, M383, A384, R385, E387, A388, N391, R394, R395, T433, V440, **K447**, Q450, I451, S453, Q454, T457, H458, R460, V461, R465

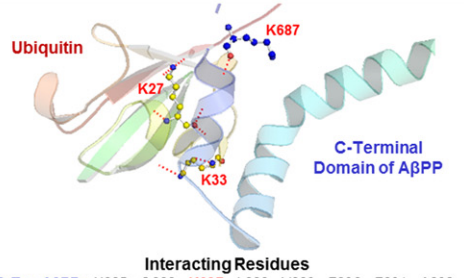
Ubiquitin: **K6**, T7, L8, T9, G10, **K11**, T12, I13, T14, Q31, D32, **K33**, E34, G35, I36, P37, Q40, L69, L71, L73



Interacting Residues

Aβ42: Y10, E11, H13, H14, L17, V18, F20, A21, E22, V24, G25, **K28**, L34, M35, G37, G38, V39, I41, A42

Ubiquitin: F4, **K6**, T9, G10, **K11**, T12, T14, E34, F45, A46, G47, **K48**, D58, Y59, N60, Q62, **K63**, E64, S65, T66, H68



Interacting Residues

C-Ter AβPP: H685, Q686, **K687**, L688, V689, F690, F691, A692, E693, D694, V695, G700, I703, G704, L705, V707, G708, V710, V711, I712, V715

Ubiquitin: T22, E24, N25, **K27**, A28, Q31, D32, **K33**, E34, G35, P37, P38, D39, Q40, Q49, D52, R72, R74

Fig. 6. Lysine residues in Ubiquitin and AβPP Interactions. A) *Prediction of AβPP–Ubiquitin Interactions:* The strongest evidence for AβPP–Ubiquitin interactions was reported by S_{Net}, i.e., sum of edge weights along the shortest path between homologous proteins in a protein-protein interaction network followed by overall interactions (S_{All}), statistical propensities of domain-domain interactions (S_{Dom}), and sequence similarities to a known interacting protein pair (S_{Seq}). B) *Important Lysine for AβPP–Ubiquitin Interactions:* The AβPP-Ubiquitin interactions revealed the role of diverse lysine residues in the interaction of different AβPP domains and Ubiquitin including K6, K11, K33, K48, and K63 in ubiquitin and K66, K99, K155, K447, K687 (K16 in Aβ), and K699 (K28 in Aβ) in AβPP.

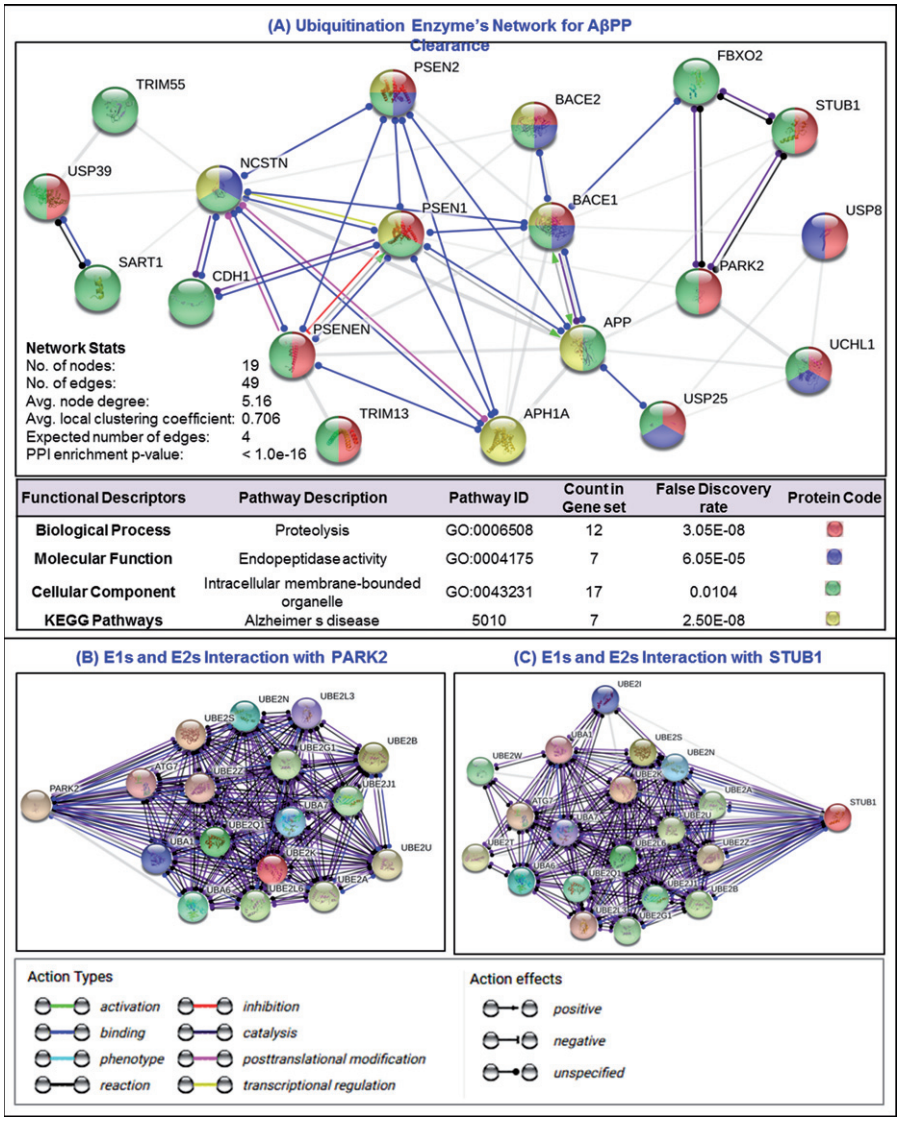


Fig. 7. Protein-Protein Interaction Network. A) *Ubiquitination Enzyme Network for AβPP:* The interaction network of AβPP and amyloid cascade proteins (BACE1, BACE2, PSEN1, PSEN2, PSENEN, NCSTN, APH1A) with the ubiquitin E3 ligases are shown in molecular action view. Ubiquitin E3 ligases, Park2 and STUB1 along with deubiquitinases, USP25 and UCHL1 interact with AβPP to regulate its ubiquitination process. Other E3 s including FBXO2, CDH1, TRIM13, TRIM55, and SART1 along with deubiquitinases USP8, USP25, USP39, and UCHL1 regulate the ubiquitination of amyloid cascade enzymes β-secretase (BACE1) and γ-subunit complex (PSEN1, PSNEN, NCSTN). The network's whole genome statistical analysis identified the interacting proteins associated with the proteolysis process, endopeptidase activity, intracellular membrane-bound organelle cellular component and AD pathway. B) *E1s and E2s Interaction with PARK2:* The network identified different ubiquitin E2 conjugating enzymes, UBE2A, UBE2B, UBE2G1, UBE2J1, UBE2K, UBE2L3, UBE2L6, UBE2N, UBE2Q1, UBE2S, UBE2U, and UBE2Z; and ubiquitin E1 activating enzymes, UBA1, UBA6, UBA7, and ATG7 associated with the conjugation of lysine to Park2. C) *E1s and E2s Interaction with STUB1:* The network identified different ubiquitin E2 conjugating enzymes- UBE2A, UBE2B, UBE2G1, UBE2I, UBE2J1, UBE2K, UBE2L3, UBE2L6, UBE2N, UBE2Q1, UBE2S, UBE2T, UBE2U, UBE2W, UBE2Z; and ubiquitin E1 activating enzymes- UBA1, UBA6, UBA7, ATG7 associated with the conjugation of lysine to STUB1. STUB1-STIP1 homology and U-box containing protein 1; PSENEN-Presenilin enhancer 2 homolog; CDH1-Cadherin 1; UCHL1-Ubiquitin carboxyl-terminal esterase L1; APP-Amyloid beta (A4) precursor protein; USP25-Ubiquitin specific peptidase 25; NCSTN-Nicastrin; TRIM13-Tripartite motif containing 13; USP8-Ubiquitin specific peptidase 8; SART1-Squamous cell carcinoma antigen recognized by T cells; USP39-Ubiquitin specific peptidase 39; BACE1-Beta-site AβPP-cleaving enzyme 1; TRIM55-Tripartite motif containing 55; PSEN1-Presenilin 1; BACE2-Beta-site AβPP-cleaving enzyme 2; FBXO2-F-box protein 2; PSEN2-Presenilin 2; PARK2-Parkinson protein 2; APH1A-Anterior pharynx defective 1 homolog A.

network of E1s, E2s, E3s, and DUBs enzymes for the clearance of AβPP in AD biology. The network revealed Parkin and STUB1 to be the key ubiquitin E3 ligases and USP25 and UCHL1 to be the key deubiquitinases directly involved in the ubiquitination of AβPP along with more than a dozen of E2 conjugating and E1 activating enzymes. While other E3 ligases such as FBXO2, TRIM13, CDH1, TRIM55, and SART1 are reported to regulate the ubiquitination of BACE1, PSENEN, PSEN1, and NCSTN, respectively. Similarly, other deubiquitinases, like USP8 and USP39, regulate BACE1 and NCSTN deubiquitination, respectively. In summary, the indepth studies pertaining to the lysine potential in AβPP processing, stability, interaction, ubiquitination, and other functions are provided herein that needed further investigations *in vivo* at the molecular level to devise novel therapeutic modalities against AD.

Conclusions

The Aβ synthesis is the consequence of AβPP processing by the β- and γ-secretases in neurons through amyloidogenic pathway, which is tightly regulated by the ubiquitination process. Moreover, any disruption in the ubiquitination of AβPP and Aβ, selectively amplify Aβ level that subsequently triggers AD pathogenesis. The identification of key lysine governing ubiquitination is not only crucial for regulating Aβ level in neurons but also for determining the mechanism of AβPP's subcellular trafficking and processing. Further identification of potential E3 ligases for directly (Parkin and STUB1) or indirectly (FBXO2, TRIM13, CDH1, TRIM55, and SART1) restraining Aβ production served as key therapeutic candidates for targeting neurodegenerative pathologies. Moreover, their identification is also crucial for developing chimera products like PROTACs for achieving selective protein degradation in the diseased state, which is an interesting area of research for the scientist [68]. Moreover, the finding of conserved residues near ubiquitination sites will aid in better understanding the mechanism behind lysine selection by E3 ligases and their interactions for ubiquitin positioning to govern lysine specific (K6, K11, K27, K29, K33, K48, K63) polyubiquitination. Additionally, we also reported that lysine residue of target protein may be involved in the ubiquitin positioning for determining the type of poly-ubiquitination chain during ubiquitin attachment. The present research has revealed the dynamics of Aβ or AβPP ubiquitination, which is essential to device, the strategies to regulate the AβPP metabolism. This would enable us to avoid the complications arisen from the elimination of secretases mediated biological functions of AβPP fragments: ectodomain- sAβPPα, sAβPPβ, N-terminal- APP-NTFs, and intracellular- AICD in regulating gene transcriptions [69]. Moreover, AβPP is proposed to be a cell-surface receptor [70]; therefore, AβPP ubiquitination may also serve as a signaling event for some unknown cellular processes apart from merely a signal for degradation. Further investigations are required to develop the prospective therapeutic agents that can address the clearance of such toxic proteins (Aβ) or their progenitors (AβPP) in a regulated way to ameliorate the neurodegenerative diseases like AD globally.

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-181219.

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Aβ, Tau, and α-Synuclein aggregation and integrated role of PARK2 in the regulation and clearance of toxic peptides

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Ubiquitination

ABSTRACT

Alzheimer's and Parkinson's diseases are one of the world's leading causes of death. > 50 million people throughout the world are suffering with these diseases. They are two distinct progressive neurodegenerative disorders affecting different regions of the brain with diverse symptoms, including memory and motor loss respectively, but with the advancement of diseases, both affect the whole brain and exhibit some common biological symptoms. For instance, > 50% PD patients develop dementia in their later stages, though it is a hallmark of Alzheimer's disease. In fact, latest research has suggested the involvement of some common pathophysiological and genetic links between these diseases, including the deposition of pathological Aβ, Tau, and α-synuclein in both the cases. Therefore, it is pertinent to diagnose the shared biomarkers, their aggregation mechanism, their intricate relationships in the pathophysiology of disease and therapeutic markers to target them. This would enable us to identify novel markers for the early detection of disease and targets for the future therapies. Herein, we investigated molecular aspects of Aβ, Tau, and α-Synuclein aggregation, and characterized their functional partners involved in the pathology of AD and PD. Moreover, we identified the molecular-crosstalk between AD and PD associated with their pathogenic proteins- Aβ, Tau, and α-Synuclein. Furthermore, we characterized their ubiquitinational enzymes and associated interaction network regulating the proteasomal clearance of these pathological proteins.

1. Introduction

The aggregation of certain misfolded proteins is the chief pathogenic event that evokes neurotoxicity in many neurodegenerative disorders like Alzheimer's and Parkinson's disease (Jellinger, 2010). The major structural changes that take place are the rise in β-sheet conformation in misfolded protein that promotes oligomerization and amyloid like fibril formation (Relini et al., 2013). Moreover, the aggregation mechanism involves a crucial step of seed-nucleus formation, where the monomers form a smallest aggregate, termed as 'nucleus' that grows faster by the addition of monomers in comparison to its dissociation back into smaller aggregates and free monomers. The primary nucleation event that triggers the oligomer formation is followed by the secondary nucleation events, where a nucleus formation on the surface of previously existing aggregate, direct a fast increase in the number of oligomers. Then, they attain a fibrillar form known as "fibrillar oligomers" and result into a fibril formation, i.e. seed, which leads to a rapid generation of new fibrils with same morphology and chirality (Linse, 2019). These oligomeric forms are more toxic and can be targeted by certain oligomer eliminating compounds (Dunkelmann et al., 2018). Thus, the seed-nucleation phenomenon governs a series of misfolding, and protein-protein interaction events that exaggerate the protein aggregation process (Breydo and Uversky, 2014). Consequently, aggregated proteins become resistant to proteolysis and cellular clearance that cause chronic endoplasmic reticulum stress, mitochondrial dysfunction, reactive oxygen species formation, intense tissue inflammation and activation of apoptotic pathways leading to the neuronal loss (Rutkowski and Kaufman, 2004; Morimoto, 2008).

Although AD and PD exhibit heterogeneity at the genetic and clinical level which is affecting different regions of the brain, including acetyl-cholinergic neurons in hippocampus and dopaminergic neurons in *substantia nigra pars compacta*. Recent studies revealed significant similarity in their overlapping role of pathological proteins, including amyloid-beta, tau protein, and α-synuclein and suggestive familial link in their pathogenesis (Jellinger, 2012). For instance, above 50% of patients, suffering from AD revealed amyloid like alpha-synuclein peptide aggregates (Marsh and Blurton-Jones, 2012) while PD patients displayed frequent tau deposits (Lei et al., 2010). In addition, it has

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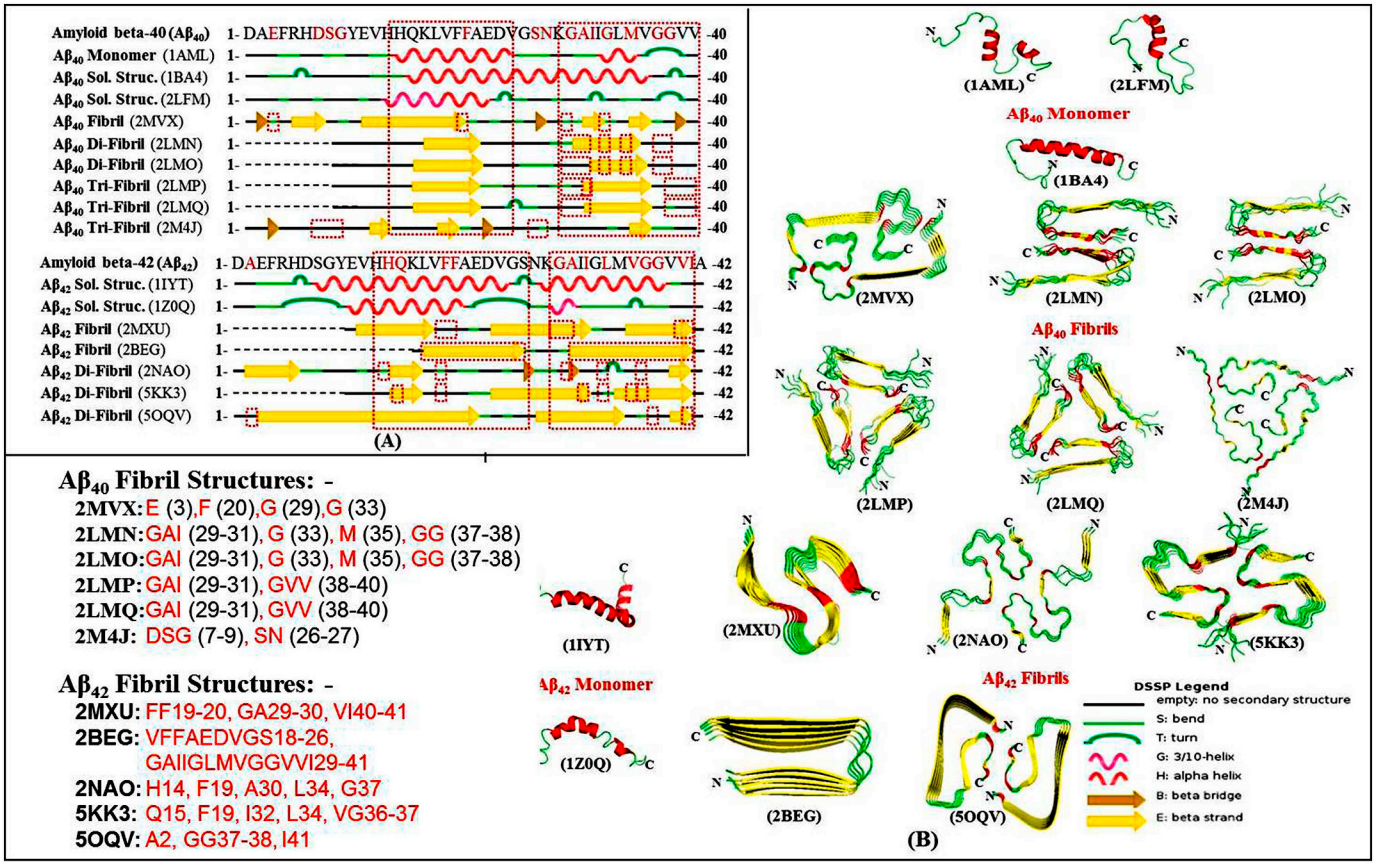


Fig. 1. Molecular basis of Amyloid beta peptide aggregation- (A) Structural changes in $A\beta_{40}/A\beta_{42}$ pathology: Helical conformation of amyloid-beta peptide gets distorted into the fibril structure rich in beta-strands and few beta-bridges. Polypeptide regions, including HQKLVFFAEDV (14-24) and GAIIGLMVGGVV (29-40) are responsible for the intra- and intermolecular fibril formation and consequent aggregation of $A\beta_{40}$ peptides. Similarly, HQKLVFFAEDVGS (14-26) and GAIIGLMVGGVVI (29-41) regions are responsible for intra- and intermolecular fibril formation and consequent aggregation of $A\beta_{42}$ peptides. Moreover, fibril-forming peptides are highlighted in red color, fibril-specific aggregation regions with red rectangles that presented the sequence motif responsible for aggregation of amyloid beta peptide. (B) 3D Structure of $A\beta_{40}/A\beta_{42}$ peptides: Aβ40 monomers (1AML, 2LFM, 1BA4) and Aβ42 monomers (1IYT, 1Z0Q) are shown in its cartoon view with aggregation prone region highlighted in red. The reported $A\beta_{40}/A\beta_{42}$ fibrils are shown in cartoon view with their aggregation prone regions in red color respectively. (2MVX: E3, F20, G29, G33; 2LMN: GAI29-31, G33, M35, GG37-38; 2LMO: GAI29-31, G33, M35, GG37-38; 2LMP: GAI29-31, GVV38-40; 2LMQ: GAI29-31, GVV38-40; 2M4J: DSG7-9, SN26-27/ 2MXU: FF19-20, GA29-30, VI40-41; 2BEG: VFFAEDVGS18-26, GAIIGLMVGGVVI29-41; 2NAO: H14, F19, A30, L34, G37; 5KK3: Q15, F19, I32, L34, VG36-37, 5OQV: A2, GG37-38, I41). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

demonstrated that the accumulation of tau proteins could take place with the help of specific strains of alpha-synuclein (Guo et al., 2013). Another study identified that Aβ Seeding escalates the formation of big sized α-synuclein oligomers that efficiently hampered neuronal SNARE-mediated vesicle fusion at synaptic junctions (Choi et al., 2015). Apart from proteinopathic similarities, some recent breakthroughs suggested a possible genetic link between these diseases in their advanced stages (Bailey et al., 2013; Singleton and Hardy, 2016; Ibanez et al., 2018; Zeng et al., 2018). For instance, LRRK2 mutant G2019S mediated phosphorylation of AβPP at T668, promoted APP intracellular domain's (AICD) nuclear translocation and consequent neurotoxicity in PD (Zeng et al., 2018). Likewise, LRRK2 is reported to directly phosphorylate tau at T149 and T153 signifying their interaction in a disease relevant manner (Bailey et al., 2013). Another study identified pathogenic mutations in AD causing genes- PSEN1 and PSEN2 in sporadic PD patients (Ibanez et al., 2018). Therefore, future research is required to establish well-defined pathological links between them.

However, these pathogenic proteins differ in their structural and functional biology, but they may share their protein-misfolding events and interact together to aggravate the disease symptoms (Barage and Sonawane, 2015; Cuanalo-Contreras et al., 2013). Moreover, they also interact with other ubiquitinational markers to relieve the proteotoxic burden inside neurons, via refolding or targeting proteins to ubiquitin proteasome system for degradation (Ciechanover and Kwon, 2015). Such a crucial ubiquitinational marker is PARK2, which is found to govern the proteasomal clearance of a wide range of substrates belonging to the nuclear proteins, cytoskeleton proteins, cell cycle regulators, heat shock proteins, neurotransmitters, and the cell signaling proteins. Some of these substrates include ataxin, Bcl-2, cyclin E, dopamine transporter, Hsp70, α-synuclein, synphilin-1, and α/β tubulin that regulates a variety of functions in different neurodegenerative disorders (Zhang et al., 2016). For instance, PARK2 regulates mitochondrial trafficking, endosomal sorting, synaptic transmission, programmed necrosis, ER stress, inflammation and cellular homeostasis. (Choong and Mochizuki, 2017; Sassone et al., 2017; Singh et al., 2018; Williams et al., 2018; Sun et al., 2019). Altogether, these findings highlight the need for a clear understanding of precise molecular mechanism behind pathogenic protein aggregation and clearance, and their interaction with other proteins to devise better diagnostic and treatment options for such neurodegenerative disorders.

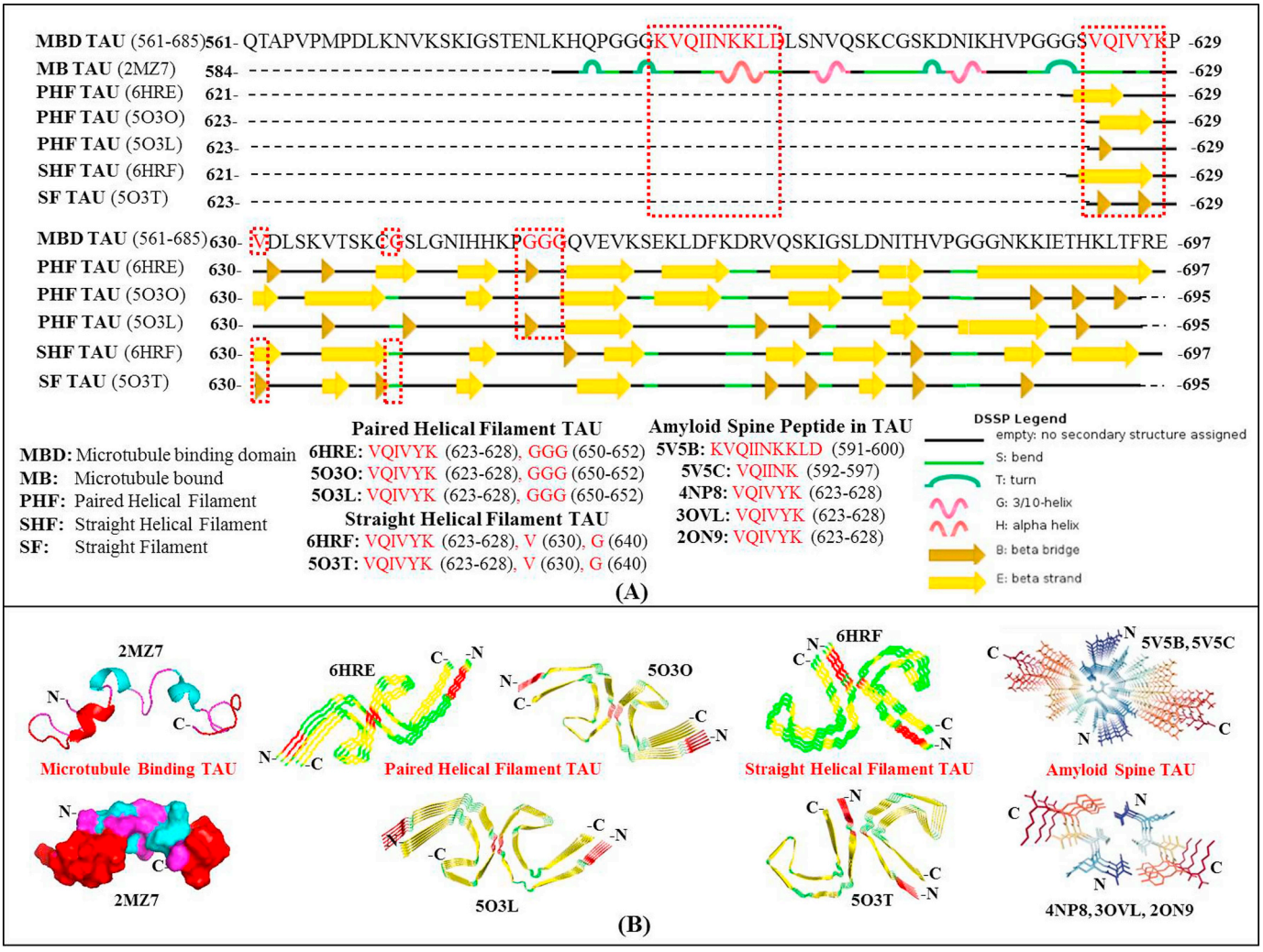


Fig. 2. Molecular basis of TAU protein aggregation- (A) Structural changes in TAU pathology: Helical conformation of microtubule binding domain is distorted into the filament structure (paired helical/ straight helical/ straight) rich in beta-strands and beta-bridges. Tripeptide glycine (650–652; Red) is responsible for paired-helical filament while valine (630; Red) and glycine (640; Red) are accountable for straight helical filament formation and consequent aggregation. Moreover, amyloid spines forming peptides are highlighted in red color that showed the sequence motif VQI(IN)/(VY)K responsible for aggregation of TAU protein in Alzheimer's disease. (B) 3D Structure of TAU protein: Microtubule binding domain of TAU (2MZ7) is shown in its cartoon and surface view with the aggregation-prone region highlighted in red. Paired-helical filaments of TAU (6HRE, 5O3O, 5O3L) reported in AD are shown in ribbon/cartoon view with their aggregation-prone regions in red color. Likewise, Straight-helical filaments of TAU (6HRF, 5O3T) reported in AD are shown in ribbon/cartoon view with their aggregation prone regions in red color. Furthermore, amyloid-spine forming TAU peptide models KVQIINKKLD and VQIVYK are shown in Licorice view that formed amyloid aggregate with help of interactions between valine and Isoleucine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Material and methods

2.1. Investigation of the molecular basis of Aβ$_{40/42}$, Tau, and α-Synuclein aggregation

The present sequences for the neurotoxic proteins were analysed for their secondary structures, and their available monomer and fibrillar structures were annotated for their hydrophobic residues responsible for aggregation.

2.1.1. Structural determination of Aβ$_{40/42}$, Tau, and α-Synuclein

The peptide sequence of Aβ$_{40/42}$, Tau and α-Synuclein were obtained from protein data bank (RCSB PDB: www.rcsb.org; Berman et al., 2000) and processed for the determination of its secondary structure by Dictionary of protein secondary structure (DSSP) database (Touw et al., 2015).

2.1.2. Macromolecular structure design and hydrophobicity annotation

The macromolecular monomeric and fibrillar structures of Aβ$_{40/42}$, Tau, and α-Synuclein proteins have been analysed for the hydrophobic or aggregation prone residues. These sites were annotated to the available 3D-structures with help of NGL viewer (http://proteinformatics.charite.de/ngl) (Rose and Hildebrand, 2015) and Pymol software (DeLano, 2002).

2.2. Characterization of the functional partners of Aβ$_{40/42}$, Tau, and α-Synuclein involved in the pathology of Alzheimer's and Parkinson's disease

The top interacting partners of the neurotoxic proteins have predicted and analysed for their role in disease pathogenesis.

2.2.1. Prediction of interacting partners of AβPP, Tau, and α-Synuclein

The interacting partners of AβPP, Tau, and α-Synuclein was determined by functional protein association networks tool called STRING, online available at https://string-db.org/. The top interactors

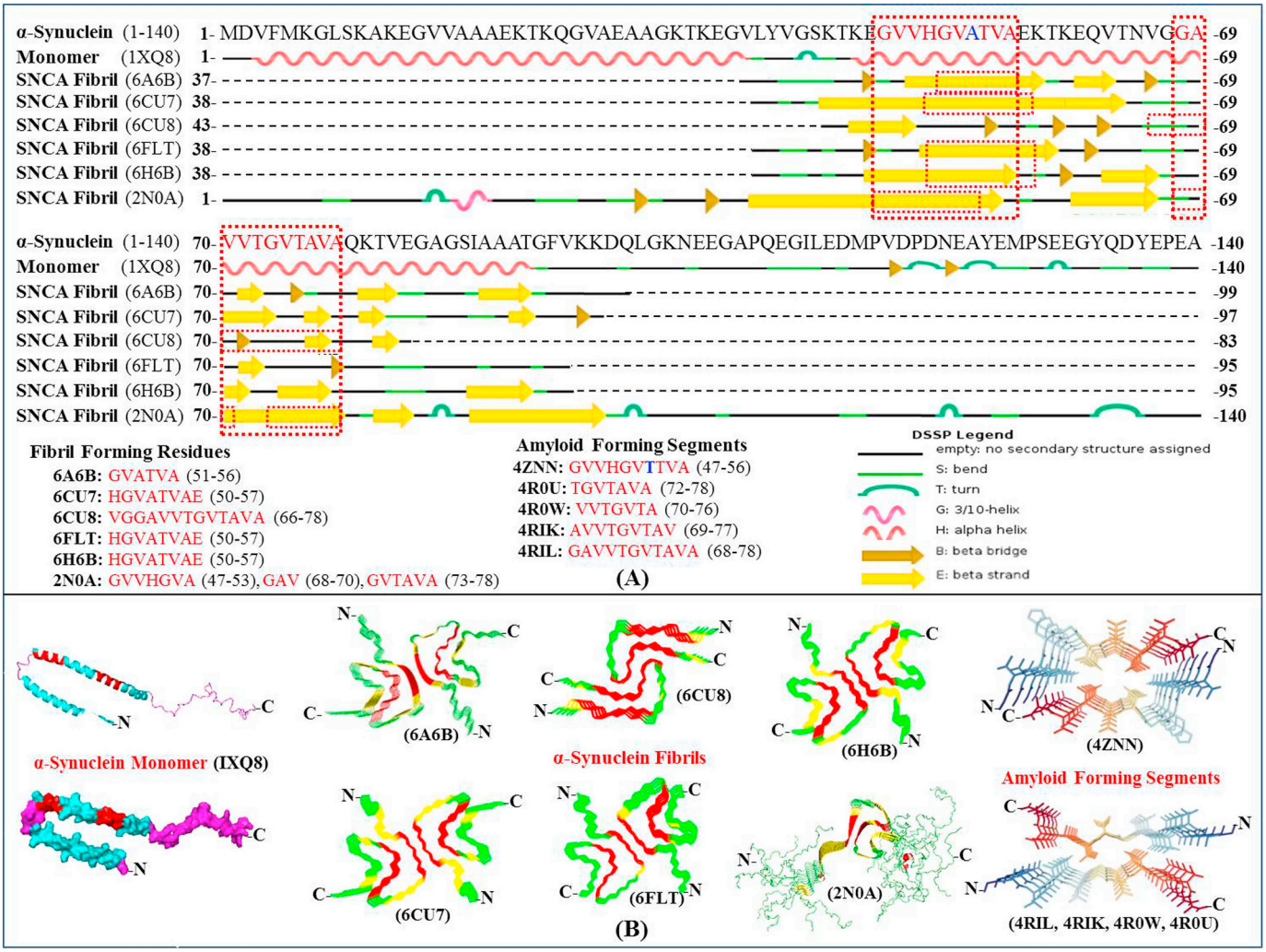


Fig. 3. Molecular basis of α-Synuclein protein aggregation- (A) Structural changes in α-Synuclein pathology: Helical conformations of synuclein monomer are distorted into the fibril structure rich in beta-strands and beta-bridges. Polypeptide regions, including GVVHGVATVAE (47-57) and GAVVTGVTAVA (68-78) are responsible for the intra- and intermolecular fibril formation and consequent aggregation of SNCA protein. Moreover, amyloid forming peptides are highlighted in red color that showed the sequence motif accountable for aggregation of SNCA protein in Alzheimer's disease. Moreover, the fibril-specific aggregation regions are shown with green rectangles. (B) 3D Structure of α-Synuclein protein: SNCA monomer (IXQ8) is shown in its cartoon and surface view with aggregation prone region highlighted in red. Reported alpha synuclein fibrils (6A6B, 6CU7, 6CU8, 6FLT, 6H6B, 2N0A) are shown in ribbon/cartoon view with their aggregation-prone regions in red color. Likewise, Straight helical filaments of TAU (6HRF, 5O3T) reported in AD are shown in ribbon/cartoon view with their aggregation prone regions in red color. Furthermore, amyloid-forming peptide models 4ZNN and 4RIL is shown in Licorice view that formed amyloid aggregate with help of interactions between their intermittent residues, glycine and valine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were identified with high confidence at the threshold of interaction score ≥ 0.700, and the network were generated without clustering and evidence based upon text mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence (Jensen et al., 2009).

2.2.2. *Protein interaction network analysis for Alzheimer's and Parkinson's disease*

Top hundred interacting partners of the AβPP, Tau, and α-Synuclein proteins were mapped on the pathways for Alzheimer's and Parkinson's disease from KEGG Pathways database and analysed for their functional association with the disease pathogenesis (Kanehisa et al., 2017).

2.3. *Investigation of the molecular crosstalk between AD and PD*

The combined AD and PD-related top-interactions of AβPP, Tau, and α-Synuclein was iterated for their interactions with another top hundred interacting partner. Those proteins that qualified their AD and PD incidence were selected and identified for their interactions with AβPP, Tau, and α-Synuclein at a high confidence threshold ≥ 0.7 with STRING tool, i.e. a Search Tool for the Retrieval of Interacting Genes/Proteins based on the evidence from text mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence (Szklarczyk et al., 2015). The interacting partner's prediction was followed by the Venn diagram analysis. Thus, the obtained proteins common to both Alzheimer's and Parkinson's disease, and were interacting with AβPP, Tau, and α-Synuclein, were screened as the key marker for AD-PD crosstalk.

2.4. *Identification of ubiquitination enzymes regulating the clearance of $A\beta_{40/42}$, Tau, and α-Synuclein*

The potential ubiquitination enzymes regulating the biology of Aβ, AβPP, Tau, and α-Synuclein proteasomal clearances were identified by determining the interaction among all the ubiquitin E1-activating enzymes, E2-conjugating enzymes, E3-ligating enzymes and deubiquitinating enzymes with AD-PD crosstalk proteins- AβPP, CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2, SNCA, and UBB at different confidences.

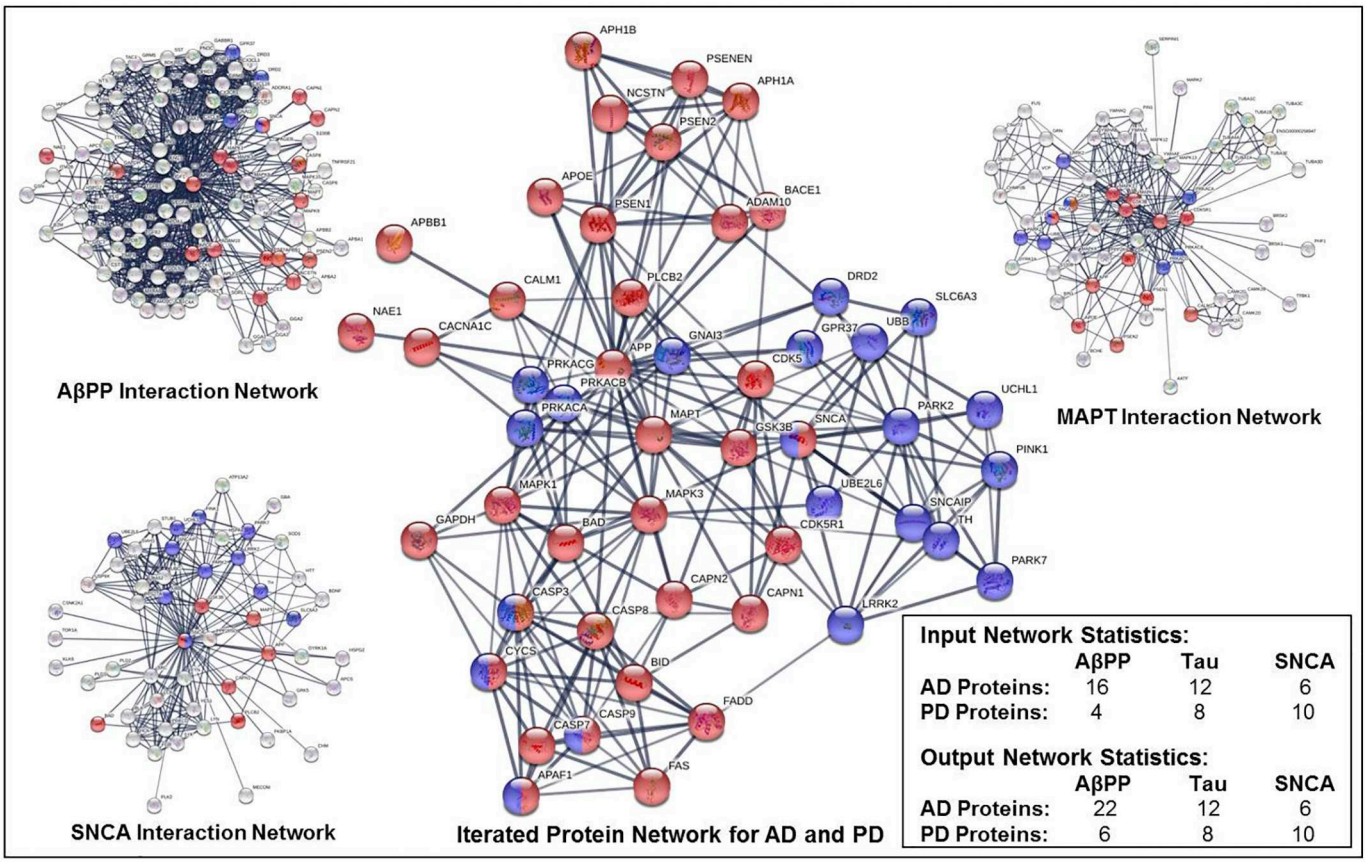


Fig. 4. Functional association network of AβPP, Tau and α-Synuclein interacting-proteins in AD and PD- The prime 100 amyloid-beta precursor interactor input proteins involved 16 AD-related proteins- ADAM10, APBB1, APOE, BACE1, CAPN1, CAPN2, CASP8, GAPDH, MAPK1, MAPK3, MAPT, NAE1, NCSTN, PSEN1, PSEN2, SNCA and 4 PD-related proteins- DRD2, GNAI3, GPR37, SNCA. Similarly, top 100 tau interactor input proteins involved 12 AD-related proteins- APOE, APP, CALM1, CASP3, CDK5, CDK5R1, GSK3B, MAPK1, MAPK3, PSEN1, PSEN2, SNCA and 8 PD-related proteins- CASP3, LRRK2, PRKACA, PRKACB, PRKACG, SNCA, PARK2, UBB. Likewise, leading 100 α-Synuclein interactor input proteins involved 6 AD-related proteins- APP, BAD, CAPN1, GSK3B, MAPT, PLCB2 and 10 PD-related proteins- LRRK2, PARK2, PARK7, PINK1, SLC6A3, SNCAIP, TH, UBB, UBE2L6, UCHL1. Moreover, the top 100 iterated interaction network of AβPP, Tau, and α-Synuclein involved the following proteins. (ADAM10-Disintegrin and metalloproteinase domain-containing protein 10; APBB1-Amyloid-beta A4 precursor protein-binding family B member 1; APH1A-Gamma-secretase subunit APH-1A; APOE-Apolipoprotein E; BACE1-Beta-secretase 1; BAD-Bcl2-associated agonist of cell death; CALM1-Calmodulin-1; CAPN1-Calpain-1 catalytic subunit; CAPN2-Calpain-2 catalytic subunit; CASP3-Caspase-3; CDK5-Cyclin-dependent-like kinase 5; CDK5R1-Cyclin-dependent kinase 5 activator 1; DRD2-D(2) dopamine receptor; GAPDH-Glyceraldehyde-3-phosphate dehydrogenase; GNAI3-Guanine nucleotide-binding protein G(k) subunit alpha; GPR37-Prosaposin receptor GPR37; GSK3B-Glycogen synthase kinase-3 beta; LRRK2-Leucine-rich repeat serine/threonine-protein kinase 2; MAPK1-Mitogen-activated protein kinase 1; MAPK3-Mitogen-activated protein kinase 3; MAPT-Microtubule-associated protein tau; NAE1-NEDD8-activating enzyme E1 regulatory subunit; NCSTN-Nicastrin; PARK2-E3 ubiquitin-protein ligase parkin; PARK7-Protein/nucleic acid deglycase DJ-1; PINK1-Serine/threonine-protein kinase PINK1; PLCB2-1-phosphatidylinositol 4;5-bisphosphate phosphodiesterase beta-2; PRKACA-cAMP-dependent protein kinase catalytic subunit alpha; PRKACB-cAMP-dependent protein kinase catalytic subunit beta; PRKACG-cAMP-dependent protein kinase catalytic subunit gamma; PSEN1-Presenilin-1; PSEN2-Presenilin-2; PSENEN-Gamma-secretase subunit PEN-2; SLC6A3-Sodium-dependent dopamine transporter; SNCAIP-Synphilin-1; TH-Tyrosine 3-monooxygenase; UBB-Polyubiquitin-B; UBE2L6-Ubiquitin/ISG15-conjugating enzyme E2 L6; UCHL1-Ubiquitin carboxyl-terminal hydrolase isozyme L1.)

Furthermore, the protein-protein interactional network among the identified proteins was designed by functional protein-association network prediction STRING tool (Szklarczyk et al., 2015).

2.5. Functional annotation of the AD-PD cross-talk and ubiquitination markers

The analysis of the biological processes, reactome pathways, molecular functions, and the protein domains of the predicted AD-PD cross talk markers and the UPS enzymes- E1s, E2s, E3s, and DUBs were performed with the help of a functional enrichment analysis tool "FunRich" version 3.1.3 (Pathan et al., 2015). It is a tool for the enrichment and interaction network analysis of genes and proteins based on data mining from the available databases, including FunRich, Uniprot, Reactome and Custom. The Unirot and reactome databases have been explored to obtain the best scoring results at very high significant *P* value, i.e. $P < .001$.

3. Results

3.1. Hydrophobic interactions are the basis of neurotoxic protein aggregation

The pathological peptides and proteins, including $A\beta_{40}$, $A\beta_{42}$, Tau, and α-synuclein in AD and PD were studied for their aggregation sites to analyze the protein folding dynamics in its diseased state. The analysis of their secondary structures revealed the transformation of their helical conformations into beta strands from their monomer to fibrillar state respectively. However, their tertiary structures have revealed the presence of crucial hydrophobic sites responsible for their intra- and inter-molecular interactions governing protein aggregation.

3.1.1. Amyloid beta peptide

The amyloid peptide existed in the two common isoforms in the brain, including $A\beta_{40}$ and $A\beta_{42}$ responsible for the senile plaques in

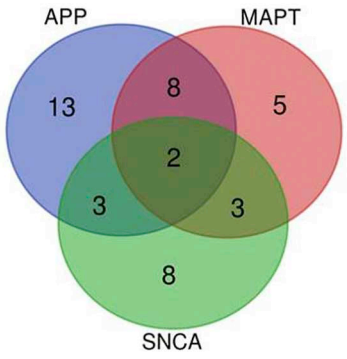
Table 1
Interacting partners of AβPP, Tau and α-Synuclein proteins and their corresponding functions.

Interacting proteins		Molecular functions
ADAM10	Disintegrin and metalloproteinase domain-containing protein 10	Responsible for the proteolytic release of TNF-alpha and several other cell-surface proteins, including heparin-binding epidermal growth-like factor, ephrin-A2, CD44, CDH2 and for constitutive and regulated alpha-secretase cleavage of amyloid precursor protein (APP)
APBB1	Amyloid-beta A4 precursor protein-binding family B member 1	Transcription coregulator with both coactivator and corepressor functions that forms a transcriptionally active complex with the gamma-secretase-derived amyloid precursor protein (APP) intracellular domain play a role in DNA damage response
APH1A	Gamma-secretase subunit APH-1A	Endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP
APOE	Apolipoprotein E	Mediates the binding, internalization, and catabolism of lipoprotein particles
BACE1	Beta-secretase 1	Responsible for the proteolytic processing of the amyloid precursor protein (APP)
BAD	Bcl2-associated agonist of cell death	Promotes cell death and appears to act as a link between growth factor receptor signaling and the apoptotic pathways
CALM1	Calmodulin-1	Mediates the control of a large number of enzymes, ion channels, aquaporins and other proteins through calcium-binding
CAPN1	Calpain-1 catalytic subunit	Catalyzes limited proteolysis of substrates involved in cytoskeletal remodeling and signal transduction
CAPN2	Calpain-2 catalytic subunit	Catalyzes limited proteolysis of substrates involved in cytoskeletal remodeling and signal transduction
CASP3	Caspase-3	Involved in the activation cascade of caspases responsible for apoptosis execution
CDK5	Cyclin-dependent-like kinase 5	Essential for neuronal cell cycle arrest and differentiation and may be involved in apoptotic cell death in neuronal diseases by triggering abortive cell cycle re-entry
CDK5R1	Cyclin-dependent kinase 5 activator 1	Neuron specific activator of CDK5 involved in dendritic spine morphogenesis and required for neurite outgrowth and cortical lamination
DRD2	D(2) dopamine receptor	Dopamine receptor whose activity is mediated by G proteins which inhibit adenylyl cyclase and promote cell proliferation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Play a role in glycolysis and nuclear functions via participating in transcription, RNA transport, DNA replication and apoptosis
GNAI3	Guanine nucleotide-binding protein G(k) subunit alpha	Function as transducers downstream of G protein-coupled receptors (GPCRs) in numerous signaling cascades
GPR37	Prosaposin receptor GPR37	Receptor for the neuroprotective and glioprotective factor prosaposin where ligand binding induces endocytosis, followed by an ERK phosphorylation cascade
GSK3B	Glycogen synthase kinase-3 beta	Acts as a negative regulator in the hormonal control of glucose homeostasis, Wnt signaling and regulation of transcription factors and microtubules
LRRK2	Leucine-rich repeat serine/threonine-protein kinase 2	Positively regulates autophagy through a calcium- dependent activation of the CaMKK/AMPK signaling pathway
MAPK1	Mitogen-activated protein kinase 1	Acts as an essential component of the MAP kinase signal transduction pathway and mediates diverse biological functions such as cell growth, adhesion, survival and differentiation through the regulation of transcription, translation, cytoskeletal rearrangements
MAPK3	Mitogen-activated protein kinase 3	Acts as an essential component of the MAP kinase signal transduction pathway and mediates diverse biological functions such as cell growth, adhesion, survival and differentiation through the regulation of transcription, translation, cytoskeletal rearrangements
NAE1	NEDD8-activating enzyme E1 regulatory subunit	Regulatory subunit of the dimeric UBA3-NAE1 E1 enzyme
NCSTN	Nicastrin	Catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein)
PARK2	E3 ubiquitin-protein ligase parkin	Functions within a multiprotein E3 ubiquitin ligase complex, catalyzing the covalent attachment of ubiquitin moieties onto substrate proteins
PARK7	Protein/nucleic acid deglycase DJ-1	Catalyzes the deglycation of the Maillard adducts formed between amino groups of proteins or nucleotides and reactive carbonyl groups of glyoxals and functions as a protein deglycase that repairs methylglyoxal- and glyoxal-glycated proteins, and releases repaired proteins and lactate or glycolate, respectively
PINK1	Serine/threonine-protein kinase PINK1	Protects against mitochondrial dysfunction during cellular stress by phosphorylating mitochondrial proteins and triggering selective autophagy (mitophagy) by mediating activation and translocation of Parkin
PLCB2	1-phosphatidylinositol 4;5-bisphosphate phosphodiesterase beta-2	Involved in the production of the second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3)
PRKACA	cAMP-dependent protein kinase catalytic subunit alpha	Phosphorylates a large number of substrates in the cytoplasm and the nucleus and regulates the abundance of compartmentalized pools of its regulatory subunits
PRKACB	cAMP-dependent protein kinase catalytic subunit beta	Mediates cAMP-dependent signaling triggered by receptor binding to GPCRs that regulates diverse cellular processes such as cell proliferation, the cell cycle, differentiation and regulation of microtubule dynamics, chromatin condensation and decondensation, nuclear envelope disassembly and reassembly
PRKACG	cAMP-dependent protein kinase catalytic subunit gamma	Phosphorylates a large number of substrates in the cytoplasm and the nucleus
PSEN1	Presenilin-1	Presenilin-2; Probable catalytic subunit of the gamma-secretase complex, an endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein) and may play a role in intracellular signaling and gene expression or in linking chromatin to the nuclear membrane
PSEN2	Presenilin-2	Presenilin-2; Probable catalytic subunit of the gamma-secretase complex, an endoprotease complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein) and may play a role in intracellular signaling and gene expression or in linking chromatin to the nuclear membrane
PSENEN	Gamma-secretase subunit PEN-2	Catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP (amyloid-beta precursor protein) and modulates both endoproteolysis of presenilin and gamma-secretase activity
SLC6A3	Sodium-dependent dopamine transporter	Terminates the action of dopamine by its high affinity sodium-dependent reuptake into presynaptic terminals
SNCAIP	Synphilin-1	Isoform 2 inhibits the ubiquitin ligase activity of SIAH1 and inhibits proteasomal degradation of target proteins
TH	Tyrosine 3-monooxygenase	Plays an important role in the physiology of adrenergic neurons

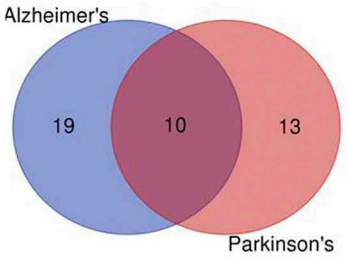
(*continued on next page*)

Table 1 (*continued*)

Interacting proteins		Molecular functions
UBB	Polyubiquitin-B	Form polyubiquitin chains on target proteins and regulate different functions depending on the Lys residue of the ubiquitin that is linked
UBE2L6	Ubiquitin/ISG15-conjugating enzyme E2 L6	Catalyzes the covalent attachment of ubiquitin or ISG15 to other proteins
UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Ubiquitin-protein hydrolase involved both in the processing of ubiquitin precursors and of ubiquitinated proteins



Names	total	elements
APP MAPT SNCA	2	GSK3B PARK2
APP MAPT	8	PSEN2 MAPK1 CDK5 SNCA CASP3 MAPK3 APOE PSEN1
APP SNCA	3	PLCB2 CAPN1 MAPT
MAPT SNCA	3	UBB APP LRRK2
APP	13	PSENEN BACE1 ADAM10 APH1A GNAI3 APBB1 NCSTN DRD2 CAPN2 GPR37 CASP8 GAPDH NAE1
MAPT	5	CDK5R1 PRKACA PRKACB PRKACG CALM1
SNCA	8	PINK1 SLC6A3 BAD PARK7 UBE2L6 SNCAIP TH UCHL1



Names	total	elements
Alzheimer's Parkinson's	10	PLCB2 UBB APP GSK3B SNCA CAPN1 CASP3 PARK2 LRRK2 MAPT
Alzheimer's	19	PSENEN BACE1 ADAM10 CASSP8 APH1A APBB1 PSEN2 MAPK1 NCSTN BAD CDK5 CDK5R1 CAPN2 MAPK3 APOE CALM1 GAPDH PSEN1 NAE1
Parkinson's	13	PINK1 GNAI3 SLC6A3 DRD2 PARK7 PRKACA PRKACB UBE2L6 PRKACG GPR37 SNCAIP TH UCHL1

Key markers for AD and PD crosstalk:

APP- Amyloid beta precursor protein
GSK3B- Glycogen synthase kinase 3 beta
MAPT- Microtubule associated protein Tau
PLCB2- Phospholipase C Beta 2
CAPN1- Calpain-1 catalytic subunit
LRRK2- Leucine-rich repeat kinase 2
PARK2- Parkin
SNCA- Synuclein Alpha

Fig. 5. Crosstalk markers involved in the pathology of Alzheimer's and Parkinson's disease- The first Venn diagram analysis highlighted the common interacting partners of Aβ, Tau and α-Synuclein while the second Venn diagram analysis showed the disease incidences of their interacting proteins into Alzheimer's and Parkinson's disease.

Alzheimer's disease. We reported high content of alpha helical structure in $A\beta_{42}$ isoforms than $A\beta_{40}$. However, $A\beta_{42}$ found to exist in mono-fibril to di-fibril state while $A\beta_{40}$ reported up to tri fibrillar state in terms of their complexity. Moreover, they shared their aggregation regions, rich in hydrophobic residues with some additional amino acids. For instance, the aggregation sites responsible for intra- and intermolecular fibril formation in $A\beta_{40}$ and $A\beta_{42}$ were HQKLVFFAEDV (14-24)/GAIIGLMVGGVV (29-40) and HQKLVFFAEDVGS (14-26)/GAIIGLMVGGVVI (29-41) respectively, which were almost similar with only few residue differences. However, the sequence specific aggregation sites for individual fibrillar peptides have shown in Fig. 1 that was populated with glycine, alanine, valine, leucine, isoleucine and phenylalanine.

3.1.2. Microtubule associated Tau protein

The MAPT is a 758 amino acid residue long protein whose microtubule-binding domain spans from 561 to 697 amino acids. It is hydrophilic, unstructured and dynamic in its aggregation. Therefore, it binds to microtubule in a random coil like fashion. The amyloid spine forming TAU (5V5B, 5V5C, 4NP8, 3OVL, 2ON9) revealed the KVQIINKKLD (591-600), VQIINK (592-597) and VQIVYK (623-628) sequence motif crucial for its aggregation. In addition, Tau's fibrillar form (6HRE, 5O3O, 5O3L) identified two types of helical filaments, including paired-helical and straight-helical form. The paired-helical filaments reported intra- and inter-molecular interactions with help of VQIVYK (623-628) and GGG (650-652) residues while straight-helical filaments with help of VQIVYK (623-628) and Valine (630), and Glycine (640) residues. Moreover, the protein-specific aggregation sites and the structural changes in their secondary-structures have shown in Fig. 2.

3.1.3. Alpha-Synuclein

The α-Synuclein is a small protein of 140 amino acids chief among other isoforms, i.e. β- and γ-synuclein. The conformational changes in its native structure take place to form pre-fibrillar oligomers and consequent fibril formation. It is also reported to attain amyloid like conformations (4R0U, 4R0W, 4RIK, 4RIL) that are identified to be formed by GVVHGVTTVA (47-56), TGVTAVA (72-78), VVTGVTA (70-76), AVVTGVTAV (69-77), and GAVVTGVTAVA (68-78) segments. Furthermore, the polypeptide regions- GVVHGVATVAE (47-57) and

Table 2
The key ubiquitin E3 ligases regulating the clearance of AD-PD crosstalk markers.

Direct regulators for AβPP, Tau, and α-Synuclein clearance

Names	Total	Elements
APP, LRRK2, MAPT, PARK2, SNCA	1	STUB1
APP, LRRK2, MAPT, SNCA	1	PARK2
GSK3B, PARK2, SNCA	2	FBXW7, SIAH1
LRRK2, PARK2, SNCA	1	FBXO7
APP, CAPN1	1	WDTC1
APP, MAPT	1	SYVN1
GSK3B, SNCA	1	TRAF6
PARK2, SNCA	4	RNF19A, SIAH2, TRIM32, NEDD4
APP	1	FBXL2
MAPT	1	MARCH7
SNCA	1	FBXO45

Indirect regulators for AβPP, Tau, and α-Synuclein clearance

Names	Total	Elements
GSK3B, LRRK2, PARK2	1	TRAF2
CAPN1, GSK3B	1	CDH1
CAPN1, PARK2	1	TRIM63
GSK3B, PARK2	5	CUL3, SKP1, RBX1, CUL1, UBE3A
LRRK2, PARK2	4	WSB1, RANBP2, HERC2, HACE1
PARK2, PLCB2	1	RNF41
GSK3B	9	UBR5, TRIM29, BIRC2, MAP3K1, PIAS1, XIAP, NHLRC1, MDM2, APC2
LRRK2	4	ERCC8, TRIM23, RHOBTB1, RHOBTB3
PARK2	149	TRAF7, MUL1, RNF114, SPSB2, RNF217, HEWE1, DET1, ASB7, HERC1, BTBD1, VPRBP, TRIM69, HECW2, FBXL18, RNF115, ASB14, HERC3, ASB11, LRRC41, FBXW4, RNF182, KLHL2, UBR1, SKP2, ASB17, RNF25, TCEB1, FBXL16, UBE3C, ASB4, RNF34, FBXL5, FBXO4, FBXL12, FBXO21, FBXO6, CDC20, MYLIP, TRIM9, TRIM11, FBXL22, KBTBD6, UBOX5, KBTBD8, ASB16, NEDD4L, ASB6, KLHL22, CDC23, RNF14, HERC4, RBCK1, ASB1, KLHL21, TCEB2, HECTD3, ASB9, FBXL3, SOCS3, ASB12, FBXW8, TRIP12, FBXL15, CUL2, ZBTB16, MGRN1, FBXL4, RNF7, CUL7, RNF19B, SPSB1, FBXO41, RNF123, RNF31, KLHL25, ITCH, HECTD1, RNF138, ARIH1, FBXO2, RFWD2, AMFR, TRIM36, UBR4, FBXL13, LRR1, MARCH5, TRIM39, KLHL20, CCNF, CUL5, FBXL19, TRIM71, SOCS1, WWP1, FBXL8, LNX1, ASB13, FBXO27, RNF111, RCHY1, TRIM21, PJA2, FBXO22, PJA1, TRIM4, FBXO17, LRSAM1, FBXO10, UBE3B, ASB8, FBXO30, SH3RF1, FBXO44, FBXW10, ASB18, RNF130, VHL, FBXO15, DZIP3, FBXW2, TRIM37, ASB5, ZNF645, FBXO9, FBXW9, ZNRF1, UBE4A, UBE3D, CDC26, ASB10, UBR2, ASB2, FBXW5, FBXL7, ASB15, FBXL14, TRIM50, ARIH2, HECTD2, FBXO11, FBXO31, FBXO40, KLHL9, KLHL11, RBBP6, TRIM41, RLIM, TRAIP

Table 3
Ubiquitinational markers for AD-PD crosstalk proteins.

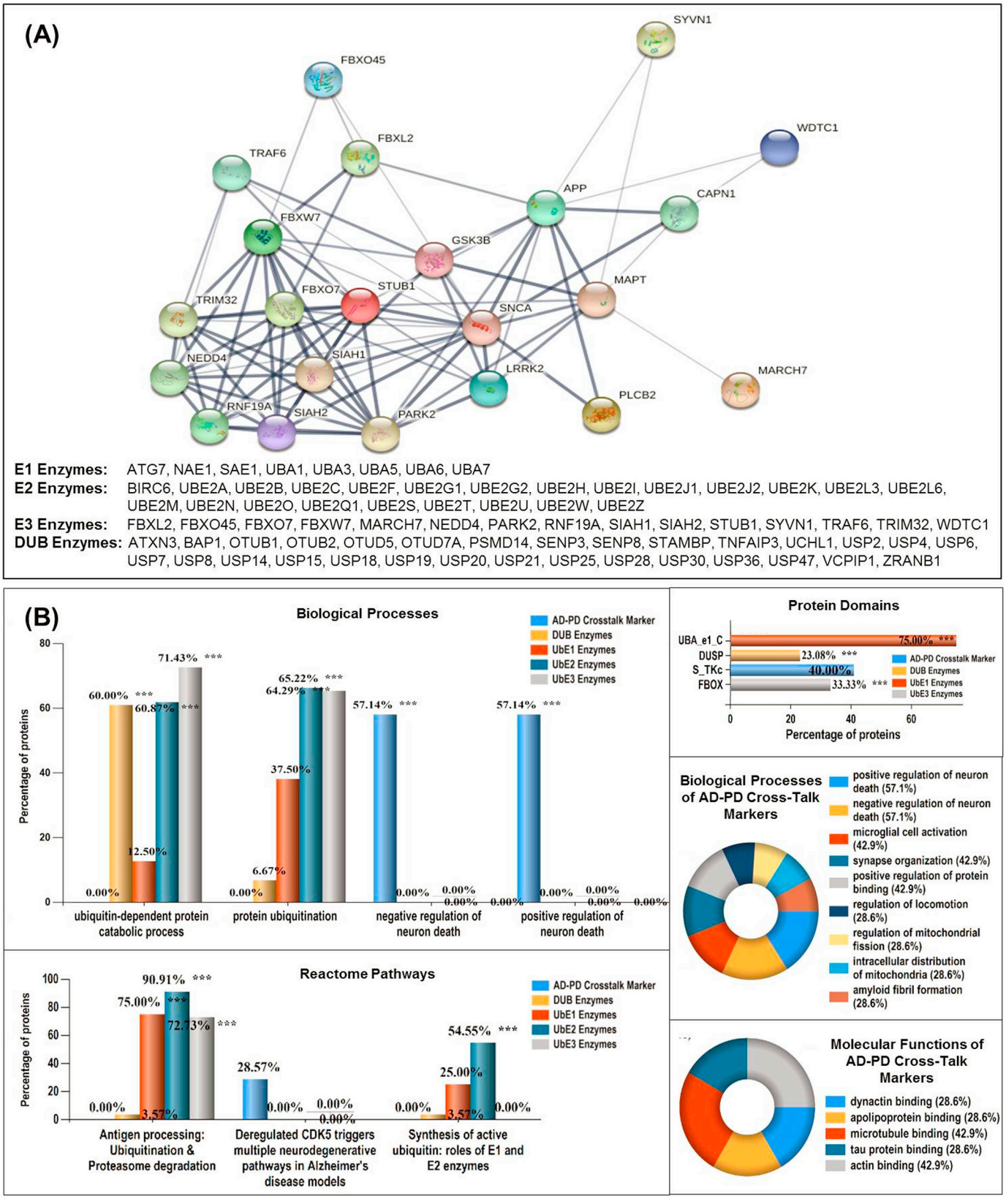
Pathological Targets	Key Ubiquitin E3 ligases	Ubiquitinating E2 Conjugating Enzymes	Ubiquitin E1 Activating Enzymes	Deubiquitinating Enzymes
AβPP, Tau, α-Synuclein, LRRK2, PARK2	**STUB1**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2L3, BIRC6, UBE2I, UBE2T	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	ATXN3, USP8, USP7, USP19, PSMD14, SENP3
AβPP, Tau, α-Synuclein, LRRK2	**PARK2**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2L3	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	ATXN3, USP8, USP30, UCHL1, BAP1, PSMD14, USP15
AβPP, α-Synuclein, LRRK2, PARK2	**FBXO7 (FBXL2, FBXO45)**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP47,UCHL1
α-Synuclein, GSK3B, PARK2	**FBXW7**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP47, USP28, USP7, USP36
α-Synuclein, GSK3B, PARK2	**SIAH1**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H,UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2I	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP19, USP4, USP6, USP15, USP20
AβPP, Tau	**SYVN1**	UBE2G2, UBE2J1, ATG3, UBE2J2, UBE2K, UBE2G1, UBE2S	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	ATXN3, PSMD14, USP19, VCPIP1
α-Synuclein, PARK2	**NEDD4**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2I, UBE2J1, UBE2J2, UBE2K, UBE2L3,	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5,	USP2, USP7, USP8, USP14, USP18, USP20, USP25,
		UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2T, UBE2U, UBE2W, UBE2Z	UBA6, UBA7	USP28, STAMBP
-Synuclein, PARK2	**RNF19A**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2L3	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	–
α-Synuclein, PARK2	**SIAH2**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z, UBE2I	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	USP19, USP4, USP6, USP15, USP20
α-Synuclein, PARK2	**TRIM32**	UBE2A, UBE2B, UBE2C, UBE2F, UBE2G1, UBE2G2, UBE2H, UBE2J1, UBE2J2, UBE2K, UBE2L6, UBE2M, UBE2N, UBE2O, UBE2Q1, UBE2S, UBE2U, UBE2W, UBE2Z	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	–
AβPP, CAPN1	**WDTC1**	UBE2M	ATG7, NAE1, SAE1, UBA1, UBA3, UBA5, UBA6, UBA7	SENP8
α-Synuclein, GSK3β	**TRAF6**	UBE2N, UBE2I, UBE2K, UBE2L3, UBE2O, UBE2S	ATG7, UBA1, UBA3, UBA5, UBA6, UBA7	USP4, USP7, OTUB1, OTUB2, TNFAIP3, ZRANB1, OTUD5, OTUD7A, STAMBP, UCHL1, USP2, USP15, USP20, USP21, USP25
Tau	**MARCH7**	UBE2G2, UBE2K, UBE2N	ATG7, UBA1, UBA3, UBA5, UBA6, UBA7	USP7

Red- Highest Confidence; Blue- High Confidence; Green- Medium Confidence

GAVVTGVTAVA (68-78) found to interact and form intra- and inter-molecular fibril that have shown in Fig. 3. These α-synuclein fibrils are rich in beta-strands and beta-bridges with varied structural conformations that initiate the lewy body formation in Parkinson's disease.

3.2. Functional partners of Aβ, Tau, and α-Synuclein involved in Alzheimer's and Parkinson's disease pathology

The top-hundred interacting partners among Aβ, Tau, and α-Synuclein



(caption on next page)

Fig. 6. (A) Ubiquitination enzyme interaction network for the clearance of AD-PD cross talk markers including Aβ, Tau and α-synuclein: - E1-activating enzymes: ATG7- Ubiquitin-like modifier-activating enzyme ATG7; NAE1- NEDD8-activating enzyme E1 regulatory subunit; SAE1- SUMO-activating enzyme subunit 1; UBA1/3/5/6/7- Ubiquitin-like modifier-activating enzyme 1/3/5/6/7. E2 conjugating enzymes: BIRC6- Baculoviral IAP repeat-containing protein 6; **UBE2A/B/C/F/G1/G2/H/I/J1/J2/K/L3/L6/M/N/O/Q1/S/T/U/W/Z-** Ubiquitin Conjugating Enzyme E2 A/B/C/F/G1/G2/H/I/J1/J2/K/L3/L6/M/N/O/Q1/S/T/U/W/Z. E3-ligases: FBXL2- F-box/LRR-repeat protein 2; FBXO45- F-box/SPRY domain-containing protein 1; FBXO7- F-box only protein 7; FBXW7- F-box/WD repeat-containing protein 7; MARCH7- Membrane Associated Ring-CH-Type Finger 7; NEDD4- Neural precursor cell expressed developmentally down-regulated protein 4; PARK2- Parkin; RNF19A- Ring finger protein19A; SIAH1/2- Siah E3 Ubiquitin Protein Ligase 1/2; STUB1- Ubiquitin-protein ligase CHIP; SYVN1- Synoviolin; TRAF6- TNF receptor-associated factor 6; TRIM32- Tripartite Motif Containing 32. Deubiquitinases: ATXN3- Ataxin-3; BAP1- Ubiquitin carboxyl-terminal hydrolase BAP1; OTUB1/2- OTU Deubiquitinase 1/2; OTUD5/7A- OTU domain-containing protein 5/7A; PSMD14- 26S proteasome non-ATPase regulatory subunit 14; SENP3/8- Sentrin-specific protease 3/8; STAMBP- STAM-binding protein; TNFAIP3- Tumor necrosis factor alpha-induced protein 3; UCHL1- Ubiquitin carboxyl-terminal hydrolase isozyme L1; USP2/4/6/7/8/14/15/18/19/20/21/25/28/30/36/47- Ubiquitin carboxyl-terminal hydrolase 2/4/6/7/8/14/15/18/19/20/21/25/28/30/36/47; VCPIP1- Valosin Containing Protein Interacting Protein 1; WDTC1- WD and tetratricopeptide repeats protein 1; ZRANB1- Zinc Finger RANBP2-Type Containing-1. (B) Functional enrichment analysis of AD-PD cross talk markers and the ubiquitination enzymes: - It has analysed the most important biological processes, reactome pathways, molecular functions, and protein domains at high significance *P*-values i.e. $P < .001$. The bar graph compares the percentage of input proteins with their associated top scoring biological processes, reactome pathways and protein domains. Biological processes: Ubiquitin-dependent protein catalytic process (UbE1s- UBA6; UbE2s- BIRC6, UBE2A/B/C/G1/G2/H/I/K/L3/L6/N/S/Z; UbE3s- FBXL2, FBXO7/45, NEDD4, RNF19A, SIAH1/2, STUB1, SYVN1, TRIM32; DUBs- ATXN3, BAP1, PSMD14, USP2/4/6/7/8/14/15/18/20/21/25/28/30/36/47), Protein ubiquitination (UbE1s- SAE1, UBA1/6; UbE2s- BIRC6, UBE2A/B/C/G1/G2/H/J2/K/L3/N/S/T/W/Z; UbE3s- FBXL2, FBXO7/45, FBXW7, NEDD4, PARK2, STUB1, SYVN1, TRIM32, WDTC1; DUBs- USP7, VCPIP1), Negative regulation of neuron death (AD-PD cross-talk markers- APP, GSK3B, LRRK2, SNCA), Positive regulation of neuron death (AD-PD cross-talk markers- APP, GSK3B, MAPT, SNCA). Reactome pathways: Antigen processing: Ubiquitination and Proteasome degradation (UbE1s- ATG7, UBA1/3/5/6/7; UbE2s- UBE2A/B/2C/F/G1/G2/H/J1/J2/K/L3/L6/M/N/O/Q1/S/U/W/Z; UbE3s- FBXO7, FBXW7, NEDD4, RNF19A, SIAH1/2, STUB1, TRIM32; DUBs- PSMD14), Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models (AD-PD cross-talk markers- APP, CAPN1), Synthesis of active ubiquitin: roles of E1 and E2 enzymes (UbE1s- UBA1/6; UbE2s- UBE2A/B/C/G1/G2/H/K/L3/S/T/W/Z; DUBs- USP7). Protein domains: UBA_e1_C (UbE1s- UBA1/6/7), DUSP (DUBs- USP4/15/20), S_TKc (AD-PD cross-talk markers- GSK3B, LRRK2), FBOX (UbE3s- FBXL2, FBXO7/45, FBXW7). The doughnut chart depicted the biological processes and molecular functions associated with the AD-PD cross talk markers. Biological processes: Positive regulation of neuron death (APP, GSK3B, MAPT, SNCA), Negative regulation of neuron death (APP, GSK3B, LRRK2, SNCA), Microglial cell activation (APP, MAPT, SNCA), Synapse organization (APP, MAPT, SNCA), Positive regulation of protein binding (APP, GSK3B, LRRK2), Regulation of locomotion (LRRK2, SNCA), Regulation of mitochondrial fission (MAPT, LRRK2), Intracellular distribution of mitochondria (MAPT, LRRK2), Amyloid fibril formation (APP, MAPT). Molecular functions: Dynactin binding (MAPT, GSK3B), Apolipoprotein binding (APP, MAPT), Microtubule binding (LRRK2, MAPT, SNCA), Tau protein binding (GSK3B, SNCA), Actin binding (LRRK2, MAPT, SNCA).

proteins were deduced and further iterated with another leading 100 interactors at high confidence, which revealed a set of 22 AβPP interacting proteins in AD while 6 in PD. Similarly, it identified 12 Tau interacting proteins in AD and 8 in PD. Likewise, six α-Synuclein interacting proteins were reported in AD and 10 in PD. In summary, the network identified 26 AD-related proteins, including ADAM10, APBB1, APH1A, APOE, APP, BACE1, BAD, CALM1, CAPN1, CAPN2, CASP3, CASSP8, CDK5, CDK5R1, GAPDH, GSK3B, MAPK1, MAPK3, MAPT, NAE1, NCSTN, PLCB2, PSEN1, PSEN2, PSENEN, and SNCA. However, it revealed 18 PD-related proteins, including PRKACB, PRKACG, PRKACA, GNAI3, TH, DRD2, GPR37, LRRK2, SNCA, SLC6A3, SNCAIP, PARK7, PARK2, CASP3, UBE2L6, UCHL1, PINK1, and UBB. The network maps of the functional partners are shown in Fig. 4, and their corresponding functions are summarized in Table 1. Altogether, these proteins were involved in the regulation of protein catalytic activity, amyloid fiber formation and associated signaling by receptor tyrosine kinases.

3.3. GSK3B and PARK2 are key markers for AD-PD crosstalk commonly interacting with Aβ, Tau, and α-Synuclein

The Venn diagram analysis of the top interacting partners reported only two proteins, including GSK3B and PARK2 to be commonly interacting with Aβ, Tau, and α-Synuclein. Instead other markers, found to interact either any of the two or any one of the Aβ, Tau, and α-Synuclein proteins. For instance, PSEN2, MAPK1, CDK5, SNCA, CASP3, MAPK3, APOE, and PSEN1 found to interact with AβPP and Tau, while PLCB2, CAPN1, and MAPT interacting with AβPP and α-Synuclein respectively. Likewise, UBB, APP, and LRRK2 observed to interact with Tau and α-Synuclein proteins. Moreover, their disease incidence analysis reported these eight key markers, including APP, CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2, and SNCA involved in the crosstalk of Alzheimer's and Parkinson's disease at the molecular level. While other markers, specific for AD and PD interacting with any of their pathological partners- Aβ, Tau and α-Synuclein is shown in Fig. 5. Here, only PARK2 and GSK3B found to interact commonly with all diseased proteins, including Aβ, Tau and α-synuclein. Since, PARK2 is a ubiquitin E3 ligase. Therefore, its interaction with Aβ, Tau and α-synuclein would certainly regulate their levels in our body.

3.4. PARK2 and STUB1 are the key ubiquitin E3 ligases regulating the clearance of pathological markers in AD and PD

The ubiquitin E3 ligases were identified against all the AD-PD cross talk markers and were classified as direct- and indirect-regulators depending on their potential interaction with Aβ, Tau and α-Synuclein. Those E3 ligases that were involved in the ubiquitination of Aβ, Tau and α-Synuclein is classified as direct-regulators, while those involved with other pathological markers classified as indirect-regulators. We reported only PARK2 and STUB1 to be commonly interacting with most of the AD-PD cross talk markers, including AβPP, MAPT, SNCA, and LRRK2. Instead, other E3 ligases found to regulate AβPP, Tau, and α-Synuclein ubiquitination individually or in different combinations. Likewise, we reported indirect-regulators that were involved with the ubiquitination of markers other than AβPP, Tau, and α-Synuclein, such as CAPN1, GSK3B, LRRK2, PARK2, and PLCB2. Among them, TRAF2 found to regulate GSK3B, LRRK2, and PARK2 markers commonly. Similarly, we defined a spectrum of E3 ligases involved in the ubiquitination of AD-PD cross talk markers that are summarized in Table 2. The comprehensive study reported 149 regulatory ubiquitin E3 ligases for the ubiquitination of PARK2 ubiquitin E3-ligase. It suggested the involvement of PARK2 in both the pathology and clearance biology, i.e. negative and positive role in neurodegenerative disorders like AD and PD.

3.5. Ubiquitination biology of toxic Aβ, Tau, and α-synuclein protein clearance

The ubiquitination reaction of Aβ, Tau, and α-synuclein clearance is a complex biology of interactions among a series of E1-activating, E2-conjugating, E3-ligating and deubiquitinating enzymes. Here, we reported the important ubiquitination markers, including the E3 ligases- PARK2, STUB1, FBXW7 SIAH1, FBXO7, WDTC1, SYVN1, TRAF6, RNF19A, SIAH2, TRIM32, NEDD4, FBXL2, MARCH7, and FBXO45, and

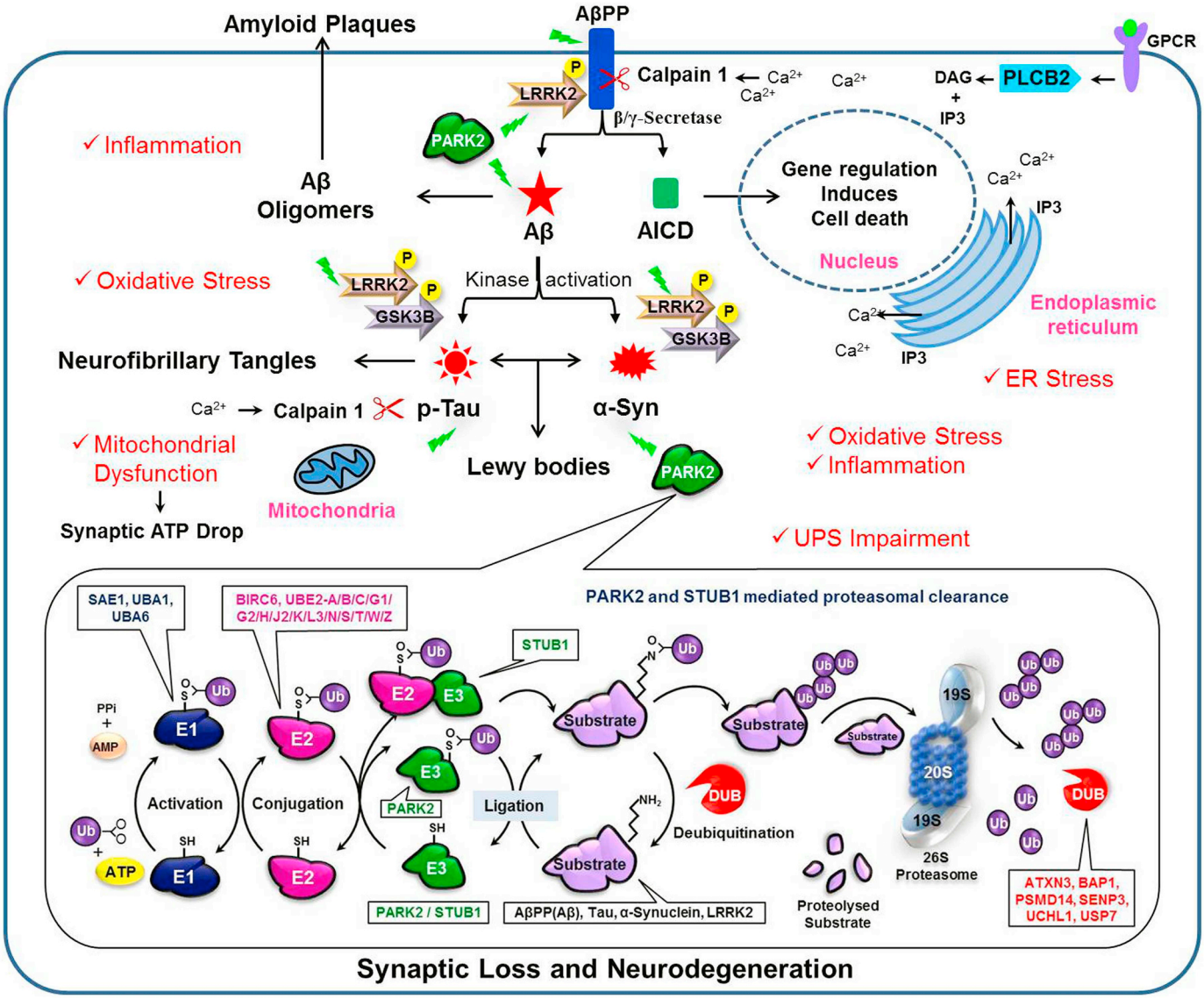


Fig. 7. Molecular mechanism of AD-PD crosstalk marker's associated neurodegeneration and their proteasomal clearance- In diseased state, proteolytic processing of AβPP leads to the production of Aβ and amyloid intracellular domain. Here, amyloid production activates the kinases like LRRK2 and GSK3B, which in turn accelerate the phosphorylation of Tau and α-Synuclein protein thereby resulting neurofibrillary tangles and lewy bodies. On the other side, AICD triggers the apoptotic gene regulation and induces cell death. Furthermore, G-protein coupled receptor activates phospholipase B (PLCB2) that triggers the calcium release from ER and consequent action of calpain 1 on their substrates, including AβPP and Tau. Here, calapin 1 mediated tau cleavage products, induces mitochondrial dysfunction and thus ATP loss. On the other side, Parkin (PARK2) as an E3 ligase acts on the pathogenic proteins- AβPP(Aβ), Tau, α-Synuclein, and LRRK2 to mark them for proteasomal degradation with the help of diverse ubiquitin activating enzymes, conjugating enzymes, and deubiquitinases. Altogether, these toxic proteins are associated with the ER stress, oxidative stress, mitochondrial dysfunction, inflammation, and UPS impairment in neurons thereby leading to synaptic loss and neurodegeneration. AICD- Amyloid intracellular domain; DAG- Diacylglycerol; PLCB2- Phospholipase B.

the deubiquitinases- ATXN3, USP8, PSMD14, and UCHL1 directly regulating the clearance of Aβ, Tau and α-synuclein. Furthermore, identified their corresponding ubiquitin E2-conjugating enzymes- UBE2A/B/C/F/G1/G2/H/J1/J2/K/L6/M/N/O/Q1/S/U/W/Z and ubiquitin E1-activating enzymes- ATG7, NAE1, SAE1, UBA1/3/5/6/7 involved in the ubiquitination biology of all these protein aggregates. In addition, we reported the majority with a similar set of E2s and E1s with crucial differences in their deubiquitinases. The important ubiquitination enzymes for the clearance of AD-PD cross talk markers are summarized in Table 3. Moreover, the enzymatic regulation of ubiquitination reaction for Aβ, Tau, and α-synuclein with their specific ubiquitin E3-ligases are mapped in Fig. 6A. These UPS enzymes along with their target AD-PD cross-talk markers were further investigated for their functional annotations, including the biological processes, reactome pathways, protein domains, and molecular functions (Fig. 6B). The biological process analysis revealed only the UPS enzymes including **UbE1s-***SAE1, UBA1/6;***UbE2s-***BIRC6, UBE2A/B/C/G1/G2/H/J2/K/L3/N/S/T/W/Z;***UbE3s-***FBXL2, FBXO7/45, FBXW7, NEDD4, PARK2, STUB1, SYVN1, TRIM32, WDTC1;* and **DUBs-***USP7, VCPIP1* to be associated with protein ubiquitination, while **UbE1s-***UBA6;***UbE2s-***BIRC6, UBE2A/B/C/G1/G2/H/I/K/L3/L6/N/S/Z;***UbE3s-***FBXL2, FBXO7/45, NEDD4, RNF19A, SIAH1/2, STUB1, SYVN1, TRIM32,* and **DUBs-***ATXN3, BAP1, PSMD14, USP2/4/6/7/8/14/15/18/20/21/25/28/30/36/47* to be linked with the ubiquitin dependent protein catabolic process. On the other hand, AD-PD cross-talk markers were reported to be linked with both positive (APP, GSK3B, MAPT, SNCA) and negative regulation (APP, GSK3B, LRRK2, SNCA) of neuronal death. The analysis revealed the peculiarity of LRRK2 in the pathogenesis of disease. Furthermore, the reactome pathway analysis identified three pathways with most significant scores, including i) UPS associated antigen processing (**UbE1s-***ATG7,*

*UBA1/3/5/6/7;***UbE2s-***UBE2A/B/2C/F/G1/G2/H/J1/J2/K/L3/L6/M/N/O/Q1/S/U/W/Z;***UbE3s-***FBXO7, FBXW7, NEDD4, RNF19A, SIAH1/2, STUB1, TRIM32; DUBs- PSMD14*), ii) CDK5 linked neurodegeneration (AD-PD cross-talk markers- APP, CAPN1), and iii) synthesis of active ubiquitin protein (**UbE1s-***UBA1/6*; **UbE2s-***UBE2A/B/C/G1/G2/H/K/L3/S/T/W/Z;***DUBs-***USP7*). In addition, the Protein domain analysis revealed the majority of ubiquitin E1 activating enzymes with UBA_e1_C domain, while ubiquitin E3 ligases with FBOX domain. Likewise, it reported DUSP domain in most of the deubiquitinases, while S_TKc domain in the majority of the AD-PD crosstalk markers. Moreover, the molecular functions and biological processes analysis of AD-PD crosstalk markers identified their roles in microglial activation, synapse organization, mitochondrial fission, amyloid fibril formation and binding with various cellular proteins, including actin, dynactin, apolipoprotein, tau and microtubule. Altogether, these findings have provided new insights into the molecular mechanisms for neurotoxic protein clearance in AD and PD.

3.6. Molecular cross talk among neurotoxic proteins and their clearance in Alzheimer's and Parkinson's disease

The functional enrichment analysis of the UPS enzymes has provided valuable insights for the choice of potential ubiquitination enzymes important for the proteasomal clearance of pathogenic proteins, while that of AD-PD cross talk markers revealed their role in protein aggregation. However, ubiquitination is a complex process of targeted protein degradation with the help of an array of ubiquitinational enzymes, especially the ubiquitin E3 ligases that are crucial for imparting the substrate specificity, but the obtained results have revealed the facts to a greater extent. Based on the evidence from our findings, we can hypothesize the molecular mechanism of the toxic protein aggregation and their clearance through the ubiquitination process for better understanding the medical state of Alzheimer's and Parkinson's disease (Fig. 7). The clinical reports suggest that the identified AD-PD cross-talk markers have their pathogenic role in both the diseases, but CAPN1 and PLCB2 are two novel markers whose roles need to be investigated in both diseases, although they are known to play some role in Alzheimer's disease. Moreover, future research is required to translate these findings to devise better diagnostic and therapeutic avenues for life management and care for the patients suffering with Alzheimer's and Parkinson's disease.

4. Discussion

The soluble forms of amyloid beta peptides- $A\beta_{40}$ and $A\beta_{42,}$ microtubule associated tau protein, and the α-synuclein protein is well evident to cause neurotoxicity in both Alzheimer's and Parkinson's disease pathology. Here, we studied their aggregation sites to analyze the protein folding dynamics in its diseased state. We found that these proteins transform their secondary structural helical conformations into beta strands upon their transition from monomer to fibrillar state. Recently, Balupuri et al. (2019) has also shown the occurrence of α-strand in the monomer to drive sheet formation in the oligomers that initiates and promotes α-synuclein aggregation and fibrillation (Balupuri et al., 2019). Moreover, the tertiary structures of amyloid beta have revealed the hydrobhobic sites HQKLVFFAEDV (14-24)/ GAIIGLMVGGVV (29-40) and HQKLVFFAEDVGS (14-26)/ GAIIGLMVGGVVI (29-41) in $A\beta_{40}$ and $A\beta_{42}$ respectively that are responsible for the intra- and inter-molecular interactions during protein aggregation. The hydrophobic residues at these sites rearrange their non-covalent interactions via disrupting their previous secondary structures and resulting in a seed-nucleus formation. It provides a surface for a series of misfolding events and molecular interactions with other proteins to form oligomers and consequent protofibrils (Marinko et al., 2019). Interestingly, Cox et al. (2018) also confirmed that protein aggregation is a biological driven process that evades out the intrinsic hydrophobic regions due to mutations or protein misfolding events (Cox et al., 2018). Moreover, Hao et al. (2010) demonstrated that $A\beta_{20–29}$ peptide blocks apoE-Aβ interaction through competitive binding at c-terminal domain of apoE and consequently, reduces full-length $A\beta_{40/42}$ fibril formation and cytotoxicity (Hao et al., 2010). This indicates that the proximal aggregation sites HQKLVFFAEDV (14-24) in $A\beta_{40}$ and HQKLVFFAEDVGS (14-26) in $A\beta_{42}$ as shown in our study has unique ability for self-aggregation as well as interaction with other proteins like apoE to facilitate its own aggregation.

Furthermore, the aggregation sites in amyloid beta peptides have revealed the spanning lysine residues that was also reported by Sinha et al. (2012) who have shown the role of lysine residues (K16) in amyloid beta folding, assembly and toxicity (Sinha et al., 2012). Moreover, the aggregation sequences in Aβ, Tau, and α-synuclein protein was highly populated with glycine and hydrophobic residues predominating alanine, valine, isoleucine and phenylalanine. Matsui et al. (2017) has also demonstrated the α-helix rules i.e. hydrophobicity of amino acids in the α-helix structure as a potential rationale for aggregation hotspot prediction (Matsui et al., 2017). Moreover, the arrangement of monomer in antiparallel fashion led to cooperative formation of β-sheet conformation (Lovas et al., 2013), and they attained different topologies based on the diversity of their intra- and inter-chain interactions. In fact, NMR studies have also shown the contribution of hydrophobic interaction and salt bridges in imparting the stability to beta-sheets and turns in protein folding and assembly (Petkova et al., 2002, 2006). However, their reverse transition from β-sheets to random coils is the governing principle for neuro-protection adopted by small peptides like NAP (NAPVSIPQ) and SAL (SALLRSIPA) in tau protein (Mokhtari et al., 2016). Interestingly, we identified VQIVYK (623–628) sequence in Tau protein responsible for its fibrillar state indicating their potential role in causing neurotoxicity that can be prevented by disrupting their specific interactions with help of certain short peptides. Moreove, the presence of glycine residue is also important for imparting flexibility to the protein structure, which can fit into both hydrophilic and hydrophobic environments due to its minimal side chain (Scott et al., 2007). Therefore, it sterically allows the protein aggregate to attain different conformations. In addition, hydrophobic regions are also responsible for the hydrophobic interactions among themselves that allow the protein to attain a variety of fibrillar forms with a common hydrophobic core (Kalinowska et al., 2017). On the other side, hydrophobic interactions with other proteins also trigger neuroinfammation. For instance, hydrophobic interactions of glycine zipper fragments of amyloid beta peptides with nitric oxide synthase facilitate the nitric oxide formation and consequent inflammation in neurons (Padayachee and Whiteley, 2013). Altogether, these findings enforce that the surface hydrophobicity guides the process of neurotoxic protein aggregation and consequent inflammation in different neurodegenerative disorders.

Alzheimer's and Parkinson's disease are two distinct neurodegenerative disorders with some pathological similarities. Therefore, numerous studies have investigated the links between them at protein level and found some protein aggregates /CSF peptides in common (Jolkkonen et al., 1991; Moskvina et al., 2013). The primary objective of this study was to investigate the shared pathogenic markers associated with Alzheimer's and Parkinson's disease. Since, the reported pathological proteins (Aβ, Tau and α-synuclein) interact synergistically to accelerate the neuropathology (Clinton et al., 2010); thus, we deduced the AD-PD cross-talk markers by identifying their functional interacting partners involved in the pathology of AD and PD. Altogether, we reported five markers apart from Aβ or AβPP, MAPT, and SNCA, including GSK3B, PARK2, LRRK2, PLCB2, and CAPN1 that are expressed in common during the diseased state. However, these markers have shown their involvement in either of the disease previously, but recent findings have shown their role in both diseases. For instance, LRRK2 variant R1628P increased the risk of AD in the population, and in-vitro findings suggested its predisposition to apoptosis (Zhao et al., 2011). On the other hand, LRRK2 mutation,

G2019S reported to promote AβPP phosphorylation and consequent AICD activity mediated neurotoxicity in PD (Chen et al., 2017). Likewise, apart from tau hyper-phosphorylation, it increased β-amyloid production and inflammatory responses in AD (Hooper et al., 2008). Moreover, GSK3B dysregulations, also contributed towards Parkinson's disease like pathology with induced phosphorylation and aggregation of Tau and α-synuclein (Credle et al., 2015), while it acts positively for neuronal growth in his health state (Yang et al., 2015). Similarly, PARK2 has shown their role in AD, since its enhancement has compensated mitophagic alterations in their pathology (Martin-Maestro et al., 2016). Here, CAPN1 and PLCB2 are two novel markers for AD-PD crosstalk whose roles are still illusive in both diseases, although they are known to play some role in Alzheimer's disease.

Interestingly, our study has reported GSK3B and PARK2 among the crosstalk markers commonly interacting with Aβ, Tau, and α-Synuclein signifying them as the potential candidates for regulating the pathophysiology of Alzheimer's and Parkinson's disease. Another objective of our study was to investigate the UPS markers crucial for the proteasomal clearance of toxic peptides from neurons. Here, we reported fifteen critical ubiquitin E3 ligases for the clearance of AD-PD crosstalk markers, including STUB1, PARK2, FBXW7, SIAH1, FBXO7, WDTC1, SYVN1, TRAF6, RNF19A, SIAH2, TRIM32, NEDD4, FBXL2, MARCH7, and FBXO45. Interestingly, only PARK2 and STUB1 are found to interact with all the key toxic proteins- Aβ, Tau, and α-Synuclein. It suggested the involvement of PARK2 in both the pathology and clearance biology, i.e. negative and positive role in neurodegenerative disorders like AD and PD. For instance, in the healthy state, HSPs, STUB1 and Parkin are known to play a critical role in refolding or targeting of these neurotoxic proteins to ubiquitin proteasome system for degradation (Yao, 2010), but the expression varies. However, in case of AD, Parkin's level is elevated along with HSPs and STUB1, but in case of PD, loss of parkin has been observed with high HSP and STUB1 level resulting in altered mitophagy and consequent pathologies (Kumar et al., 2012). Altogether, these findings reinstate that Parkin has a dual role, i.e. itself a molecular marker for AD-PD cross talk, and its role in the ubiquitination biology of toxic aggregates. Furthermore, AD-PD cross talk markers and their ubiquitination enzymes were extensively investigated for the precise molecular mechanism of Aβ, Tau, and α-Synuclein ubiquitination. It proposed the members from different classes of ubiquitination enzymes associated with the protein clearance in humans, including UbE1s- SAE1, UBA1/6; UbE2s- BIRC6, UBE2A/B/C/G1/G2/H/J2/K/L3/N/S/T/W/Z; UbE3s- FBXL2, FBXO7/45, FBXW7, NEDD4, PARK2, STUB1, SYVN1, TRIM32, WDTC1; and DUBs- USP7, VCPIP1. Moreover, the AD-PD cross talk markers are mapped on the reactome pathways and analysed for their biological functions in neurons. Based on the current findings, we hypothesized the molecular mechanism of neurotoxic protein aggregation and their proteasomal clearance in AD and PD. Overall, our study findings suggest a crucial role of PARK2 in the pathogenesis and therapeutics of neurodegenerative disorders like Alzheimer's and Parkinson's disease.

5. Conclusion

The formation of misfolded protein aggregates is the key hallmark of many neurodegenerative diseases that trigger the neurotoxicity and consequent proteostatic collapse. In addition, active research is going on to unravel the mechanism of protein folding and aggregation. Here, the distortion of helical conformation into beta-strands/bridges containing fibrils is the active principle for aggregation in amyloid-beta, Tau, and α-synuclein proteins. Moreover, aggregation sequences in $A\beta_{40/42}$: HQKLVFFAEDV (14-24), GAIIGLMVGGVV (29-40)/ HQKLVFFAEDVGS (14-26), GAIIGLMVGGVVI (29-41); Tau: GGG(650-652) and V630/G640 in paired and straight helical filaments, and VQI(I/V)(N/Y)K in amyloid-spines; α-Synuclein: GVVHGVATVAE (47-57) and GAVVTGVTAVA (68-78) was rich in glycine and hydrophobic residues-alanine, valine, isoleucine and phenylalanine. These hydrophobic residues were involved in the intra-chain and inter-chain interactions and reported to interact with other proteins involved in the pathogenesis of AD and PD. Therefore, the elucidation of aggregation sites in these pathological proteins and identification of their interacting partners would enable us to identify novel therapies for multiple disease states. Furthermore, the interactome analysis identified AβPP, CAPN1, GSK3B, LRRK2, MAPT, PARK2, PLCB2, and SNCA as key markers for AD-PD cross talk with GSK3B and PARK2 as common interactor of amyloid-beta, tau, and alpha synuclein. In addition, the identification of ubiquitinational markers, including E3 ligases- PARK2, STUB1, FBXW7, SIAH1, FBXO7, WDTC1, SYVN1, TRAF6, RNF19A, SIAH2, TRIM32, NEDD4, FBXL2, MARCH7, and FBXO45; and the deubiquitinases- ATXN3, USP8, PSMD14, and UCHL1 as direct-regulators of Aβ, Tau and α-synuclein ubiquitination, would help us to devise better therapeutic options for targeting misfolded proteins and large-scale rebalancing of proteostatic network. Moreover, the ubiquitination reaction is a complex biology of interactions among a series of E1-activating, E2-conjugating, E3-ligating and deubiquitinating enzymes that are addressed here for the clearance of AD-PD cross talk markers. Altogether, these key findings can help the scientists to accelerate the identification of novel therapeutic modalities for such age related incurable neurodegenerative pathologies, including AD and PD.

Declaration of Competing Interest

There is no conflict or competing interest declared by the authors.

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ORIGINAL RESEARCH ARTICLE

An In-Silico Investigation of Key Lysine Residues and Their Selection for Clearing off Aβ and Holo-AβPP Through Ubiquitination

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Abstract
Malicious progression of neurodegeneration is a consequence of toxic aggregates of proteins or peptides such as amyloid beta (Aβ) reported in Alzheimer's disease (AD). These aggregates hinder the electrochemical transmission at neuronal junctions and thus deteriorate neuronal-health by triggering dementia. Electrostatic and hydrophobic interactions among amino-acid residues are the governing principle behind the self-assembly of aforesaid noxious oligomers or agglomerate. Interestingly, lysine residues are crucial for these interactions and for facilitating the clearance of toxic metabolites through the ubiquitination process. The mechanisms behind lysine selectivity and modifications of target proteins are very intriguing process and an avenue to explore the clearance of unwanted proteins from neurons. Therefore, it is fascinating for the researchers to investigate the role of key lysine, their selectivity and interactions with other amino acids to clear-off toxic products in exempting the progression of Neurodegenerative disorders (NDDs). Herein, (1) we identified the aggregation prone sequence in Aβ40 and Aβ42 as 'HHQKLVFFAE' and 'SGYEVHHQKLVFFAEDVG/KGAIIGLMVGGV' respectively with critical lysine (K) at 16 and 28 for stabilizing the aggregates; (2) elucidated the interaction pattern of AβPP with other Alzheimer's related proteins BACE1, APOE, SNCA, APBB1, CASP8, NAE1, ADAM10, and PSEN1 to describe the pathophysiology; (3) found APOE as commonly interacting factor between amyloid beta and Tau for governing AD pathogenesis; (4) reported K224, K351, K363, K377, K601, K662, K751, and K763 as potential putative lysine for facilitating AβPP clearance through ubiquitination thereby arresting Aβ formation; and (5) observed conserved glutamine (Q), glutamic acid (E), and alpha-helical conformation as few crucial factors for lysine selectivity in the ubiquitination of AβPP.

Keywords Amyloid beta (Aβ) · Amyloid-beta precursor protein (AβPP) · Alzheimer's disease (AD) · Lysine (K) · Ubiquitin proteasome system (UPS) · Neurodegeneration

1 Introduction

Alzheimer's disease is a problematic state, where human's memory, thinking, and behaviour get affected which account for 60–80% dementia cases. Typically, these symptoms develop leisurely without prior notice of its actual onset and worsen over time to the extent of impeding with day-to-day tasks. Moreover, it has become a sixth leading cause of death in the United States with undefined treatment until date [1]. The researchers are striving for finding the ways to treat, delay, or prevent the onset of this dreadful disease. The prime suspect for the pathogenic events in Alzheimer's disease (AD) is the senile plaque deposition inside the brain. Here, the amyloid-β proteins $A\beta_{40}$ and $A\beta_{42}$ are the building blocks of these senile plaques that are produced by the sequential cleavage of amyloid-beta precursor protein (AβPP) by β-site AβPP-cleaving enzyme (BACE1) and γ-secretase, a multi-subunit PS1/PS2-containing integral membrane protease [2]. These plaques principally block the communications among the neurons and interfere with the proper functioning of brain. However, increased Aβ production is attributed to

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the AβPP gene duplication or base substitutions on AβPP and γ-secretase subunits PS1/PS2, but majority of AD has exhibited Aβ accumulation without these mutations [3]. This observation signifies the role of defective Aβ degradation and clearance in AD pathogenesis. Henceforth, facilitating degradation and clearance of Aβ and AβPP could be the potential approach for alleviating the disease symptoms.

Therefore, ubiquitination plays a cardinal role in the degradation of Aβ/AβPP by the 26S Proteasomal complex with help of its key lysine. The selectivity of pathogenic proteins for degradation is mediated by the specific ubiquitin E3 ligases (E3s) that ubiquitinate the substrate protein with the help of its lysine (K) residues. For example, certain UbE3s such as parkin, mouse double minute-2 homolog (Mdm2), HMG-coA Reductase Degradation 1 (HRD1), carboxy terminus of Hsc70 interacting protein (CHIP), gigaxonin, and neural precursor cell expressed developmentally down-regulated protein 8 (NEDD8) are reported in Alzheimer's disease to facilitate the ubiquitination process [4–8]. The UbE3 attaches ubiquitin (Ub) molecule to the specific lysine residue and marks it as an identification flag for degradation [9]. The ubiquitination pattern of the target protein determines the fate of that protein based on the site of ubiquitination. For instance, K11, K29, K48, and K63 poly ubiquitination (PolyU) triggers proteasomal degradation, K6 PolyU triggers DNA repair, K63 PolyU triggers signal transduction and DNA repair, while mono ubiquitination (MonoU) triggers degradation by endosomal lysosomal pathway [10–14]. Therefore, identification of key lysine in a pathological protein responsible for its UPS-mediated degradation possesses great importance for unraveling the therapeutic avenues for various neurodegenerative diseases. In addition, the roles played by respective E3s in controlling the ubiquitination pattern of pathogenic proteins are critical for their contribution in developing future therapies. Our work has provided the possible insights associated with the ubiquitination of Aβ/AβPP through important lysine residue prediction that could mediate their clearance through proteasomal degradation and also depicted the crucial region in Aβ40/42 responsible for its aggregation. Moreover, this study deduced the interacting partners of AβPP and their interaction network with their roles in the pathogenesis of disease. Another interesting finding is the crucial lysine in amyloid beta, ubiquitin, and AβPP along with their nearby conserved residues and their structural selectivity which could serve as an important factor for lysine selection in the ubiquitination process of toxic proteins.

2 Materials and Methods

2.1 Structural Determination of $A\beta_{40}$ and $A\beta_{42}$

The peptide sequence of Aβ40 and Aβ42 is obtained from the protein data bank (RCSB PDB: http://www.rcsb.org) [15] and processed for the determination of its secondary structure by dictionary of protein secondary structure (DSSP) database [16]. Thereafter, macromolecular structure has been designed and annotated by NGL viewer (http://proteinformatics.charite.de/ngl) [17].

2.2 Prediction of Interacting Partners of Aβ and AβPP

The interacting partners of Aβ/AβPP have been determined by functional protein association networks tool called STRING, online available at https://string-db.org/. Top 30 interacting proteins have been identified with highest confidence at 0.900 score, and the network has been generated without clustering and evidence based upon text mining, experiments, databases, co-expression, neighbourhood, gene fusion, and co-occurrence [18].

2.3 Ubiquitination-Site Predictions for Aβ, AβPP, and Ubiquitin

The ubiquitination sites in $A\beta_{42}$, ubiquitin, and AβPP have been predicted with the help of machine learning tools UbPred (http://www.ubpred.org/) and UbiPred (http://e045.life.nctu.edu.tw/ubipred/). Both are the sequence based prediction method tools to identify the promising ubiquitination sites.

2.3.1 UbPred

The UbPred is a ubiquitination-site predictor tool which functions on the algorithm, namely, random forest algorithm. This tool is programmed on 266 non-redundant collective set of experimentally proved sites for ubiquitination. Moreover, their class-balanced accuracy is tuned up to 72%, while the area assessed under the ROC curve is ~80% [19].

2.3.2 UbiPred

UbiPred is a support vector machine-based tool to identify the potential ubiquitinating sites using an informative physicochemical property mining algorithm with a prediction accuracy of 84.44% [20].

2.4 Lysine Site Conserved Residue Analysis

The lysine site conserved residues were identified by multiple sequence alignments of 21 window size lysine containing sequence at the centre from ubiquitin and AβPP protein using ClustalW MSA tool [21]. The 21 window size sequence has been designed by taking 10 residues at both ends of a lysine residue. Furthermore, the obtained alignment was annotated with the help of Bioedit (sequence alignment editor tool) and the conservation has been identified and shown with color-based shading of sequence identity and sequence similarity [22].

2.5 Determination of Structural Selectivity of Lysine's Ubiquitination

The secondary structure of AβPP has been determined with the help of PSIPRED (protein structure prediction server) available at http://bioinf.cs.ucl.ac.uk/psipred/ [23]. Furthermore, the obtained results were compared with the prediction results of the lysine's ubiquitination information obtained from Ub-site prediction tools—UbPred and UbiPred. The corresponding secondary structure, i.e., alpha helix, beta sheet, and turn/loop, has been correlated with its Ub-informative sites, and comparative structural selectivity has been determined.

3 Results and Discussion

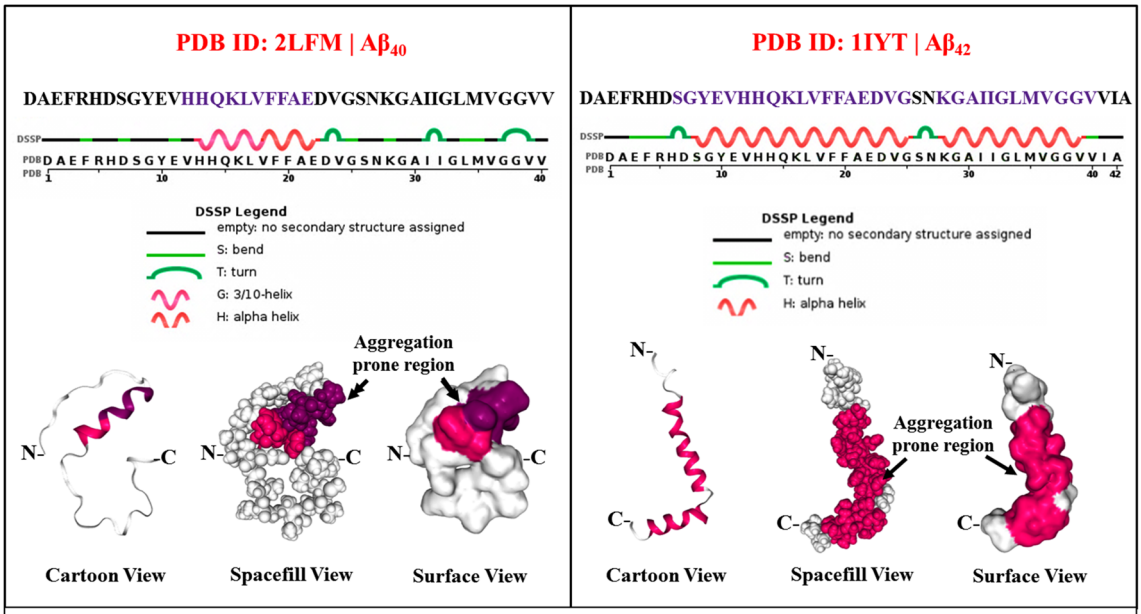
3.1 Lysine Residues K_{16} and K_{28} in Aβ Aggregation

The Amyloid beta 40 and 42 were analysed for their aggregation sites responsible for fibril formation with the help of the secondary structure prediction tool DSSP. The obtained results have identified one sequence motif HHQKLVFFAE in Aβ40 and two sequence motifs SGYEVHHQKLVFFAEDVG and KGAIIGLMVGGV in Aβ42. The obtained motif is found to code for both 3–10 helix and alpha helix in Aβ40 while only for alpha helix in Aβ42 (Fig. 1a). Interestingly, these motifs are spanned with lysine residues, namely, K16 in Aβ40, while K16 and K28 in Aβ42. These lysine residues are crucial for imparting self-assembling property to the Aβ Sequence via stabilizing the aggregates through their inherent potential to have salt bridges, hydrogen bonding, electrostatic, and hydrophobic interactions. Sinha et al. have also reported the role of lysine residues (K16) in Aβ folding; assembly, and toxicity [24]. In this connection, NMR studies have shown the contribution of hydrophobic interaction and salt bridges in imparting the stability to the beta sheets and turns in amyloid-beta folding and assembly [25, 26]. Therefore, disrupting these interactions of Lys via ubiquitination could not only perturb its assembly, but also provide an avenue to clear the burden of toxic proteins in the cell.

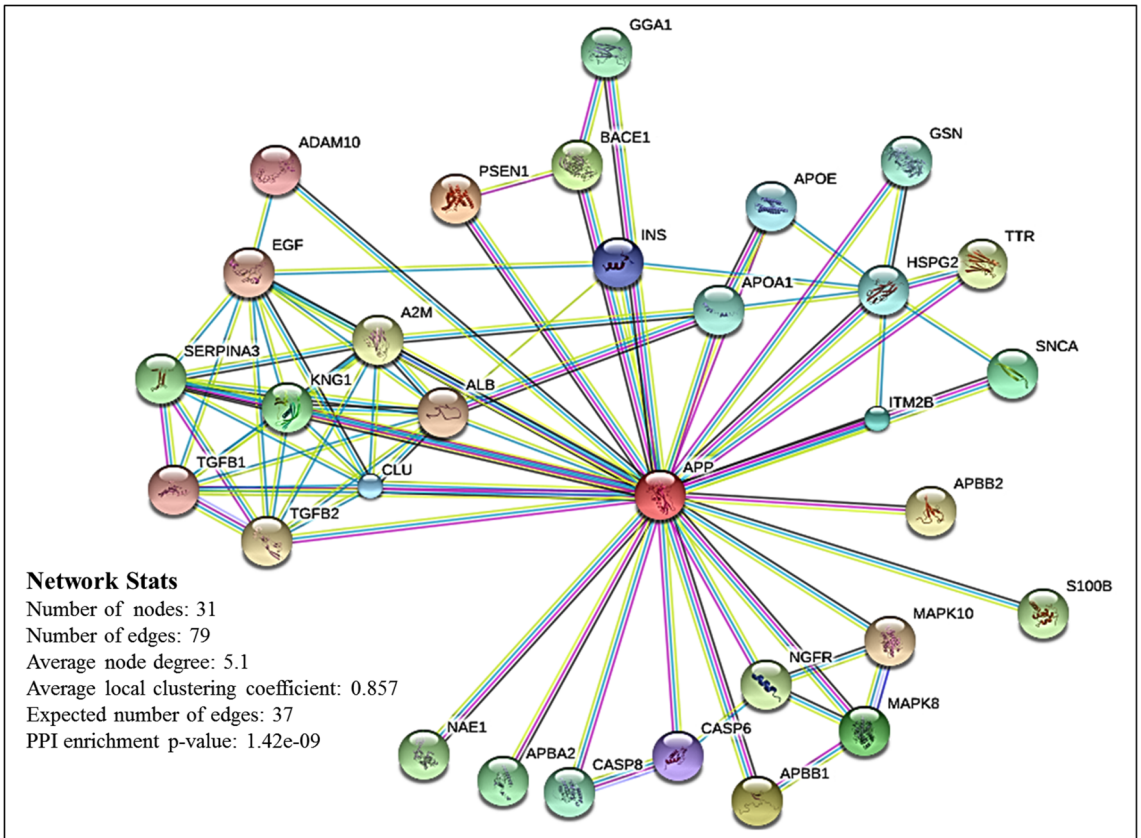
Moreover, secondary structural analysis results revealed 25% helical (2 helices; 10 residues) regions in Aβ40 and 71% helical (2 helices; 30 residues) regions in Aβ42 with rest as coils. The larger helical content in Aβ42 and additional hydrophobic residues at its C-terminal contribute towards its higher aggregation as reported in familial AD patients and imparts towards toxicity to the neurons [27, 28]. Interestingly, the participation of K28 only in the aggregation of Aβ42 as proposed by our results also corresponds to the results of Vandersteen et al., who observed higher oligomer accumulation in Aβ42 peptides than in Aβ40 [29].

3.2 Functional Partners of AβPP with APOE as Common Interactor of Aβ and TAU

The functional interaction network of amyloid-beta precursor protein is mined to understand the pathophysiology of AD which has been shown in Fig. 1b. The network revealed 30 potential interacting partners with 9 promising AD expressed proteins, i.e., BACE1, APOE, SNCA, APBB1, CASP8, NAE1, ADAM10, PSEN1, and AβPP. These proteins are reported to catalyze the formation of amyloid beta and trigger signaling pathways to activate the defense mechanisms against toxic agglomerate. Further network analysis identified 9 Aβ interacting proteins, including BACE1, APOE, APOA1, ITM2B, APBB2, NGFR, APBB1, APBA2, and TGFB2 along with three Tau-binding proteins, i.e., APOE, SNCA, and S100B playing a crucial role in the pathology of Alzheimer's disease. The clustering analysis of the network revealed the proteins to be involved in various cellular processes like cell activation, extracellular matrix organization, exocytosis, platelet activation, and degranulation with Aβ and Tau-binding molecular function. Moreover, the comprehensive analysis of AβPP interacting proteins identified APOE as a commonly interacting protein binding with both Aβ and Tau, screening it as a key target for future therapies. Furthermore, the detailed functional roles of interacting protein in the pathophysiology of disease progression with their interaction scores are summarized in Table 1 and the AβPP interacting partners identified by us were also consistent with the results obtained by Perreau et al., about interaction network of amyloid-beta precursor protein [30].


PDB ID: 2LFM | Aβ40
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
DSSP
PDB
DSSP Legend
empty: no secondary structure assigned
S: bend
T: turn
G: 3/10-helix
H: alpha helix
Aggregation prone region
N-
-C
Cartoon View
Spacefill View
Surface View
PDB ID: 1IYT | Aβ42
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
DSSP Legend
empty: no secondary structure assigned
S: bend
T: turn
H: alpha helix
C-
Aggregation prone region
Cartoon View
Spacefill View
Surface View

(a)


GGA1
ADAM10
BACE1
GSN
PSEN1
APOE
EGF
INS
TTR
HSPG2
APOA1
A2M
SERPINA3
SNCA
KNG1
ALB
ITM2B
TGFB1
CLU
APP
APBB2
TGFB2
S100B
MAPK10
NGFR
MAPK8
NAE1
CASP6
APBA2
CASP8
APBB1
Network Stats
Number of nodes: 31
Number of edges: 79
Average node degree: 5.1
Average local clustering coefficient: 0.857
Expected number of edges: 37
PPI enrichment p-value: 1.42e-09

(b)

◂**Fig. 1** **a** Primary sequence of Aβ40 (PDB ID: 2LFM) and Aβ42 (PDB ID: 1IYT) with secondary and tertiary structures: primary sequence is highlighted for the aggregation prone sequence. Secondary structure is shown over the sequence based on DSSP prediction. Tertiary structure is shown in its three views, i.e., cartoon, spacefill, and surface to depict the aggregation prone region responsible for fibril formation, **b** predicted functional partners of amyloid-beta precursor protein (AβPP): *PSEN1* presenilin 1, *APBB1* amyloid-beta (A4) precursor protein binding, family B, member 1, *BACE1* beta-site AβPP-cleaving enzyme 1, *MAPK8* mitogen-activated protein kinase 8, *SNCA* α-synuclein, *ITM2B* integral membrane protein 2B, *CLU* clusterin, *INS* insulin, *CASP6* caspase 6, *ADAM10* ADAM metallopeptidase domain 10, *TGFB1* transforming growth factor, beta 1, *EGF* epidermal growth factor, *ALB* albumin, *MAPK10* mitogen-activated protein kinase 10, *TGFB2* transforming growth factor, beta 2, *APBB2* amyloid-beta (A4) precursor protein binding, family B, member 2, *A2M* alpha-2-macroglobulin, *TTR* transthyretin, *NGFR* nerve growth factor receptor, *S100B* S100 calcium binding protein B, *NAE1* NEDD8 activating enzyme E1 subunit 1, *SERPINA3* serpin peptidase inhibitor, clade A (alpha-1 anti-proteinase, antitrypsin), member 3, *KNG1* kininogen 1, *APBA2* amyloid-beta (A4) precursor protein binding, family A, member 2, *GGA1* golgi-associated, gamma adaptin ear containing, ARF-binding protein 1, *CASP8* caspase 8, *GSN* gelsolin, *APOA1* apolipoprotein A-I, *HSPG2* heparan sulfate proteoglycan 2, *APOE* apolipoprotein E

3.3 Potential Lysine for Triggering Ubiquitination

3.3.1 Amyloid beta 42

Aβ42 peptide sequence has two lysine at positions 16 and 28. The respective ubiquitination-site prediction scores by UbPred and UbiPred machine learning tools were K16: 0.52 and 0.24 while K28: 0.50 and 0.44, respectively. However, computationally, both the tools were failed to identify the significant score for ubiquitin attachment, but comparatively K28 was shown the more probable scores for ubiquitination (Fig. 2a) which corresponds to the structural studies suggesting its involvement in intra- or intermolecular contacts, while K16 exposed to solvent. Interestingly toxicological studies identified greater impact of K16 on Aβ40 toxicity as compared with Aβ42, while K28 has a greater impact on folding and assembly of both Aβ40 and Aβ42 [31, 32].

3.3.2 Ubiquitin

Ubiquitin protein contains 7 lysine at positions 6, 11, 27, 29, 33, 48, and 63. The predicted sites for ubiquitination by UbPred and UbiPred machine learning tools were K27 and K33, and K48 respectively. The significant score obtained for K27 by UbPred is 0.64 and K33, and K48 by UbiPred is 0.53 and 0.83, respectively. Comparatively, K48 is shown to have the highest probable score for ubiquitination site, as shown in Fig. 2b. Moreover, K48 poly ubiquitin chain marks a signal for targeting the proteins towards proteasome degradation. The significant high score for K48 corresponds well for the selection of UbPred and UbiPred machine learning tools for predicting key lysine ubiquitination with higher confidence for proteasome degradation of target protein.

3.3.3 Amyloid-Beta Precursor Protein

AβPP sequence analysis identified 41 lysine residues in total spanned throughout the sequence, where UbPred analysis reported 16 potential sites. Among them, five sites K224, K377, K393, K395, and K438 are with high confidence, eight sites K51, K60, K161, K351, K601, K662, K751, and K763 are with medium confidence, while three sites K155, K363, and K568 are with low confidence. The most significant score obtained was for **K224**, i.e., 0.97 [DTDYADGSED**K**VVEVAEEEEV], while other relevant lysine site scores were K377-0.94, K438-0.89, K395-0.88, K393-0.86, K601-0.81, K662-0.81, K351-0.79, K751-0.74, K60-0.72, K51-0.71, K161-0.70, and K763-0.70 (Fig. 3a). Likewise, 19 potential lysine sites were predicted by UbiPred for ubiquitination, including K103, K224, K351, K363, K377, K401, K428, K429, K495, K496, K503, K510, K521, K522, K601, K662, K670, K751, and K763. Here, the most significant score obtained was for **K351**, i.e., 0.91 [CGSAMSQSLL**K**TTQEPLARDP], while other relevant lysine site scores were K521-0.72, K503-0.71, K601-0.71, K377-0.69, K510-0.69, K363-0.67, K522-0.66, K429-0.61, K495-0.61, K401-0.60, K751-0.59, K428-0.57, K496-0.57, K224-0.56, K662-0.56, K670-0.55, K103-0.54, and K763-0.54 (Fig. 3b). Moreover, the significant sites were neighbored by the glutamine and glutamic acid residues signifying their role in lysine selection. In summary, the ubiquitination site predicted by UbPred and UbiPred identified eight sites in common for AβPP ubiquitination which includes K224, K351, K363, K377, K601, K662, K751, and K763 summarized in Table 2. Since there is very little information available regarding the ubiquitination sites of AβPP, our in-silico analysis could be a stepping stone in providing an avenue for identifying the ubiquitination patterns of AβPP for restricting Aβ production.

3.4 Glutamine and Glutamic Acid: Key Residues Conserved at Lysine Site for Ubiquitination

The functionally predicted ubiquitination sites were scanned for the conserved residues which could be critical for providing lysine selectivity for the ubiquitination process. The multiple sequence analyses of potential ubiquitination

Table 1 Functional role of AβPP interacting proteins in the pathophysiology of disease

S.No.	Protein	Full Name	Length (amino acid)	Molecular Function	Score
1	AβPP	Amyloid beta Precursor protein	770	N-AβPP binds TNFRSF21 triggering caspase activation and degeneration of both neuronal cell bodies (via caspase-3) and axons (via caspase-6)	Input
2	PSEN1	Presenilin 1	467	Catalytic subunit of g-secretase complex that catalyzes the intramembrane cleavage of integral membrane proteins such as Notch receptors and AβPP	0.999
3	APBB1	Amyloid beta (A4) precursor protein-binding, family B, member 1	708	It acts like an adapter protein that forms transcriptionally active complex with g-secretase-derived amyloid β-precursor protein intracellular domain	0.999
4	BACE1	Beta-site AβPP-cleaving enzyme 1	501	Proteolytically process AβPP and cleaves at N-terminus of the Aβ peptide sequence, between 671-672 residues of AβPP to generate soluble AβPP and corresponding cell-associated C-terminal fragment	0.995
5	MAPK8	Mitogen-activated protein kinase 8	427	Involved in various processes such as cell proliferation, differentiation, migration, transformation and programmed cell death	0.985
6	SNCA	α-Synuclein	140	Involved in the regulation of dopamine release and transport and induces fibrillization of microtubule-associated protein tau	0.984
7	ITM2B	Integral membrane protein 2B	266	It has a regulatory role in the processing of AβPP and acts as an inhibitor of Aβ peptide aggregation and fibrils deposition	0.983
8	CLU	Clusterin	449	Functions as extracellular chaperone which prevents aggregation of nonnative proteins and inhibits formation of amyloid fibrils by AβPP, APOC2, B2M, CALCA, CSN3, SNCA and aggregation-prone LYZ variants (in vitro)	0.979
9	INS	Insulin	110	It decreases blood glucose concentration and increases cell permeability to monosaccharides, amino acids and fatty acid	0.976
10	CASP6	Caspase 6	293	Involved in the activation cascade of caspases responsible for apoptosis execution	0.975
11	ADAM10	ADAM metallopeptidase domain 10	748	Responsible for the proteolytic release of several cell-surface proteins, including heparin-binding epidermal growth- like factor, ephrin-A2 and for constitutive and regulated α-secretase cleavage of AβPP	0.972
12	TGFB1	Transforming growth factor, beta 1	390	Multifunctional protein that controls proliferation, differentiation and other functions in many cell types	0.972
13	EGF	Epidermal growth factor	1207	EGF stimulates the growth of various epidermal and epithelial tissues in vivo and in vitro and of some fibroblasts in cell culture	0.97
14	ALB	Albumin	609	It is a main protein of plasma that has a good binding capacity for water, Ca(2+), Na(+), K(+), fatty acids, hormones, bilirubin and drugs. Moreover, it functions for the regulation of the colloidal osmotic pressure of blood	0.969

sites in ubiquitin and AβPP revealed the conservation of uncharged glutamine (Q) and negative-charged glutamic acid (E) (marked with green arrow) in close proximity with the positively charged lysine residue, as shown in Fig. 4. These conserved residues could serve as a ubiquitin-interacting motif important for imparting lysine selectivity for ubiquitin attachment via providing favourable environment, i.e., suitable charge or interacting potential. Further investigations are required to validate their role in the ubiquitination process of AβPP.

3.5 Alpha-Helical Structural Selectivity for Lysine's Ubiquitination

The potential ubiquitination sites were analysed for their structural selectivity for lysine's recognition and ubiquitin attachment that has been summarized in Table 3. The structural incidence of the putative ubiquitination sites revealed the presence of both alpha helix and turn/loop regions. In-depth analysis of both UbPred and UbiPred prediction results showed alpha-helical region in the majority of the ubiquitination sites while turn/loop region in the majority of non-ubiquitination sites. However, the ubiquitination

Table 1 (continued)

15	MAPK10	Mitogen-activated protein kinase 10	464	It is a serine/threonine-protein kinase involved in various processes such as neuronal proliferation, differentiation, migration and programmed cell death	0.968
16	TGFB2	Transforming growth factor, beta 2	442	It is a cytokine which performs many cellular functions especially during embryonic development	0.964
17	APBB2	Amyloid beta (A4) precursor protein-binding, family B, member 2	759	It modulate the internalization of AβPP	0.963
18	A2M	Alpha-2-macroglobulin	1474	It is able to inhibit all four classes of proteinases by a unique 'trapping' mechanism. This protein has a peptide stretch, called 'bait region' which contains specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced in the protein which traps the proteinase	0.961
19	TTR	Transthyretin	147	It is a thyroid hormone-binding protein that transports thyroxine from bloodstream to the brain	0.961
20	NGFR	Nerve growth factor receptor	427	It plays a role in the regulation of GLUT4 translocation to the cell surface in adipocytes and skeletal muscles in response to insulin. It can mediate cell survival as well as cell death of neural cells	0.961
21	S100B	S100 calcium binding protein B	92	It binds and initiates the activation of STK38 by releasing autoinhibitory intramolecular interactions within the kinase and its interaction with AGER after myocardial infarction may play a role in myocyte apoptosis by activating ERK1/2 and p53/TP53 signaling	0.96
22	NAE1	NEDD8 activating enzyme E1 subunit 1	534	Activates NEDD8 and involved in regulating cell death	0.959
23	SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	423	Although its physiological function is unclear, but it is known to inhibit neutrophil cathepsin G and mast cell chymase, both of which can convert angiotensin-1 to the active angiotensin-2	0.958
24	KNG1	Kininogen 1	644	Kininogens are inhibitors of thiol proteases and plays an important role in blood coagulation, influence smooth muscle contraction, induce hypotension, natriuresis and diuresis	0.956
25	APBA2	Amyloid beta (A4) precursor protein-binding, family A, member 2	749	Putative function in synaptic vesicle exocytosis by binding to STXBP1, an essential component of the synaptic vesicle exocytotic machinery and may modulate processing of AβPP and hence formation of Aβ	0.956
26	GGA1	Golgi-associated, gamma adaptin ear containing, ARF binding protein 1	639	It plays a role in protein sorting and trafficking between the trans-Golgi network and endosomes	0.954
27	CASP8	Caspase 8	538	It is an apoptosis-related cysteine peptidase and is the most upstream protease of the activation cascade of caspases responsible for the TNFRSF6/FAS mediated and TNFRSF1A induced cell death	0.954
28	GSN	Gelsolin	782	Calcium-regulated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange (end-blocking or capping) and promote the assembly of monomers into filaments (nucleation)	0.951
29	APOA1	Apolipoprotein A-I	267	Participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase	0.951
30	HSPG2	Heparan sulfate proteoglycan 2	4391	Integral component of basement membranes providing fixed negative electrostatic membrane charge, and thus provides a barrier by both size- and charge-selective. It also serves as an attachment substrate for cells and plays essential roles in vascularization	0.949
31	APOE	Apolipoprotein E	317	Mediates the binding, internalization, and catabolism of lipoprotein particles and serve as a ligand for the LDL (apo B/E) receptor and for the specific apo-E receptor (chylomicron remnant) of hepatic tissues	0.948

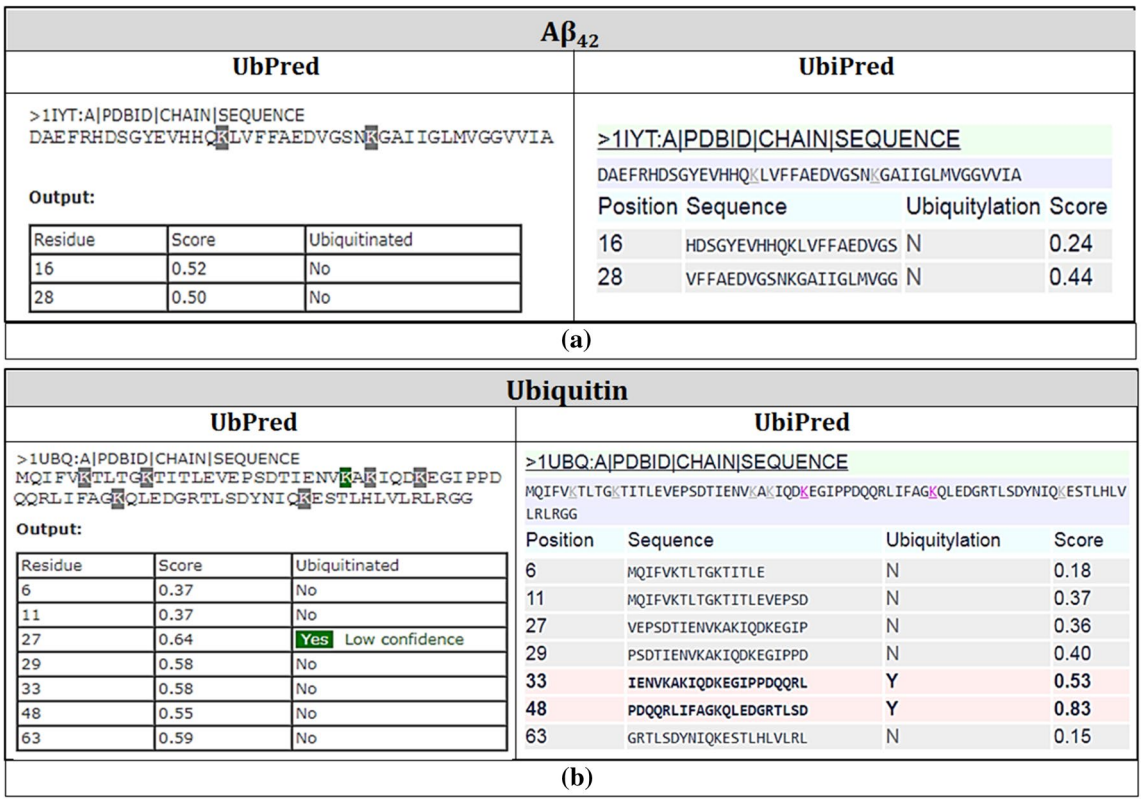


Fig. 2 Ubiquitination-site predictions using UbPred and UbiPred: **a** $A\beta_{42,}$ **b** ubiquitin: there are two lysine residues in Aβ at positions 16 and 28 with a confidence score of 0.52 and 0.50 by UbPred while 0.24 and 0.44 by UbiPred, respectively. Likewise, among seven lysine residues in ubiquitin (6, 11, 27, 29, 33, 48, 63) UbPred identified K27 (green) with confidence score 0.64, while UbiPred detected K33 and K48 (pink) with a confidence score of 0.53 and 0.83, respectively

Table 2 Key lysine sites commonly predicted by UbPred and UbiPred for ubiquitination

Substrate protein	Site	Sequence	Ubiquitination status
Amyloid beta precursor Protein (AβPP)	224	DTDYADGSED**K**VVEVAEEEEV	Yes
	351	CGSAMSQSLL**K**TTQEPLARDP	Yes
	363	TQEPLARDPV**K**LPTTAASTPD	Yes
	377	TAASTPDAVD**K**YLETPGDENE	Yes
	601	DALMPSLTET**K**TTVELLPVNG	Yes
	662	TRPGSGLTNI**K**TEEISEVKMD	Yes
	751	AVTPEERHLS**K**MQQNGYENPT	Yes
	763	QQNGYENPTY**K**FFEQMQN	Yes

process was not exactly found to be structural selective, but the results signify the importance of alpha helix in ubiquitination that need further investigations along with the role of ubiquitination in structural detriment to take proteasomal degradation in effect.

Although, the ubiquitination is an intriguing process involving the role of a wide range of factors for the successful clearance of toxic proteins, but the inferred results have revealed the facts to an extent. Based on the obtained results, we can hypothesize the ubiquitination mechanism associated with the clearance of Aβ or AβPP (Fig. 5) that requires further researches for better understanding the ubiquitination process.

4 Conclusions

In spite of intensive research in the pathogenesis of neurodegenerative disorders such as AD, a clear understanding about its treatment remained rudimentary. Undeveloped or unidirectional symptomatic medication approaches showed adverse results in treatment trials.

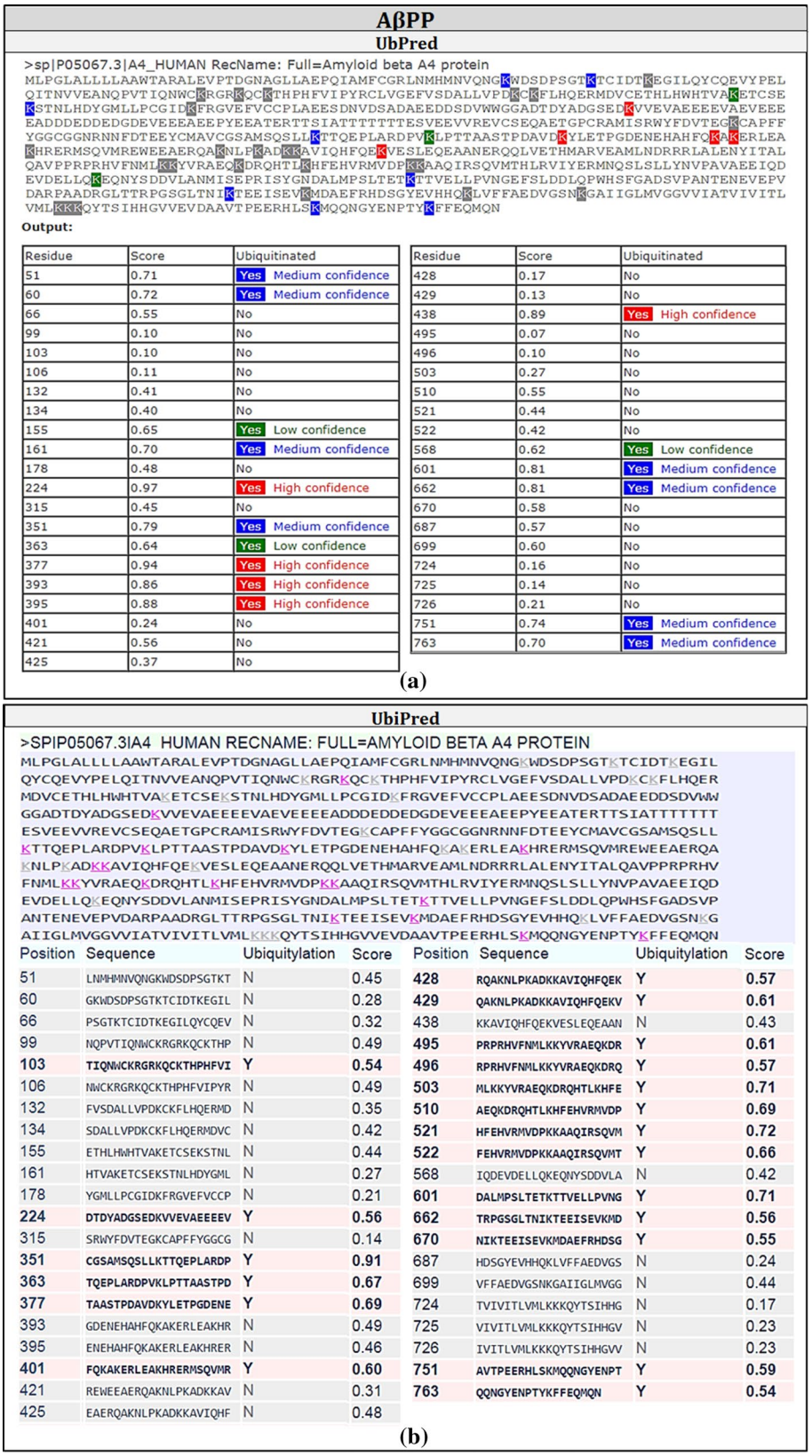


Fig. 3 Ubiquitination sites in AβPP predicted by **a** UbPred, **b** UbiPred: among the 41 lysine residues UbPred identified 16 sites in total including 5 high confidence sites K224, K377, K393, K395, and K438 (red); 8 medium confidence sites K51, K60, K161, K351, K601, K662, K751, and K763 (blue); and 3 low confidence sites K155, K363, and K568 (green), while UbiPred identified 19 potential ubiquitination sites K103, K224, K351, K363, K377, K401, K428, K429, K495, K496, K503, K510, K521, K522, K601, K662, K670, K751, and K763 (pink)

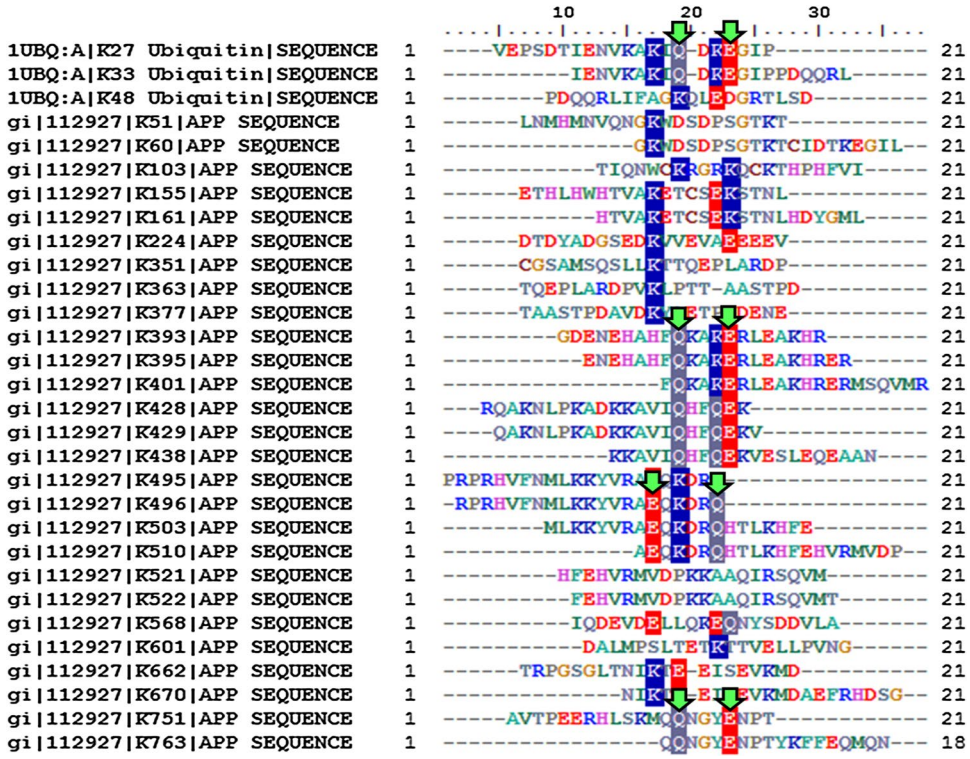


Fig. 4 Multiple sequence alignment of 21 window size lysine sites of ubiquitin and AβPP predicted by UbPred and UbiPred: the sequence conservation analysis revealed the conservation of an uncharged amino-acid glutamine (Q; grey) and a negatively-charged amino-acid glutamic acid (E; red) marked with green arrow in the close proximity of positively charged amino-acid lysine (K; blue) imparting overall neutrality to the site of ubiquitination as a basis of lysine selectivity for ubiquitination

New approaches that can target it at the molecular level could be the best possible method to control the disease progression. Such an effective method is the triggering of ubiquitination, i.e., self-defense mechanism of cell to overcome the protein burden inside it. Therefore, the molecular-level understanding of Aβ or AβPP ubiquitination is necessary for developing the prospective therapeutic agents to address the clearance of such toxic proteins or toxin progenitor proteins for ameliorating the neurodegenerative diseases like AD. Here, we identified the highly potential lysine residues for facilitating the ubiquitination process involving K27, K33, and K48 in ubiquitin, while K224, K351, K363, K377, K601, K662, K751, and K763 in AβPP. Moreover, the aggregation prone region in Aβ40 and Aβ42 was identified to be HHQKLVFFAE and SGYEVHHQKLVFFAEDVG/KGAIIGLMVGGV, respectively. However, significant scores were not obtained for K16 and K28 in Aβ, but both lysines were crucial for aggregation and clearance through ubiquitination that requires further studies. Further interaction studies identified 9 AD-related proteins AβPP, BACE1, APOE, SNCA, APBB1, CASP8, NAE1, ADAM10, and PSEN1 along with 9 Aβ-binding BACE1, APOE, APOA1, ITM2B, APBB2, NGFR, APBB1, APBA2, TGFB2, and 3 Tau-binding proteins APOE, SNCA, and S100B with APOE as commonly interacting partner with Aβ and TAU. Apart from this, we reported glutamine (Q) and glutamic acid (E) as K site conserved residues that could be crucial for lysine selection in ubiquitination. These amino acids overall impart a negative charge in proximity to the positively charged lysine residue and could aid in its selection for ubiquitination by the respective ubiquitin E3 ligase enzymes. Interestingly, lysine responsible for ubiquitination was found to be mostly present in alpha-helical region, signifying ubiquitination's role in structural disruption, while non-ubiquitination lysine residues were in turn/loop region. Altogether, the lysine selection and Aβ/AβPP ubiquitination are crucial for addressing the pathogenesis of AD and that has been revealed here to a certain extent. Further researches are required to generate the novel potential therapeutic avenues to treat the malicious progression of AD worldwide.

Table 3 Secondary structures of the non-ubiquitination and ubiquitination sites predicted by UbPred and UbiPred

Non ubiquitinated sites

UbPred sites

Protein	Lysine residue	Ubiquitination	Secondary structure
Ubiquitin	6	No	Beta Strand
Ubiquitin	11	No	Turn/Loop
Ubiquitin	29	No	Alpha Helix
Ubiquitin	33	No	Alpha Helix
Ubiquitin	48	No	Beta Strand
Ubiquitin	63	No	Turn/Loop
AβPP	66	No	Turn/Loop
AβPP	99	No	Turn/Loop
AβPP	103	No	Turn/Loop
AβPP	106	No	Turn/Loop
AβPP	132	No	Turn/Loop
AβPP	134	No	Turn/Loop
AβPP	178	No	Turn/Loop
AβPP	315	No	Turn/Loop
AβPP	401	No	Alpha Helix
AβPP	421	No	Turn/Loop
AβPP	425	No	Turn/Loop
AβPP	428	No	Alpha Helix
AβPP	429	No	Alpha Helix
AβPP	495	No	Alpha Helix
AβPP	496	No	Alpha Helix
AβPP	503	No	Alpha Helix
AβPP	510	No	Alpha Helix
AβPP	521	No	Alpha Helix
AβPP	522	No	Alpha Helix
AβPP	670	No	Turn/Loop
AβPP	687	No	Turn/Loop
AβPP	699	No	Turn/Loop
AβPP	724	No	Turn/Loop
AβPP	725	No	Turn/Loop
AβPP	726	No	Turn/Loop

UbiPred sites

Protein	Lysine residue	Ubiquitination	Secondary structure
Ubiquitin	6	No	Beta Strand
Ubiquitin	11	No	Turn/Loop
Ubiquitin	27	No	Alpha Helix
Ubiquitin	29	No	Alpha Helix
Ubiquitin	63	No	Turn/Loop
AβPP	51	No	Turn/Loop
AβPP	60	No	Turn/Loop
AβPP	66	No	Turn/Loop
AβPP	99	No	Turn/Loop
AβPP	106	No	Turn/Loop
AβPP	132	No	Turn/Loop
AβPP	134	No	Turn/Loop
AβPP	155	No	Alpha Helix
AβPP	161	No	Turn/Loop
AβPP	178	No	Turn/Loop
AβPP	315	No	Turn/Loop
AβPP	393	No	Alpha Helix
AβPP	395	No	Alpha Helix
AβPP	421	No	Turn/Loop
AβPP	425	No	Turn/Loop
AβPP	438	No	Alpha Helix
AβPP	568	No	Alpha Helix
AβPP	687	No	Turn/Loop
AβPP	699	No	Turn/Loop
AβPP	724	No	Turn/Loop
AβPP	725	No	Turn/Loop
AβPP	726	No	Turn/Loop

Ubiquitinated sites

UbPred sites

Protein	Lysine residue	Ubiquitination	Secondary structure
Ubiquitin	27	Yes	Alpha Helix
AβPP	51	Yes	Turn/Loop
AβPP	60	Yes	Turn/Loop
AβPP	155	Yes	Alpha Helix
AβPP	161	Yes	Turn/Loop
AβPP	224	Yes	Alpha Helix
AβPP	351	Yes	Turn/Loop
AβPP	363	Yes	Turn/Loop
AβPP	377	Yes	Turn/Loop
AβPP	393	Yes	Alpha Helix
AβPP	395	Yes	Alpha Helix
AβPP	438	Yes	Alpha Helix
AβPP	568	Yes	Alpha Helix
AβPP	601	Yes	Turn/Loop
AβPP	662	Yes	Turn/Loop
AβPP	751	Yes	Alpha Helix
AβPP	763	Yes	Alpha Helix

UbiPred sites

Protein	Lysine residue	Ubiquitination	Secondary structure
Ubiquitin	33	Yes	Alpha Helix
Ubiquitin	48	Yes	Beta Strand
AβPP	103	Yes	Turn/Loop
AβPP	224	Yes	Alpha Helix
AβPP	351	Yes	Turn/Loop
AβPP	363	Yes	Turn/Loop
AβPP	377	Yes	Turn/Loop
AβPP	401	Yes	Alpha Helix
AβPP	428	Yes	Alpha Helix
AβPP	429	Yes	Alpha Helix
AβPP	495	Yes	Alpha Helix
AβPP	496	Yes	Alpha Helix
AβPP	503	Yes	Alpha Helix
AβPP	510	Yes	Alpha Helix
AβPP	521	Yes	Alpha Helix
AβPP	522	Yes	Alpha Helix
AβPP	601	Yes	Turn/Loop
AβPP	662	Yes	Turn/Loop
AβPP	670	Yes	Turn/Loop
AβPP	751	Yes	Alpha Helix
AβPP	763	Yes	Alpha Helix

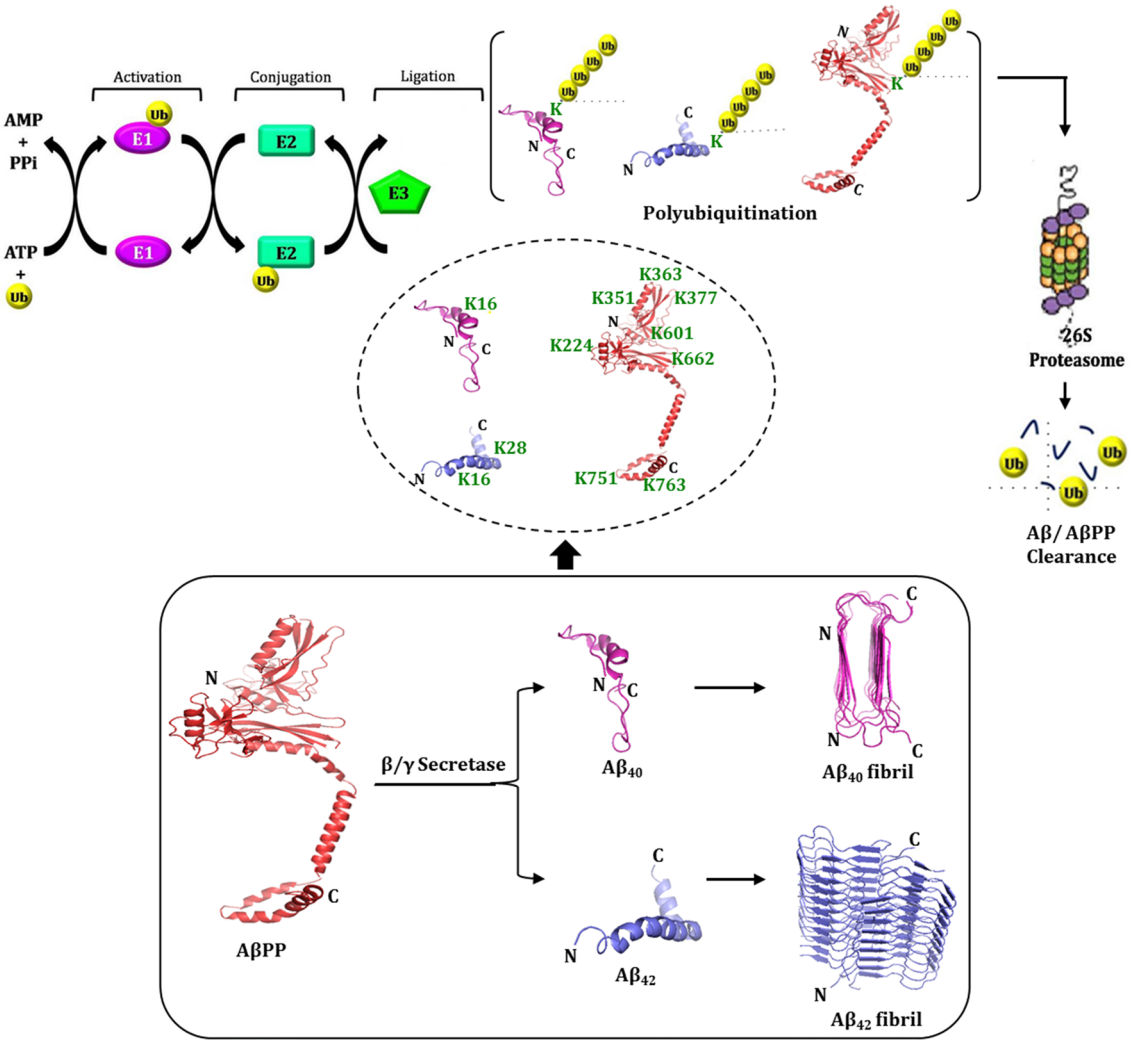


Fig. 5 Amyloid-beta clearance mechanism: AβPP is enzymatically cleaved by the β- and γ-secretases to form soluble Aβ monomers that assembles to form oligomers, fibrils, and consequent plaques, which are further processed via the ubiquitin proteasome system with help of selective lysine residue. In ubiquitination, E1 ubiquitin activating enzyme triggers the ubiquitin molecules and E2 conjugating enzyme attach this activated ubiquitin either directly to the substrate protein with the help of ubiquitin E3 ligase or to the substrate through conjugation with ubiquitin E3 ligase itself at specific lysine K16 for $A\beta_{40}$ or K16/K28 for $A\beta_{42}$ or K224/K351/K363/K377/K601/K662/K751/K763 for AβPP. Thus, poly-ubiquitinated substrate, e.g., $A\beta_{40/42}$ or AβPP, is processed for proteolytic degradation by 26S proteasome to clear the toxic Aβ plaques

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Compliance with Ethical Standards

Conflict of interest There is no conflict or competing interest declared by the authors.

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Role of Wnt-p53-Nox Signaling Pathway in Cancer Development and Progression

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Authors' contributions

This work was carried out in collaboration between all authors. The concept and idea behind this paper has been conceived by author RKA. Data and literature review is done by authors DK, SS and SV. The artwork has been carried out by authors DK and PK. Paper is written by author RKA. Final editing is done by all the authors. All authors read and approved the final manuscript

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Review Article

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ABSTRACT

Signaling pathways play an intricate role in regulating the homeostasis of a normal cell and any chronically altered activity in such signaling pathways causes cancer. Such aberrantly activated Wnt and vanished p53 signaling contribute to the development of various carcinomas. Majority of cancer cells exhibit elevated production of reactive oxygen species (ROS) in an NADPH oxidases dependent manner that further enhances cellular damage. However, Nox family enzymes regulate various physiological functions; for instance, gene regulation, cellular signaling, host defense and cell differentiation. All of these processes get affected in cancer thereby signifying the role of Nox in controlling various signaling pathways such as Wnt and p53. Therefore, unraveling of complex signaling pathways underlying tumorigenesis is enforcing the development of next-generation anticancer drugs directed against specific molecular targets.
This review provides an insight about Nox in regulation of Wnt and p53 pathway to govern the pathogenesis of cancer. Therefore, implementation of NOX inhibitors for inhibiting aberrant Wnt and p53 signaling could provide novel opportunities for therapeutic intervention.

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REVIEW

Molecular Signalling Saga in Tumour Biology

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ABSTRACT

'Cancer' is a broad term used for malignant tumours that has spread throughout the body. The initiation and progression of tumour is driven by complex signaling pathway that can be majorly categorized as oncogenic signal and anti-oncogenic signal. The development of tumour is regulated by abnormal oncogenic signal. The therapy of tumour is designed via targeting the blockage of oncogenic signal or activation of anti-oncogenic signal. This review is an illustration of different signaling pathway involved in tumour biology and the timeline historical review of progress made in the field of tumour biology.

Key words: Cancer; Tumour; Signalling in Tumour; Molecular Basis of Tumour development

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INTRODUCTION

1. WHAT IS CANCER?

'Cancer' is a broad term used to describe a group of more than hundred diseases characterized by abnormal cells giving rise to a lump or mass of tissue called as Tumor or Neoplasm. The term 'carcinos' or 'carcinoma' was coined by Hippocrates (Father of Medicine) to describe cancer. Development of cancer is a result of persistent unregulated cell proliferation in the body of affected person[1]. Here cells do not respond properly to the signals corresponding to normal cell behavior and surpass the 'programmed cell death' which involves self destruction of damaged and worn out cells or when a cell no longer continues to function under the restraints of the cell cycle and proliferate continuously[2]. As a consequence tumor formation takes place which can be categorized as Benign or Malignant depending on the invasion potential of a cell (Figure 1). When a tumor is restricted to one place showing limited growth then it is termed as Benign but if the tumor starts to invade nearby tissues via lymphatic system or circulatory system (blood and lymph) then the tumor is said to be malignant and the process is termed as Metastasis[3]. The subsequent loss of growth control is the ultimate result of accumulated aberrations in numerous cell regulatory systems that affects cell behavior and discriminate cancer cells from normal cells.

2. TYPES OF CANCER

There are more than hundred distinct types of cancer based on the originating cell types. The affected cells differ widely in their response and behavior towards the applied treatments. Any anomalous cell proliferation is a tumor that can be benign or malignant as discussed above. However, both types of tumor possess characteristic feature of unregulated cell proliferation but only malignant tumors are designated as cancer as it is much more dangerous than benign tumors. It is tough to treat malignant tumors

Invited review for BBA-Molecular Basis of Diseases

Re-expression of cell cycle markers in aged neurons and muscles: Whether cells should divide or die?

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Abstract

Emerging evidence revealed that abrogated cell cycle entry into highly differentiated mature neurons and muscles is having detrimental consequences in response to cell cycle checkpoints disruption, altered signaling cascades, pathophysiological and external stimuli, for instance, Aβ, Parkin, p-tau, α-synuclein, impairment in TRK, Akt/GSK3β, MAPK/Hsp90, and oxidative stress. These factors, reinitiate undesired cell division by triggering new DNA synthesis, replication, and thus exquisitely forced mature cell to enter intoa disturbed and vulnerable state that often leads to death as reported in many neuro-and myodegenerative disorders. A pertinent question arises how to reverse this unwanted pathophysiological phenomenon is attributed to the usage of cell cycle inhibitors to prevent the degradation of crucial cell cycle arresting proteins, cyclin inhibitors, chaperones and E3 ligases. Herein, we identified the major culprits behind the forceful cell cycle re-entry, elucidated the cyclin re-expression based on disturbed signaling mechanisms in neuromuscular degeneration together with plausible therapeutic strategies.

CHAPTER THREE

Ion Channels in Neurological Disorders

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Contents

Abstract

The convergent endeavors of the neuroscientist to establish a link between clinical neurology, genetics, loss of function of an important protein, and channelopathies behind neurological disorders are quite intriguing. Growing evidence reveals the impact of ion channels dysfunctioning in neurodegenerative disorders (NDDs). Many neurological/neuromuscular disorders, viz, Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis, and age-related disorders are caused due to altered function or mutation in ion channels. To maintain cell homeostasis, ion channels are playing a crucial role which is a large transmembrane protein. Further, these channels are important as it determines the membrane potential and playing

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Chapter 6
Epigenesis in Colorectal Cancer: A Lethal Change in the Cell

Rashmi K. Ambasta, Dhiraj Kumar, Piyush Sawhney, Rajat Gupta, Parul Yadav, Pooja Pabari, and Pravir Kumar

6.1 Introduction

Colorectal cancer (CRC) is a heterogeneous disease characterized by progressive aggregation of genetic mutations and epigenetic alterations of the genes involved in cell cycle regulation and cell differentiation [1]. These alterations provide growth advantage for clonal expansion of these altered colons epithelial cells to transform into colon adenocarcinomas. Colorectal cancer arises as a polyp outgrowth, called an adenoma, in the colon and/or rectum lining and undergoes a malignant transformation to cause cancer [2]. It has been widely observed that colorectal cancer is initiated due to dysfunction in the signaling elements of Wingless/Wnt-signaling pathway resulting in either activation of oncogenes or silencing of tumor suppressor genes [3]. About 70–85 % of colorectal cancers are sporadic in nature, i.e. arise from somatic gene alterations. However, the heritable colorectal cancers originating from germline mutations are either familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer [4, 5]. Multiple molecular pathways have been identified for the development of colorectal cancers (CRCs) that comprised of both mutations and epigenetic alterations. For instance, tubular adenomas mostly arise in response

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Review paper

Can luteolin be a therapeutic molecule for both colon cancer and diabetes?

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Abstract

Diabetes and colon cancer are the leading cause of mortality worldwide. According to World Health Organization, the number of patients with diabetes and cancer is going to be elevated by 50% in 2020. However, several flavonoids have been known to be useful in reducing the chance of cancer/diabetes but the hunt of a single biomolecule that can act as therapeutic and preventive molecules for future epidemic continues. In this review, we aim to perform an illustration of all researches done that target molecular signaling using luteolin in cancer/diabetes and predicted target protein using PharmMapper. The search confirms that luteolin can be a remedial molecule for both cancer and diabetes via acting on variety of signaling pathway. Furthermore, we also intend to illustrate/compare the predicted and verified molecular modes of action of luteolin. Fluorescence in situ hybridization analysis confirms the expression of CCND1 in colon cancer while immunofluorescence analysis confirms the CDK4 in diabetes. Finally, an effort has been made to map docking of marker protein-luteolin at a particular site using docking software. This review gives a holistic overview about luteolin as a therapeutic molecule for cancer/diabetes via acting on multiple signaling cascade such as p53, Wnt, eNOS, iNOS, SOD and MMP9, with especial emphasis on the cyclin-CDK pathway. Altogether, the review concludes that luteolin can be a molecule for the therapy of both cancer and diabetes by acting on broad signaling pathway.

Key words: luteolin; colon cancer; diabetes; therapeutic biomolecule; CCND1; CDK4

Background

Diabetes and colon cancer are the two common diseases resulting in mortality worldwide. According to World Health Organization, diabetes is going to affect half of the United States in 2020, contributing to epidemic by 2030. The cancer survey also highlights the dramatic increase in colorectal cancer cases by 2030. There are several flavonoids which are known to have beneficial effect in treating colon cancer [1–3]. In reports, luteolin is a bioflavonoid that possesses antioxidant, anti-inflammatory, anti-angiogenic and anti-proliferative effects [4, 5]. Luteolin, 3′, 4′, 5, 7-tetrahydroxyflavone, is a flavonoid existing in many plant products and imbibing therapeutic potential for cancer and diabetes. Luteolin can induce apoptosis and inhibit cell proliferation, metastasis and angiogenesis for its anti-cancerous property while luteolin can be anti-diabetic because of its antioxidant property as persistent hyperglycemia generates an intracellular level of reactive oxygen species (ROS). Luteolin may contribute

Rashmi K. Ambasta (R.K.A.) is the Council of Scientific and Industrial Research (CSIR) scientific pool officer (under Scientist Pool Scheme) at the Department of Biotechnology, Delhi Technological University (DTU). R.K.A. conceptualized and collected relevant information and result for writing the manuscript. R.K.A. received funding for the CSIR for the project as principal investigator (PI). R.K.A. also received the funding from Science and Engineering Research Board for Young Scientist project as PI and contributed substantially for the review article.
Rohan Gupta (R.G.) is a PhD student at the DTU. R.G. operated and presented the docking part of the manuscript.
Dhiraj Kumar (D.K.) is pursuing PhD at the DTU. D.K. has meticulously drawn the figure on luteolin action on cancer signaling.
Aditi Sarkar (A.S.) and **Saurabh Bhattacharya** (S.B.) have done their PhD from Vellore Institute of Technology, Vellore, India, and they together have toiled on the fluorescence in situ hybridization data execution and compilation of raw data to imaging in the manuscript.
Pravir Kumar (P.K.) is a professor at the Department of Biotechnology and a dean (alumni) at the DTU. P.K. contributed on collection and discussion of data, art work and compiling and writing of the manuscript as senior investigator.

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Review

Stress-Induced Synaptic Dysfunction and Neurotransmitter Release in Alzheimer's Disease: Can Neurotransmitters and Neuromodulators be Potential Therapeutic Targets?

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Abstract. The communication between neurons at synaptic junctions is an intriguing process that monitors the transmission of various electro-chemical signals in the central nervous system. Albeit any aberration in the mechanisms associated with transmission of these signals leads to loss of synaptic contacts in both the neocortex and hippocampus thereby causing insidious cognitive decline and memory dysfunction. Compelling evidence suggests that soluble amyloid-β (Aβ) and hyper-phosphorylated tau serve as toxins in the dysfunction of synaptic plasticity and aberrant neurotransmitter (NT) release at synapses consequently causing a cognitive decline in Alzheimer's disease (AD). Further, an imbalance between excitatory and inhibitory neurotransmission systems induced by impaired redox signaling and altered mitochondrial integrity is also amenable for such abnormalities. Defective NT release at the synaptic junction causes several detrimental effects associated with altered activity of synaptic proteins, transcription factors, Ca^{2+} homeostasis, and other molecules critical for neuronal plasticity. These detrimental effects further disrupt the normal homeostasis of neuronal cells and thereby causing synaptic loss. Moreover, the precise mechanistic role played by impaired NTs and neuromodulators (NMs) and altered redox signaling in synaptic dysfunction remains mysterious, and their possible interlink still needs to be investigated. Therefore, this review elucidates the intricate role played by both defective NTs/NMs and altered redox signaling in synaptopathy. Further, the involvement of numerous pharmacological approaches to compensate neurotransmission imbalance has also been discussed, which may be considered as a potential therapeutic approach in synaptopathy associated with AD.

Keywords: Amyloid-β, neurotransmitters/neuromodulators, redox signaling, synaptic dysfunction, tau, therapeutics

INTRODUCTION

To maintain brain homeostasis, synapses and their associated neurotransmitters (NTs) play the role where synapses are specialized structures that form a network to transmit electrochemical signals

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Linking mitochondrial dysfunction, metabolic syndrome and stress signaling in Neurodegeneration☆

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ABSTRACT

Mounting evidence suggests a link between metabolic syndrome (MetS) such as diabetes, obesity, non-alcoholic fatty liver disease in the progression of Alzheimer's disease (AD), Parkinson's disease (PD) and other neurodegenerative diseases (NDDs). For instance, accumulated Aβ oligomer is enhancing neuronal Ca^{2+} release and neural NO where increased NO level in the brain through post translational modification is modulating the level of insulin production. It has been further confirmed that irrespective of origin; brain insulin resistance triggers a cascade of the neurodegeneration phenomenon which can be aggravated by free reactive oxygen species burden, ER stress, metabolic dysfunction, neuorinflammation, reduced cell survival and altered lipid metabolism. Moreover, several studies confirmed that MetS and diabetic sharing common mechanisms in the progression of AD and NDDs where mitochondrial dynamics playing a critical role. Any mutation in mitochondrial DNA, exposure of environmental toxin, high-calorie intake, homeostasis imbalance, glucolipotoxicity is causative factors for mitochondrial dysfunction. These cumulative pleiotropic burdens in mitochondria leads to insulin resistance, increased ROS production; enhanced stress-related enzymes that is directly linked MetS and diabetes in neurodegeneration. Since, the linkup mechanism between mitochondrial dysfunction and disease phenomenon of both MetS and NDDs is quite intriguing, therefore, it is pertinent for the researchers to identify and implement the therapeutic interventions for targeting MetS and NDDs. Herein, we elucidated the pertinent role of MetS induced mitochondrial dysfunction in neurons and their consequences in NDDs. Further, therapeutic potential of well-known biomolecules and chaperones to target altered mitochondria has been comprehensively documented. This article is part of a Special Issue entitled: Oxidative Stress and Mitochondrial Quality in Diabetes/Obesity and Critical Illness Spectrum of Diseases - edited by P. Hemachandra Reddy.

1. Introduction

For the maintenance of energy metabolism and cellular homeostasis mitochondria is an important organelle which is also known as the power house of cells and predominantly required for determining many cellular functions ranging from metabolic to catabolic activities. Mitochondria performs numerous crucial functions within the cell, which include cellular ATP production, Ca^{2+} buffering, regulation of apoptotic process and involvement in the synthesis of key metabolites. Nevertheless, it also acts as a primary source of endogenous reactive oxygen species (ROS) under oxidative stress. Additionally, mitochondria provides most of the ATP for the metabolic and cellular reaction within the cell, which is mainly coupled with electron transport system (ETS) [1,2]. However, research in the past few decades has recognized various factors, such as mutations in mitochondrial DNA and environmental toxins causing homeostatic imbalances, consequently leading to the damage of normal mitochondrial dynamics. Such alterations include altered mitophagy, decelerated ATP production, disturbed Ca^{2+} homeostasis, reduced mitochondrial membrane potential and compromised mitochondrial respiration [3]. Since, the potential mechanistic role played by altered mitochondria and their associated risk factors in MetS and NDDs remain unsettled, and their possible interlinking is still needed to be investigated. This review extensively covers the involvement of mitochondrial dysfunction in both MetS and neuronal dysfunction. Further, implementation of several biomolecules and chaperones for targeting MetS and NDDs induced by mitochondrial dysfunction has also been elaborated.

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RESEARCH Open Access

Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules

Rashmi K. Ambasta[1,2*], Saurabh Kumar Jha[1,2], Dhiraj Kumar[1], Renu Sharma[1], Niraj Kumar Jha[1,2] and Pravir Kumar[1,2,3]

Abstract

Background: Angiogenesis is a hallmark feature in the initiation, progression and growth of tumour. There are various factors for promotion of angiogenesis on one hand and on the other hand, biomolecules have been reported to inhibit cancer through anti-angiogenesis mechanism. Biomolecules, for instance, luteolin, lectin and lupeol are known to suppress cancer. This study aims to compare and evaluate the biomolecule(s) like luteolin, lupeol and lectin on CAM assay and HT-29 cell culture to understand the efficacy of these drugs.

Method: The biomolecules have been administered on CAM assay, HT-29 cell culture, cell migration assay. Furthermore, bioinformatics analysis of the identified targets of these biomolecules have been performed.

Result: Luteolin has been found to be better in inhibiting angiogenesis on CAM assay in comparison to lupeol and lectin. In line with this study when biomolecules was administered on cell migration assay via scratch assay method. We provided evidence that Luteolin was again found to be better in inhibiting HT-29 cell migration. In order to identify the target sites of luteolin for inhibition, we used software analysis for identifying the best molecular targets of luteolin. Using software analysis best target protein molecule of these biomolecules have been identified. VEGF was found to be one of the target of luteolin. Studies have found several critical point mutation in VEGF A, B and C. Hence docking analysis of all biomolecules with VEGFR have been performed. Multiple allignment result have shown that the receptors are conserved at the docking site.

Conclusion: Therefore, it can be concluded that luteolin is not only comparatively better in inhibiting blood vessel in CAM assay, HT-29 cell proliferation and cell migration assay rather the domain of VEGFR is conserved to be targeted by luteolin, lupeol and lectin.

Keywords: CAM assay, Flavonoids, HT-29 cell, Anti-angiogenesis, Luteolin, Lupeol, Lectin

Background

Angiogenesis process is regulated by several factors that have a critical role in governing the initiation and progression of tumour. Angiogenic factors such as bFGF, HGF, VEGF, hyluronatelyase, collagenase, MMP supports the formation of new blood vessels. In addition, cell cycle markers, for instance, cyclin A2, Cyclin Dependent Kinase-2, 6 and MAPK1, 14, 10 promote the tumour progression whereas caspase 3 inhibits the tumour progression. Mounting evidence is suggesting the critical role of cyclin inhibitors, and inducers of apoptotic markers in cancer therapy. Furthermore, several biomolecules elicit the anti-cancerous property such as, luteolin, lectin and lupeol but comparative studies in terms of anti-angiogenic activity remain unsettled.

Luteolin is a flavonoid; lupeol is a triterpene and lectin is a protein possessing carbohydrate. Flavonoids are polyphenols that play an important role in defending plant cells against microorganisms, insects, and UV

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Review

Impact of Insulin Degrading Enzyme and Neprilysin in Alzheimer's Disease Biology: Characterization of Putative Cognates for Therapeutic Applications

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Abstract. Alzheimer's disease (AD) is a neurodegenerative process primarily characterized by amyloid-β (Aβ) agglomeration, neuroinflammation, and cognitive dysfunction. The prominent cause for dementia is the deposition of Aβ plaques and tau-neurofibrillary tangles that hamper the neuronal organization and function. Aβ pathology further affects numerous signaling cascades that disturb the neuronal homeostasis. For instance, Aβ deposition is responsible for altered expression of insulin encoding genes that lead to insulin resistance, and thereby affecting insulin signaling pathway and glucose metabolism in the brain. As a result, the common pathology of insulin resistance between Type-2 diabetes mellitus and AD has led AD to be proposed as a form of diabetes and termed 'Type-3 diabetes'. Since accumulation of Aβ is the prominent cause of neuronal toxicity in AD, its clearance is the prime requisite for therapeutic prospects. This purpose is expertly fulfilled by the potential role of Aβ degrading enzymes such as insulin degrading enzyme (IDE) and Neprilysin (NEP). Therefore, their molecular study is important to uncover the proteolytic and regulatory mechanism of Aβ degradation. Herein, (i) *In silico* sequential and structural analysis of IDE and NEP has been performed to identify the molecular entities for proteolytic degradation of Aβ in the AD brain, (ii) to analyze their catalytic site to demonstrate the enzymatic action played by IDE and NEP, (iii) to identify their structural homologues that could behave as putative partners of IDE and NEP with similar catalytic action and (iv) to illustrate various IDE- and NEP-mediated therapeutic approaches and factors for clearing Aβ in AD.

Keywords: Alzheimer's disease, amyloid-β, insulin degrading enzyme, Neprilysin, therapeutics

INTRODUCTION

Alzheimer's disease (AD) is a neurological disorder that is characterized by neuronal death, which is caused by the abnormal burden of amyloid-β (Aβ) in the brain resulting in memory loss and cognitive decline [1–3]. The cognitive collapse in AD occurs due to neuronal dysfunction that is attributable to the extracellular Aβ

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Review

Hypoxia-Induced Signaling Activation in Neurodegenerative Diseases: Targets for New Therapeutic Strategies

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Abstract. For the maintenance of cellular homeostasis and energy metabolism, an uninterrupted supply of oxygen (O_2) is routinely required in the brain. However, under the impaired level of O_2 (hypoxia) or reduced blood flow (ischemia), the tissues are not sufficiently oxygenated, which triggers disruption of cellular homeostasis in the brain. Hypoxia is known to have a notable effect on controlling the expression of proteins involved in a broad range of biological processes varying from energy metabolism, erythropoiesis, angiogenesis, neurogenesis to mitochondrial trafficking and autophagy, thus facilitating neuronal cells to endure in deprived O_2. On the contrary, hypoxia to the brain is a major source of morbidity and mortality in humans culminating in cognitive impairment, gradual muscle weakness, loss of motor activity, speech deficit, and paralysis as well as other pathological consequences. Further, hypoxia resulting in reduced O_2 deliveries to brain tissues is supposed to cause neurodegeneration in both *in vivo* and *in vitro* models. Similarly, chronic exposure to hypoxia has also been reportedly involved in defective vessel formation. Such vascular abnormalities lead to altered blood flow, reduced nutrient delivery, and entry of otherwise restricted infiltrates, thereby limiting O_2 availability to the brain and causing neurological disabilities. Moreover, the precise mechanistic role played by hypoxia in mediating key processes of the brain and alternatively, in triggering pathological signals associated with neurodegeneration remains mysterious. Therefore, this review elucidates the intricate role played by hypoxia in modulating crucial processes of the brain and their severity in neuronal damage. Additionally, the involvement of numerous pharmacological approaches to compensate hypoxia-induced neuronal damage has also been addressed, which may be considered as a potential therapeutic approach in hypoxia-mediated neurodegeneration.

Keywords: Angiogenesis, energy metabolism, hypoxia, neurodegeneration, neurogenesis, therapeutics

INTRODUCTION

The structural and functional integrity of the brain exquisitely depends on a regular supply of O_2. In order to avoid the probable damaging outcomes due to deficient O_2 availability, the brain triggers endogenous adaptive and pro-survival mechanisms—a phenomenon known as brain hypoxic tolerance. Under brain hypoxic tolerance, the brain has an ability to tolerate either acute or chronic hypoxic challenges induced directly by environmental stressors or indirectly by physiological responses. The highly conserved hypoxia-inducible factor (HIF) family of transcription factors and their associated downstream signaling molecules are responsible

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