2
Review of Literature

The correct cumulative function of a network of thousands of cellular proteins, simultaneous degradation, and clearance of aberrant proteins generate a cellular quality control (QC) system in cells. Ubiquitin–proteasome system (UPS) governs the selective intracellular protein degradation in eukaryotic cells [Glickman and Ciechanover, 2002]. Assembly of a polyUbiquitin chain is marked for degradation of various cytosolic and nuclear proteins through UPS. The first step in ubiquitination process is covalent linkage of the small (8.5 kDa) Ubiquitin protein to the target protein. Addition of a Ubiquitin molecule to lysine residues of substrate is a multistep process. Ubiquitination of a protein is catalyzed by three classes of enzymes called Ubiquitin-activating enzyme E1, a small group of Ubiquitin-conjugating enzymes (UBCs) or E2s, and Ubiquitin-ligating enzymes (E3s). There are eight E1 enzymes that have been identified, two of them were characterized in human genome; several E2 enzymes have also been characterized. However, in this complex process, E3 Ubiquitin ligases are the enzymes that majorly determine substrate specificity to govern the ubiquitination process and exist with vast diversity [Pickart, 2001]. Polyubiquitinated proteins are, in general, targeted for degradation through proteasome and eliminated from the cellular system. There are several components of the cellular protein quality control system, among them, E3 Ubiquitin ligases can be kept at special place because they are the molecules that somehow retain the ability to recognize the misfolded proteinaceous species and subsequently ubiquitinate them.

2.1 E3 UBIQUITIN LIGASES AND PROTEIN QUALITY CONTROL MECHANISM

Loss or imbalance in the QC system leads to inability of aberrant protein degradation and chiefly contributes to the molecular pathomechanism of protein associated diseases such as Parkinson's Disease (PD), Alzheimer's Disease (AD), Amyotrophic Lateral Sclerosis (ALS), and polyglutamine-associated neurodegenerative diseases. The main players, providing specificity of aberrant protein degradation in the QC system, are E3 Ubiquitin ligases [Pickart, 2001; Hershko and Ciechanover, 1998]. However, we are far from understanding how these QC E3 Ubiquitin ligases recognize misfolded or aberrant proteins from normal proteins.

There are substantial and growing evidences which make us realize that aberrant protein aggregation leads to selective neuronal vulnerability in various neurodegenerative diseases [Ross and Poirier, 2004]. Some recent clues have appeared suggesting that the crucial cytotoxic role of damaged proteins is aggregation induced by intracellular stress stimuli in cells [Dobson, 2003; Selkoe, 2003]. A detailed understanding of cellular survival mechanism against misfolded aggregation-prone proteins is an interesting challenge for future research. In the following sections, on the basis of available literature, it is discussed that how cells specifically target aberrant protein inclusions for clearance in dense cellular pool and maintain cellular homeostasis. Protein homeostasis is perhaps the primary goal of the protein quality control system (Figure 2.1); achieving this proteastasis, may lead cells to later attain cellular homeostasis. In a cellular system, thousands of proteins are to be maintained in their required orchestrated and dynamic steady state levels for proper cellular functionality.

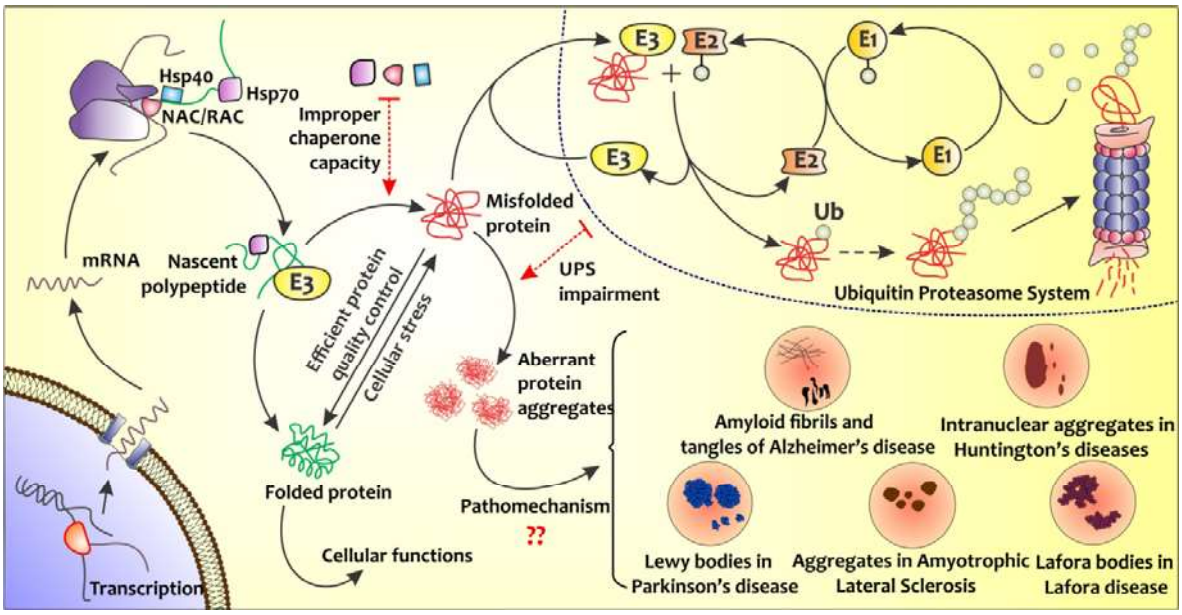

Figure 2.1 : *Cellular and molecular steps of Protein Quality Control Mechanism primarily implicated in various neurodegenerative diseases:* In living cells, DNA transcribed into messenger RNA; this information is translated into polypeptide chains. Ribosome is a large macromolecular cellular machine responsible for the synthesis of nascent polypeptide chains as per information reserved in mRNA transcripts. A newly translated emerging nascent polypeptide chains from the ribosome face a constant risk of misfolding and aggregation. To overcome this problem, molecular chaperones govern immediate protein folding into their native structure for proper cellular functions. This is a challenging task, and lack of chaperone capacity and various cellular insults generate a cumulative imbalance in protein homeostasis, which leads to misfolded protein aggregation in cells. Misfolded protein aggregation is a pivotal hallmark in several neurodegenerative diseases. In living cells, Ubiquitin proteasome pathway is responsible for the intracellular protein degradation. In this pathway, E3 Ubiquitin ligases provide various cellular strategies to select specific substrates or misfolded proteins. Neurons are post-mitotic cells that are more prone towards the aggregation of misfolded proteinaceous structures. Still, the molecular pathomechanism of various neurodegenerative diseases linked with malfunctioned/damaged proteins is not known.

2.2 ENDOPLASMIC RETICULUM STRESS AND E3 UBIQUITIN LIGASES

Endoplasmic reticulum (ER) is an important cellular organelle for the correct folding and post-translational modifications of nascent polypeptides towards their right destiny in crowded milieu of cell. Accumulation of misfolded proteins generates ER stress, which is due to the disturbance in the structure and function of ER in cells and leads to cell death [Paschen and Frandsen, 2001; Breckenridge *et al.,* 2003; Rao *et al.,* 2004]. To protect cells against ER stress, several E3 Ubiquitin ligases actively participate in the clearance of misfolded proteins through endoplasmic reticulum associated degradation (ERAD) pathway. During ER stress exposure, various ER-linked E3 Ubiquitin ligases facilitate degradation of ER-associated misfolded proteins. ERAD is an essential mechanism by which eukaryotes facilitate degradation of abnormal accumulated proteins in ER [Hampton, 2002].

In humans, SMAD-specific E3 Ubiquitin protein ligase 1 (SMURF1) gene encodes Smurf1 E3 Ubiquitin ligase [Zhu *et al.,* 1999]. Recently, it was reported that Smurf1 targets ER-localized Wolfram syndrome protein (WFS1). Mutations in WFS1 gene leads to Wolfram syndrome, an optic atrophy disease. Interaction of Smurf1 with WFS1 proteins promotes its proteasomal degradation. Depletion of Smurf1 endogenous level induces accumulation of WFS1. This finding clearly suggests that Smurf1 promotes ER-associated substrate degradation

and that its endogenous level is induced by ER stress [Guo *et al.,* 2011]. In *C. elegans*, Really Interesting New Gene (RING) finger protein 121 (RNF121) is localized into the ER membrane and retains E3 Ubiquitin ligase activity. Inactivation of RNF121 generates sensitivity against ER stress and induces unfolded protein response (UPR) in cells. Surprisingly, ER stress treatment elevates RNF-121 protein level but not at the mRNA level of RNF-121 [Darom *et al.,* 2010]. Due to ER stress exposure, when the local concentration of misfolded proteins exponentially elevates, the ERQC system releases various ER-associated E3s for clearance of ER-linked misfolded proteins. Membrane-associated ring finger (C3HC4) (MARCH) gene encodes a novel RING finger-type ER-linked E3 Ubiquitin ligase, TBE4. Here are few key E3 Ubiquitin ligases involved in ER quality control.

2.2.1 Gp78

The tumor autocrine motility factor receptor (AMFR), also known as gp78, is a transmembrane glycoprotein from murine melanoma cells and is implicated in tumor invasion and metastasis [Nabi and Raz, 1987; Nabi *et al.,* 1991]. Gp78 is a RING finger domain-dependent Ubiquitin ligase that mainly localizes in ER and is involved in ERAD of several substrates. ER membrane-anchored E3, gp78 specifically recruits murine ortholog of Ubc7p (MmUBC7), a Ubiquitin-conjugating enzyme (E2) through a different region of RING finger domain. Gp78 targets and promotes proteasomal degradation of T cell antigen receptor (TCR) CD3 subunit "CD3-δ", a well characterized ERAD substrate [Fang *et al.,* 2001]. Gp78 specifically promotes the proteasomal degradation of superoxide dismutase-1 (SOD1) and Ataxin-3 proteins, implicated in Familial Amyotrophic Lateral Sclerosis (FALS) and Machado–Joseph disease/spinocerebellar ataxia type 3 neurodegenerative diseases, respectively. Gp78 stimulates mutant SOD1 degradation, and this gp78-mediated ERAD loss of function elevates SOD1 accumulation [Ying *et al.,* 2009]. Earlier, it has been observed that gp78 can target AAT deficiency disease protein Z variant of alpha-1-antitrypsin and normal 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoA reductase) and also promotes self-ubiquitination [Song *et al.,* 2005; Shen *et al.,* 2006]. It can be speculated that gp78 is a major E3 Ubiquitin ligase of ERAD, and thereby play a significant role in cellular protein quality control.

2.2.2 Doa10

Earlier observations have shown that Doa10, a transmembrane protein Ubiquitin ligase governs ERAD function and is located in ER/nuclear envelope (NE). In cells, Ndc10 interacts with intranuclear spindle microtubules and acts as a subunit of DNA binding CBF3 complex [Muller-Reichert *et al.,* 2003]. Doa10 induces degradation of mutant Ndc10-2 kinetochore protein and a mutant NE membrane protein with the help of Ubiquitin-conjugating enzymes, Ubc6 and Ubc7, as well as the Ubc7 cofactor Cue1 in *Saccharomyces cerevisiae* [Kopski and Huffaker, 1997; Ravid *et al.,* 2006]. Human Doa10 ortholog, TEB4 (MARCHVI) is a member of the MARCH family E3 Ubiquitin ligases. It resides in ER and participates in ERAD pathway and also promotes degradation of Type 2 Iodothyronine Deiodinase [Kreft *et al.,* 2006; Zavacki *et al.,* 2009].

2.2.3 HRD1

During translocation, probable mislocalization may occur for a newly synthesized protein molecule from its final site in various cellular compartments. Human E3 Ubiquitin ligase HRD1 influences the degradation of ER-linked two classic ERAD substrates, CD3-δ and TCR-α. It was observed that HRD1 endogenous level is elevated after the treatment with ER stress inducers suggesting that it may be implicated in ERAD pathway [Kikkert *et al.,* 2004]. HRD1 is expressed in brain neurons but not in glia cells [Omura *et al.,* 2006]. This E3 Ubiquitin ligase is expressed against ER stress and generates cellular protective response against ER stress-induced apoptosis [Kaneko *et al.,* 2002; Carvalho *et al.,* 2006; Denic *et al.,* 2006; Ismail and Ng, 2006]. Earlier, it was reported that human HRD1 endogenous levels were changed after ER

stress exposure; probably upon ER stress treatment HRD1 promotes degradation of ERAD-linked substrates and enhances clearance capacity of cell through ERAD process [Kikkert *et al.,* 2004]. Recently, it has been shown that HRD1 induces ubiquitination and degradation of Huntingtin (Htt)-expanded polyglutamine proteins, Parkin-associated endothelin receptor like receptor (Pael-R), and prion protein (PrP) [Apodaca *et al.,* 2006]. HRD1 is involved in the degradation of immature nicastrin and regulates the production of amyloid beta-protein, showing indirect regulation in beta-amyloid levels [Maeda *et al.,* 2009].

2.2.4 Rfp2

Ret finger protein 2 (Rfp2), also known as tripartite motif containing 13 (TRIM13) or LEU5, belongs to RING finger, B-box, coiled coil (RBCC) family of highly conserved group proteins [Lerner *et al.,* 2007]. It acts as a novel RING domain dependent ERAD E3 Ubiquitin ligase and colocalizes with distinct ER-resident proteins, including the T cell receptor subunits CD3-δ and Ubc6. Numerous ER-resident proteins interact with Rfp2, including valosin-containing protein (VCP). Functional interaction of Rfp2 with these ERAD substrates promotes their degradation, *e.g.*, CD3-δ. Earlier studies suggest that E3 Ubiquitin ligases can determine substrate selection specificity in UPS and, on other side, single substrates may be targeted by several different E3s [Amati, 2004; Nishitani *et al.,* 2006]. Most probably to cope against ER stress exposure and to suppress multifactorial toxic effects, E3s overlap in substrate specificity and possibly try to reduce overburden of misfolded proteins in ER.

2.2.5 RMA1

Multiprotein complex initiates ubiquitination of misfolded proteins and promotes degradation through ERAD system. It is a C' terminus membrane-bound novel RING finger E3 Ubiquitin ligase, which is conserved from Arabidopsis to Human. RMA1 promotes ubiquitination of MBP–RMA1 fusion protein, but not free MBP in solution; this observation suggests that MBP recognition by Rma1 is due to its physical vicinity or localization [Matsuda, 2001]. ER membrane-linked RMA1 makes a complex that holds Ubc6e and the transmembrane QC factor Derlin-1 [Lilley and Ploegh, 2004; Ye *et al.,* 2004; Younger *et al.,* 2006]. E2 Ubc6e and RMA1 facilitate proteasomal degradation of cystic fibrosis transmembrane conductance regulator (CFTR) protein. RMA1 protein senses folding defects coincident with translation, while carboxyl terminal Hsp70-interacting protein (CHIP) sense folding defects post-translationally. This study indicates that RMA1 and CHIP sequentially detect folding defects in both normal CFTR and CFTR ΔF508 in ER membrane and cytosol, respectively. This sequential stochastic interaction with various molecules generates a well-defined, highly regulated complex pattern to govern the correct folding of CFTR and promote degradation of CFTR ΔF508 [Younger *et al.,* 2006].

Many of the above mentioned E3 Ubiquitin ligases were interestingly found to be associated in the degradation of several misfolded protein species linked to neurodegeneration through being involved in the protein quality control mechanism.

2.3 OXIDATIVE STRESS AND E3 UBIQUITIN LIGASES

Protein oxidation leads to misfolding and requires higher UPS activity to maintain cellular homeostasis under oxidative insults. Earlier, it has been reported that UPS activity is markedly increased in cells during oxidative stress and recovery states [Shang *et al.,* 1997]. Cells continuously tolerate proteotoxic threats from various kinds of stresses and always try to manage a proper cellular homeostasis. Mainly, intracellular cytosolic misfolded and aggregated proteins are targeted and degraded by UPS [Reinstein and Ciechanover, 2006]. During oxidative stress exposure, cellular proteins suffer from several forms of post-translational protein modifications including oxidation of sulfhydryl groups and oxidation of amino acids residues

[Agarwal and Sohal, 1994]. Oxidation of proteins may affect numerous cellular functions in cell, including deregulated cytoskeleton dynamics, aberrant protein synthesis, impairment in protein degradation, and lack of energy production; and this, finally, leads to apoptosis [Davies, 1987; Starke-Reed and Oliver, 1989; Stadtman, 1992; Stadtman and Levine, 2000; Squier, 2001]. Various post-translational modifications help E3s in signal recognition process. A RING finger E3 Ubiquitin ligase, heme-oxidized IRP2 Ubiquitin ligase-1 (HOIL-1) senses the oxidized form of iron regulatory protein 2 (IPR2). Probably, this function of HOIL-1 contributes to clearance of metabolized oxidized proteins [Iwai, 2003; Yamanaka *et al.,* 2003]. Here are few very important E3 Ubiquitin ligases, those that are directly involved in oxidative stress and are associated with molecular mechanism underlying human diseases.

2.3.1 CHIP E3 Ubuiquitin Ligase

C-terminus of Hsp70 interacting protein (CHIP) joins the two major cellular pathways of protein QC, the UPS and molecular chaperones. U-box domain family member CHIP retains tetratricopeptide repeat (TPR) domains that interact with the Hsp chaperones [Ballinger *et al.,* 1999; Jiang *et al.,* 2001]. Proteasomal inhibition treatment induces colocalization of CHIP with proteasome, and this functional linkage of CHIP with chaperones facilitates the ubiquitination and degradation of chaperone-anchored substrates with the help of proteasome [Connell *et al.,* 2001; Meacham *et al.,* 2001].

E3-Ubiquitin ligase activity of CHIP resides in the U-box domain. During stress conditions, CHIP can also control a chief heat shock transcriptional factor 1 (HSF1); by this function, it can actively contribute in protein QC system [Dai *et al.,* 2003]. During stress conditions, CHIP mainly targets misfolded proteins for proteasomal degradation such as denatured Luciferase protein, expanded polyglutamine proteins, CFTR, and tau [Meacham *et al.,* 2001; Murata *et al.,* 2001; Hatakeyama *et al.,* 2004; Shimura *et al.,* 2004; Jana *et al.,* 2005]. CHIP was earlier thought not to be a stress-induced E3 ligase, but Imai *et al*. have shown that ER stress affects CHIP activity [Imai *et al.,* 2002]. Under oxidative load, CHIP can regulate senescence; a demonstration of CHIP (−/−) mouse fibroblast has been observed with impaired UPS in such condition [Sisoula and Gonos, 2011]. It has also been observed that CHIP induces ubiquitylation of mutant SOD1-associated Hsc/Hsp70 molecules and thus facilitate proteasomal degradation of mutant SOD1 protein, too [Urushitani *et al.,* 2004]. Recently, it was shown that CHIP endogenous levels elevate under various stress conditions to generate an adaptive cellular protective response against various stress conditions [Dikshit and Jana, 2007].

CHIP is one of the highly explored E3 Ubiquitin ligase; its capability of binding with and regulating Hsp70 chaperone and functioning as a cochaperone, makes it a distinguished E3 ligase of protein quality control system. CHIP is also categorized in E4 enzyme.

2.3.2 Keap1–Cul3–Rbx1 E3 Ligase Complex

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor regulates the expression of many antioxidant genes [Chan and Kan, 1999]. Nrf2 is also involved in regulating a group of genes that protect cells against the harmful effects of environmental insults [Nguyen *et al.,* 2009; Leiser and Miller, 2010]. In normal conditions, Nrf2 is regulated by complex cullin-based E3 ligase Keap1(Kelch like ECH-associated protein 1)–Cul3–Rbx1, and Keap1 acts as an adaptor protein that binds to Nrf2. So under normal condition, Nrf2 activity is repressed. Upon oxidative insults, Keap1 activity is inhibited, which activates Nrf2 [Kobayashi *et al.,* 2004]. Recently, the complex has been found to be disrupted in a head and neck squamous cell carcinoma [Martinez *et al.,* 2014].

2.3.3 Park–PINK–PARK7 E3 Ligase Complex

Cumulative function of parkin, PTEN-induced putative kinase-1 (PINK1), and PARK7 most probably generates cellular protective response against oxidative stress. Functional interaction of these three proteins forms a novel E3 complex, which stimulates ubiquitination and proteasomal-mediated degradation of heat stress-stimulated parkin substrates, synphilin-1, and parkin. It has been reported that aberrant PINK1 or mutant parkin lost the proteasomal-dependent degradation ability for both parkin and synphilin-1 [Xiong *et al.,* 2009]. PARK7 gene is ubiquitously expressed and linked to PD, and the end product of this gene is DJ1 protein; mutations in this gene cause early onset of the disease with autosomal recessive inheritance [Bonifati *et al.,* 2003]. Oxidative stress exposure makes DJ1 a more acidic hydroperoxide-responsive protein, suggesting that it may act as an antioxidant protein [Mitsumoto *et al.,* 2001]. DJ-1 generates cellular protective response both in cells as well as in Drosophila against oxidative stress [Taira *et al.,* 2004; Menzies *et al.,* 2005; Meulener *et al.,* 2005]. PINK1 in Drosophila and parkin or DJ-1 inactivation in mouse leads to aberrant mitochondrial function and elevates sensitivity against oxidative stress [Ciechanover and Brundin, 2003; Park *et al.,* 2006; Wang *et al.,* 2006].

2.3.4 Cul2–VHL E3 Ligase Complex

Transcription factor hypoxia-inducible factor 1α (HIF-1α) has an important role in maintaining oxygen homeostasis. HIF-1α regulates various genes involved in reactive oxygen species (ROS) [Iyer *et al.,* 1998]. During normoxic conditions, HIF-1α endures prolyl hydroxylation; this change leads to its identification by Von Happel–Lindau (VHL) protein, a component of complex Cul2–VHL E3 Ubiquitin ligase. Identification of HIF-1α by Cul2–VHL E3 promotes its proteasomal degradation. Hydroxylation of HIF-1α prevents its identification by Cul2–VHL E3 complex, and thus, it results in no degradation of HIF-1α under hypoxic condition [Cockman *et al.,* 2000].

2.4 E3 UBIQUITIN LIGASES IMPLICATED IN NEURODEGENERATION

There are several E3 Ubiquitin ligases that are associated with various misfolded proteins; few of them are however, well characterized as protein quality control E3 Ubiquitin ligases (QCE3s). Following are such QCE3s and their role in neurodegeneration; whereas table 2.1 shows different E3 Ubiquitin ligases reported to be associated with misfolded proteins or similar species which strengthen the possibilities of their role in protein quality control mechanism.

Table 2.1 : A Unified List of Several E3 Ubiquitin Ligases (shaded in green) that Actively Interact or Recruit with Various Misfolded Proteinaceous Bodies and Implicated in Diseases Caused by Protein Aggregation in Neuronal Cells

E3 Ubiquitin Ligase	Aggregated Protein	Aggresome	Misfolded Protein	Amyloid	Inclusion Bodies	References
RNF146						Callow *et al.*, 2011
Parkin						Imai *et al.*, 2000; Choi *et al.*, 2001; Ardley *et al.*, 2003; Bandopadhyay *et al.*, 2005; Burns *et al.*, 2009; Chin *et al.*, 2010, Kawajiri *et al.*, 2010
E6-AP						Mishra *et al.*, 2008; Mishra *et al.*, 2009; Mulherkar *et al.*, 2009
Malin						Mittal *et al.*, 2007; Garyali *et al.*, 2009
Rfp2						Lerner *et al.*, 2007
Dorfin						Niwa *et al.*, 2001; Niwa *et al.*, 2002; Hishikawa *et al.*, 2003; Ishigaki *et al.*, 2007
CHIP						Meacham *et al.*, 2001; Jana *et al.*, 2005; Miller *et al.*, 2005; Kumar *et al.*, 2007; Sha *et al.*, 2009; Löffek *et al.*, 2010
RNF5						Delaunay *et al.*, 2008
Ubr2						Nillegoda *et al.*, 2010
Ubr1						Heck *et al.*, 2010; Nillegoda *et al.*, 2010
Hul5						Fang *et al.*, 2011
NEDL1						Miyazaki *et al.*, 2004
Tul1						Reggiori and Pelham, 2002
BAR						Rong *et al.*, 2011
TRAF6						Zucchelli *et al.*, 2010
Gp78						Ying *et al.*, 2009
San1						Heck *et al.*, 2010, Rosenbaum *et al.*, 2011
Doa10						Wyttenbach *et al.*, 2002, Ravid *et al.*, 2006
HRD1						Kaneko *et al.*, 2002, Kikkert *et al.*, 2004, Carvalho *et al.*, 2006, Denic *et al.*, 2006, Maeda *et al.*, 2009, Burr *et al.*, 2011

2.4.1 Parkin

Parkin is a RING finger E3 Ubiquitin ligase that plays a key role in the molecular pathomechanism of PD [Mizuno *et al.,* 2001]. Overexpression of parkin generates cellular protective response against oxidative stress through reduction in the intracellular load of oxyradicals [Jiang *et al.,* 2004]. Alteration in the cysteine residues of parkin by oxygen radical impairs the function of parkin, and probably, this oxidative stress inactivates parkin and generates misfolded parkin protein [Winklhofer *et al.,* 2003]. In the context of PD, Lewy bodies are proteinaceous cytoplasmic inclusions that are well-characterized hallmark in PD patients [Lowe *et al.,* 1988; Lang and Lozano, 1998]. Parkin E3 Ubiquitin ligase retains in Lewy bodies deposits of fibrous tissue found in patients with PD [Shimura *et al.,* 2000; Ren *et al.,* 2003]. Recently, molecular function of parkin in aggresome-autophagy pathway was reviewed by [Chin *et al.,* 2010]. This report provides evidences of how parkin differentially contributes to both Lys63-linked polyubiquitination aggresome formation and Lys63-linked polyubiquitination autophagy pathways. α-Synuclein gene encodes a presynaptic protein, the

chief constituent of Levy bodies [Spillantini *et al.,* 1998]. Two point mutations (Ala53Thr and Ala30Pro) in α-synuclein gene cause familial autosomal-dominant PD [Polymeropoulos *et al.,* 1997; Krüger *et al.,* 1998]. Several reports demonstrated that overexpression of mutant α-synuclein inhibits proteasomal activity in living cells and significantly induces cell death mediated by mutant α-synuclein protein [Stefanis *et al.,* 2001; Tanaka *et al.,* 2001]. Overexpression of parkin alleviates cell death against toxicity directly linked with proteasome inhibition. Parkin is also involved in the ubiquitination of misfolded proteins derived from ER and protects against neurotoxicity stimulated by unfolded protein stresses [Imai *et al.,* 2000].

2.4.2 Malin

Lafora Disease (LD) is caused by mutations in the protein laforin, encoded by EPM2A gene [Minassian *et al.,* 1998; Serratosa *et al.,* 1999; Ganesh *et al.,* 2000]. A mutated form of NHL repeat containing 1 (NHLRC1), encodes an aberrant malin, and a Ubiquitin ligase may be one of the factors of LD pathogenesis [Chan *et al.,* 2003; Gentry *et al.,* 2005]. Malin is a RING finger Ubiquitin ligase and laforin protein, a dual specificity phosphatase that is recruited towards aggresomes during proteasomal inhibition stress condition [Mittal *et al.,* 2007]. Malin also interacts with laforin and functionally promotes laforin degradation by UPS in cells [Gentry *et al.,* 2005]. Aberrant malin or laforin leads to the accumulation of misfolded proteins, suggesting its involvement in the clearance of misfolded proteins through UPS [Garyali *et al.,* 2009]. It may be possible that functional interaction of malin and laforin together contributes in the pathogenesis of LD [Ganesh *et al.,* 2006]. The presence of Ubiquitin-positive protein aggregates of malin suggests the dysfunction in UPS. Malin–laforin complex together with Hsp70 alleviates the cellular toxicity generated by misfolded proteins, and this functional complex could be targeted as a potential therapeutic strategy against neuronal cytotoxic proteins [Garyali *et al.,* 2009].

2.4.3 E6-AP

An end product of UBE3A gene is a homologous to E6-AP C terminus (HECT) domain E3 Ubiquitin ligase known as E6-associated protein (E6-AP). Mutations in the UBE3A gene or aberrant form of E6-AP protein are considered as a prime factor for Angelman syndrome (AS) mental retardation neuro-developmental disorders. Growing evidences suggest that some E3s are associated with chaperones and directly implicated in cellular QC system including regulation of neurogenesis [Cyr *et al.,* 2002; Stegmuller and Bonni, 2010]. Recently, it has been reported that UBE3A is actively involved in synapse development and also plays an important role in experience dependent synaptic plasticity [Yashiro *et al.,* 2009]. An AS mice model study clearly demonstrates that E6-AP loss of function does not affect normal cellular architecture in the brain but leads to dendritic abnormalities related to shape, size, and density of spines. This study suggests that E6-AP probably contributes to the regulation of spine development and is actively involved in the development of synaptic plasticity [Dindot *et al.,* 2008]. UBE3A promotes the ubiquitination and degradation of synaptic protein Arc and regulates synaptic functions. Loss of function of UBE3A results in the accumulation of Arc synaptic protein in neurons. Accumulated Arc stimulates the excessive internalization of AMPA receptors at synapse and thus, finally, disturbs normal synaptic functions in neurons [Greer *et al.,* 2010]. E6-AP also promotes the degradation of expanded polyglutamine proteins via UPS and suppresses protein aggregation-mediated cellular toxicity [Mishra *et al.,* 2008]. It is also reported that AS possesses PD-like symptoms [Harbord, 2001]. E6-AP was also found to be a component of Lewy bodies linked with PD [Mulherkar *et al.,* 2009]. It has been observed that E6-AP interacts with Hsp70 and promotes the clearance of misfolded proteins anchored by Hsp70 chaperone. Proteasomal inhibition induces recruitment of E6-AP at the site of microtubule organizing center (MTOC) and CFTR aggresomes. Under various cellular insults such as oxidative stress and ER stress, E6-AP endogenous levels are found to be induced [Mishra *et al.,* 2009].

2.5 GLOBAL IMPAIRMENT DUE TO PROTEIN AGGREGATION

Is Hunting of Misfolded Protein by E3 Ubiquitin Ligases a Reward or Cost?

Cells always keep doing their regular and efficient efforts to maintain a proper proteostasis balance via quality control mechanism [Powers *et al.,* 2009]. Nascent polypeptide generation and replacement of old proteins are common events in living cells. In an entire lifespan from nascent to mature state, polypeptide chains always stand with a persistent cytotoxic threat of misfolding and aggregation [Goldberg, 2003]. Under stress conditions, protein misfolding vulnerability and aggregation propensity rise exponentially in cells [Grune *et al.,* 2004]. To avoid such cytotoxic potential, cells continuously try to fold nonnative proteins and degrade unsolved misfolded proteins through UPS [Paul, 2008]. Aggregated proteinaceous structures represent a defective or exhaustive cellular quality control system in the cells. Inefficient degradation leads to overburden of misfolded proteins and finally crosses the refolding or chaperone capacity of a cell. This failure of protein quality control mechanism leads to frequent probability of sequestration of noncomplex polypeptides that are more prone towards aggregation [Dobson, 2003]. We do not know whether recognition of an aberrant protein by E3 Ubiquitin ligases is a real beneficiary challenge or, probably, a mysterious risk. It may be possible that, after recognition, recruitment of E3 Ubiquitin ligases towards the site of aggregation from the site of action unknowingly progresses to another side, *i.e.* ultrasensitive sequestration.

Apart from chaperones and UPS components, several other essential proteins such as transcription factors, *e.g.*, heat shock transcription factor 1, CREB-binding protein (CBP), NF-Y transcriptional factor, tumor suppressor protein p53, TATA-binding protein, specificity protein (Sp1), and transcription initiation factor TFIID subunit 4 (TAFII130), sequester with misfolded proteinaceous species and consequently affect entire cellular proteostasis [Sullivan *et al.,* 2001; Harjes and Wanker, 2003; Yamanaka *et al.,* 2008]. Recently, in a quantitative proteomic analysis, it was observed that numerous metastable proteins, including pre-existing and newly synthesized proteins, sequester with amyloidogenic aggregates. The same study suggests that numerous proteins responsible for various cellular functions interact with amyloid-like aggregates and thereby generate toxicity and successive failure of critical cellular functions [Olzscha *et al.,* 2011].

Under stress conditions, misfolded protein generation load dramatically increases, and due to insufficient chaperone capacity, cells become inable to cope against these disastrous effects. Consequently, misfolded and damaged proteins get actively sequestered in a pericentriolar structure known as aggresomes [Kopito, 2000; Olzmann *et al.,* 2008]. EPM2B gene encodes an end product malin protein which serves as really interesting new gene (RING) finger domain E3 Ubiquitin ligase. Missense mutations in malin were reported with an autosomal recessive neurodegenerative epilepsy disorder. Malin loss of function impairs the degradation of laforin [Gentry *et al.,* 2005]. Recently, it was investigated that ubiquitinated Lafora bodies are co-localized with Hsc/Hsp70 chaperones, 20S proteasome, and mutant malin. Association of mutant malin, chaperones, and proteasome components with Lafora bodies indicates failure of the quality control system and one of the possible reasons of disease progression [Rao *et al.,* 2010]. Mutated form of laforin protein and E3 Ubiquitin ligase malin makes perinuclear-like structures and is nicely co-localized with ribosomes [Singh *et al.,* 2008].

Earlier studies suggested that few E3 Ubiquitin ligases' aberrant function would generate serious imbalance in cellular quality control events [Kahle and Haass, 2004; Lenartowski *et al.,* 2008]. On beneficiary side, E3 Ubiquitin ligases try to solve the problem of aggregation and play a major role in protein quality control mechanism, but in the same pool, absence at the site of origin due to major recruitment with an earlier aggregate possibly leads to accumulation of unattended client proteins. It is not sure, but most probably, those unattended

accumulated substrates are more prone to sequestration with preformed aggregates which are already targeted by respective E3 Ubiquitin ligase. This situation is likely to be aggravated because of the loss of function of E3 Ubiquitin ligase due to sequestration with preformed aggregates. In support of our proposed model, there is another interesting study which states that, like expanded polyglutamine aggregates, chimera GFP170* protein also forms cytoplasmic and nuclear aggregates. GFP170* protein recruits promyelocytic leukemia protein (bodies), chaperones, proteasomal components, SUMO-1, and transcription factors, *e.g.*, CBP and p53 [Fu *et al.,* 2005]. E3 Ubiquitin ligase E6–AP regulates the turnover of expanded polyglutamine, p53 and p27 proteins, and acts as a quality control E3 Ubiquitin ligase [Mishra *et al.,* 2008; Mishra and Jana, 2008; Mishra *et al.,* 2009]. However, earlier reports suggest that various essential cellular proteins get attracted towards misfolded protein inclusions such as vimentin [Johnston *et al.,* 1998], tubulin [Wigley *et al.,* 1999], elongation factor 1 alpha [Mitsui *et al.,* 2002] and Ubiquitin protein [Li *et al.,* 2010].

In support of cellular defense line mechanism, recently, it was shown that a stress response lipid mediator neuroprotection D1 (NPD1) attenuates ataxin-1 poly(Q)-induced proteotoxic stress, and aspirin triggered NPD1 treatment suppresses cerebral ischemic injury [Bazan *et al.,* 2012; Calandria *et al.,* 2012]. It may be possible that stress response-mediated chaperones and other proteins also participate in sequestration process. Still, it is not clear that what typical molecular features or signatures are responsible for discrimination between misfolded protein and native protein.

2.6 MISFOLDED PROTEINS RECOGNITION TACTICS OF E3 UBIQUITIN LIGASES

Possibly, without much disturbing normal cellular homeostasis, cells apply different strategies for identification and degradation of a damaged protein in crowded milieu of cells. E3 Ubiquitin ligases are found to be associated with proteins at various levels starting from their synthesis; this indicates their importance and fidelity of keen targeting. Here is a discussion of various E3 Ubiquitin ligases based on cellular strategies for the clearance of misfolded proteins to avoid interference with normal cellular functions (Figure 2.2).

2.6.1 Conformational Plasticity of Disorder

Eukaryotic cells achieve intracellular degradation through Ubiquitin proteasome system [Glickman and Ciechanover, 2002]. E3s enzymes provide specificity for protein degradation in ubiquitination-based quality control (QC) system. In crowded cellular environment, nearly 30% of the nascent polypeptide chains are misfolded due to posttranslational slips related with folding process [Yewdell *et al.,* 1996; Schubert *et al.,* 2000]. Because disordered proteins tend to form cytotoxic aggregates, cellular quality control system immediately recruits E3 Ubiquitin ligases for clearances of misfolded protein via UPS [Berke and Paulson, 2003], but how these E3 Ubiquitin ligases discriminate between normal and misfolded proteins is not clear. Recently, it was shown that Sir Antagonist 1 (San1) E3 Ubiquitin ligase contributes in both nuclear and cytoplasmic protein quality control mechanisms [Heck *et al.,* 2010; Matsuo *et al.,* 2011]. Mostly, functional proteins are natively organized into three-dimensional structure with well-ordered structural motifs, thus easily accessible for recognition by other interacting proteins, but misfolded proteins lose their native structures and adopt irregular shapes, so how is it possible to recognize and separate them from crowded environment? Interestingly, a recent report suggests that E3 Ubiquitin ligase San1 applies a novel conformational plasticity strategy in the recognition of toxic abnormal proteins. San1 targets misfolded substrates through highly disordered N- and C-terminal regions that retain substrates identification modules [Hampton, 2011; Rosenbaum *et al.,* 2011]. The strategies of such E3 Ubiquitin ligases provide an understanding to their functionality.

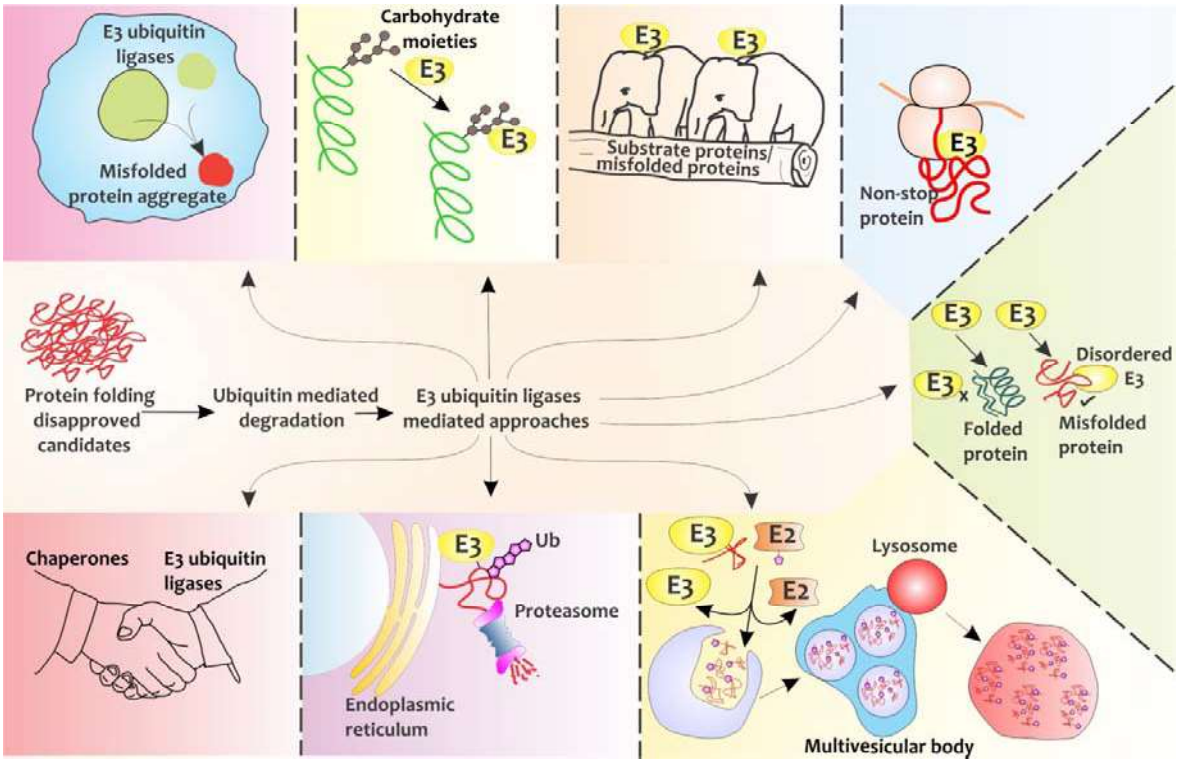

Figure 2.2 : *Proposed diagrammatic representation of comprehend cellular tactics adopted by various E3 Ubiquitin ligases implicated in the clearance of misfolded and other client proteins*. Newly synthesized protein folds into their correct three-dimensional structure and transported into various cellular compartments or extracellular environment for normal cellular functions. Improperly or poorly folded proteins need immediate assistance from chaperones to further fold into their native form. Absence of sufficient chaperone capacity leads to protein misfolding and aggregation. Most likely, E3 Ubiquitin ligases can assist various protein quality control disapproved candidates/client proteins in different manners according to their specificity and subcellular localization. Caption of various E3 Ubiquitin ligases tactics as shown in the figure include the following: (1) conformational plasticity of disorder, (2) ribosomal association of quality control mechanism, (3) harmonious interaction of E3 Ubiquitin ligases with chaperones, (4) disposal of endoplasmic reticulum anchored misfolded proteins, (5) hand to hand coordination, (6) modulated recruitment with misfolded proteins, (7) sugar chains recognition.

2.6.2 Ribosomal Association of Quality Control Mechanism

Protein synthesis cellular machinery, *i.e.*, ribosomes, translates messenger RNA (mRNA) transcript into nascent polypeptide chains. Ribosomes hold about 30 % of entire cell bulk, and approximately 10^5 and 10^6 ribosomes exist in bacterial and mammalian cells, respectively [Bashan and Yonath, 2008]. Polysomes arrange in such a fashion that polypeptide exit faces outward and thereby, probably inhibits unwanted interactions among them [Brandt *et al.,* 2009]. In addition to great arrangement to minimize unfavorable early misfolding and aggregation, cells have evolved ribosomal-associated chaperones, which promote protein folding [Koplin *et al.,* 2010]. In bacteria, trigger factor, the archaeal and eukaryotic nascent polypeptide-associated complex, and particular heat shock proteins serve as ribosomal associated chaperones [Hartl and Hayer-Hartl, 2002; Rauch *et al.,* 2005; Kramer *et al.,* 2009].

Recently, it has been demonstrated that ribosomal-associated Ltn1, a really interesting new gene domain containing E3 Ubiquitin ligase, provides quality control mechanism against newly synthesized nonstop proteins [Bengtson and Joazeiro, 2010]. Recently, it was shown that

mutations in LISTERIN, which is a homolog of Ltn1, cause neurodegeneration in mice and critically involved in embryonic development [Chu *et al.,* 2009]. It was reported that proteasomal subunit 19S coimmunoprecipitates with Ubiquitin ligases [Verma *et al.,* 2000]. *Saccharomyces cerevisiae* Not4 RING finger domain E3 Ubiquitin ligase, involved in transcriptional regulation and transcriptional elongation, has been detectable in polysome fractions [Collart, 2003; Panasenko and Collart, 2012]. These studies provide a clue that protein quality control mechanism and degradation of nascent polypeptide chains are possible during protein synthesis.

2.6.3 Harmonious Interaction of E3 Ubiquitin Ligases with Chaperones

Capacity of refolding of misfolded proteins via chaperones or degradation through E3 Ubiquitin ligases determines the overall efficiency of cellular quality control system in cells [Gao and Hu, 2008]. During stress conditions, cells need extensive protein folding capacity through chaperones to overcome the exponential load of misfolded proteins [Ellis, 1988]. Because all folding attempts are not successful in one go and to avoid unsolicited aggregation of this failure, UPS immediately governs QC E3 Ubiquitin ligases for intracellular degradation of misfolded proteins. Under such improper protein-folding capacity, it is not well known that how chaperones help E3 Ubiquitin ligases in aberrant protein recognition process and make their job easier. Earlier studies and models suggest that the best instantaneously available help is possible through chaperones in this triage process [Lee *et al.,* 1996; Bercovich *et al.,* 1997; Plemper *et al.,* 1997; McClellan and Frydman, 2001]. CHIP (C-terminus of Hsc70-interacting protein) is a tetratricopeptide repeat containing co-chaperone protein, which controls chaperone activities of Hsc70 [Ballinger *et al.,* 1999]. This U-box domain containing E3 Ubiquitin ligase CHIP cooperates with HSP family chaperones and facilitates the ubiquitylation of unfolded proteins [Murata *et al.,* 2001]. Cullin5 RING E3 Ubiquitin ligase interacts with Hsp90 chaperone complex and promotes the degradation of its client proteins, *i.e.*, ErbB2 and HIF1-α [Ehrlich *et al.,* 2009]. Similarly, another homologous to E6-AP C-terminus (HECT) domain E3 Ubiquitin ligase E6-AP also promotes the ubiquitylation of misfolded proteins captured by Hsp70 molecular chaperone [Mishra *et al.,* 2009]. Undoubtedly, these observations clearly indicate that E3 Ubiquitin ligases take help from various chaperones. Most possibly, interaction of various E3 Ubiquitin ligases with chaperones facilitate their misfolded proteins recognition mechanism and these cumulative efforts design an accurate scheme for the clearance of misfolded proteins.

2.6.4 Disposal of Endoplasmic Reticulum-Anchored Misfolded Proteins

Endoplasmic reticulum (ER) is an important cell organelle for the posttranslational modifications of nascent polypeptide chains synthesized by ribosomes. ER membrane-anchored gp78 RING finger domain E3 Ubiquitin ligase is encoded by tumor autocrine motility factor receptor gene. Gp78 targets Ataxin-3 and superoxide dismutase-1 for proteasomal degradation involved in Machado–Joseph disease/spinocerebellar ataxia type 3 and familial amyotrophic lateral sclerosis neurodegenerative disease, respectively [Ying *et al.,* 2009]. ER-associated gp78 E3 Ubiquitin ligase also recognizes CFTRΔF508 and promotes their polyubiquitylation [Morito *et al.,* 2008]. Apart from correct folding of nascent polypeptide chains, to ensure proper function of a protein, ER cellular network helps in the placement of polypeptide chains into their correct subcellular localization. Gene SMAD ubiquitination regulatory factor 1 (Smurf1) encodes Smurf1 E3 Ubiquitin ligase [Zhu *et al.,* 1999]. Smurf1 targets Wolfram syndrome (WFS1) protein for proteasomal degradation at ER, and its endogenous levels are elevated against ER stress condition [Guo *et al.,* 2011]. Misfolded protein aggregation interrupts the normal structure and function of ER and generates ER stress, which consequently leads to cell death governed by proteasome [Xu *et al.,* 2005; Egger *et al.,* 2007]. BAX-induced apoptosis inhibitor, bifunctional apoptosis regulator, is an ER-linked RING finger type E3 Ubiquitin ligase, which is mainly expressed in neurons and protects cell death against various cell death stimuli, *e.g.*, ER stress [Roth *et al.,* 2003]. In the same context, ER-linked E3 Ubiquitin ligase Der3/Hrd1p contains six

transmembrane domains, and membrane topology of Hrd1p is implicated in endoplasmic reticulum-associated degradation (ERAD) pathway [Deak and Wolf, 2001].

Under ER stress conditions, to avoid aggravation of proteotoxicity, cells actively govern ER-associated E3s for the clearance of damaged proteins through endoplasmic reticulum-associated degradation pathway. ERAD-E3 Ubiquitin ligase ret finger protein 2 is a family member of RBCC (RING finger, B-box, and coiled-coil) proteins that associate with valosin-containing protein (VCP), T cell receptor subunit CD3-δ, and Ubc6 [Lerner *et al.,* 2007]. Several E3 Ubiquitin ligases have been found to be involved in mammalian endoplasmic reticulum-associated degradation pathway. RING finger protein 103 Kf-1 is an ER-localized E3 Ubiquitin ligase, and its expression level has been found increased in Alzheimer's disease patient; Kf-1 interacts with Derlin–VCP complex and acts as component of ERAD pathway [Yasojima *et al.,* 1997; Maruyama *et al.,* 2008]. Human ER membrane E3 Ubiquitin ligase TEB4 (MARCH-VI) is an ortholog of Doa10 and induces the degradation of type 2 iodothyronine deiodinase [Kreft *et al.,* 2006; Zavacki *et al.,* 2009]. In conclusion, it can be said that ER serves as a major cytoplasmic compartment for protein quality control.

2.6.5 Hand to Hand Coordination

Deregulation of Ubiquitin proteasome system can lead to intracellular accumulation of damaged or abnormal proteins and consequently leads to neurodegeneration [Shah and Di Napoli, 2007]. To challenge this condition, cells possess highly evolved protein quality control system which is well organized with various quality control E3 Ubiquitin ligases. Interestingly, few studies suggest that cooperation of various E3 ligases among themselves may play a critical role in substrate recognition and, most probably, promotes an efficient degradation via Ubiquitin system. Ubr1 gene encodes UBR box-containing Ubr1 (Ubiquitin-protein ligase E3 component n-recognin 1), a 225-kDa RING finger domain protein [Kwon *et al.,* 1998]. Lack of function of UBR1 Ubiquitin ligase causes mental retardation, Johanson–Blizzard syndrome [Zenker *et al.,* 2005]. Ufd4 is a HECT domain 168-kDa E3 Ubiquitin ligase, joins Ubiquitin carrier 4 (Ubc4) or Ubiquitin carrier 5 (Ubc5) E2 enzymes for functional activity in Ubiquitin-fusion degradation pathway [Johnson *et al.,* 1995; Koegl *et al.,* 1999]. Dual proteolytic pathways co-target Mgt1 O6meG-DNA alkyltransferase protein for polyubiquitylation, mediated by both Ubr1 and Ufd4 E3 Ubiquitin ligases, and this cooperation enhances the yield of polyubiquitylated Mgt1 [Hwang *et al.,* 2009]. Physical and functional interaction complexes of HECT type Ufd4 and RING-type Ubr1 are more effective to produce longer polyUbiquitin chain linked with substrates with their E2 Ubiquitin carrier enzymes Ubc4/Ubc5 and Rad6, respectively [Hwang *et al.,* 2010].

To reduce proteotoxicity, misfolded protein degradation emerges as a cellular adaptability for clearance of unwanted aggregates in cells. Ubr1 and Ubr2 Ubiquitin ligases promote the ubiquitylation of unfolded polypeptides and stimulate the degradation of damaged proteins. Ubr1 specifically promotes the ubiquitylation of damaged/aberrant proteins [Eisele and Wolf, 2008; Nillegoda *et al.,* 2010]. To ensure correct cellular functioning, three-dimensional natively folded protein structures are essential in crowded milieu. E3 Ubiquitin ligase San1 degrades misfolded nuclear proteins, and Ubr1 is involved in "N-end rule" pathway [Varshavsky, 2000; Wilhovsky *et al.,* 2000; Vashist *et al.,* 2001]. Recently, it was shown that both Ubr1 and San1 regulate proteotoxic stress via different approaches for cytoplasmic QC process. San1 needs chaperones function for nuclear delivery of substrates, unlike the Ubr1 that governs chaperones for direct substrates ubiquitination [Heck *et al.,* 2010].

2.6.6 Modulated Recruitment with Misfolded Proteins

Protein misfolding, amyloid fibrils accumulation and aggregation are well-known conformation changes in protein associated neurodegenerative diseases [Ross and Poirier, 2004]. Recent evidence suggests that aggravation of these aberrant proteinaceous species cause neuronal apoptosis [Nakamura and Lipton, 2009]. But how protein misfolding initiates neurodegeneration and what other factors are recruited with those preformed aggregates are not completely known. Earlier studies indicate that the most suspicious interacting proteins are chaperones, a component of UPS, and transcriptional factors [Kubota, 2009]. As it is known that few QC E3 Ubiquitin ligases are dedicated for misfolded protein clearance process, but the reason of recruitment with misfolded protein is not known yet. Still, it is a big question of debate whether QC E3 Ubiquitin ligase recruitment with damaged proteins facilitates their degradation or may unknowingly generate a mysterious problem.

UBE3A gene encodes a conserved HECT domain family E3 Ubiquitin ligase, *i.e.*, E6-AP, mutated in Angelman mental retardation syndrome [Kishino *et al.,* 1997]. Recently, it was observed that E6-AP retains QC properties and actively recruits with cystic fibrosis transmembrane conductance regulator aggresomes. E6-AP recruitment/co-localization facilitates the ubiquitination of misfolded proteins anchored by Hsp70 chaperone [Mishra *et al.,* 2009]. In another study, it was demonstrated that E6-AP recruits to neuronal intranuclear inclusions in Huntington's disease transgenic mice model. E6-AP alleviates proteoxicity mediated by expanded polyglutamine proteins via degradation through ubiquitination [Mishra *et al.,* 2008]. Lafora disease is a progressive neurodegenerative disease caused by mutation in NHLRC1 gene, which encodes RING finger malin E3 Ubiquitin ligase [Chan *et al.,* 2003; Gentry *et al.,* 2005]. Several studies report that parkin is another E3 Ubiquitin ligase, which co-localizes with aggresome after proteasomal inhibition [Junn *et al.,* 2002; Zhao *et al.,* 2003; Muqit *et al.,* 2004].

2.6.7 Sugar Chains Recognition

In protein quality control process, newly synthesized proteins enter into ER through a channel termed as "translocon" for N-glycosylation [Fiedler and Simons, 1995; Ellgaard *et al.,* 1999; Helenius, 2001]. Earlier, it has been reported that many E3s are implicated in the ER-associated degradation pathway. E3 Ubiquitin ligases also help in the selective elimination of glycoproteins. However, the molecular mechanism underlying the ability of E3 Ubiquitin ligases to recognize target glycoproteins remains to be understood. Recently, a novel approach adopted by a few Ubiquitin ligases in the recognition and ubiquitylation of N-linked glycoproteins that act as major players in ERAD pathway has been highlighted. Skp1-Cullin1-Fbx2-Roc1 (SCFFbx2) Ubiquitin ligase complex utilizes N-glycan signal for degradation. In this complex Ubiquitin ligase, Fbs2 protein interacts with endogenous N-linked high-mannose oligosaccharides containing glycoproteins and promotes their proteasomal degradation [Yoshida *et al.,* 2002]. Fbx2 expression levels are very high in the organ of Corti [Thalmann *et al.,* 1997]. Fbx2 lack of function leads to degeneration in epithelial support cells of the organ of Corti, and hearing loss in Fbxo2-/- mice began, which may be due to aberrant quality control mechanism of glycoprotein [Nelson *et al.,* 2007]. Another F-box protein Fbs2 acts as E3 Ubiquitin ligase, which is bound with N-glycan of T cell receptor α subunit, and promotes its degradation through ERAD pathway [Yoshida *et al.,* 2003]. In SCFFbs1.2 Ubiquitin ligase complex, Fbs1 and Fbs2 proteins preferentially associate with denatured glycoproteins as compared to properly folded proteins. This study suggests that, due to exposed chitobiose structure, Fbs can recognize and discriminate folded glycoproteins over unfolded glycoproteins [Yoshida *et al.,* 2005]. Interaction of HRD1 E3 Ubiquitin ligase and SCFFbs2 Ubiquitin ligase complex with uncleaved precursor of asialoglycoprotein receptor H2a promotes its degradation [Groisman *et al.,* 2006].

2.7 MODEL MISFOLDED PROTEINS

There is a range of genes that after expression eventually or sometimes under specific conditions, make misfolded protein species. Such genes can be used as tools in cell cultures as a source of misfolded proteinaceous species; this is done through overexpression of these genes in cells. Such genes can be clones in high expression vectors (often tagged with fluorescent markers) and then can be transfected into mammalian cells in order to analyze. Here are few such genes/proteins that have been used in the experiments conducted for the present study.

2.7.1 Heat-denatured Luciferase Protein

Luciferases refer to class of enzymes that produce bioluminescence through oxidation. In biotechnological approaches Luciferase enzyme has been explored for various purposes, perhaps, the most popular use of Luciferase has been implicated in reporter gene assay. In reporter gene assay, firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) are used. A desired gene can be fused with Luciferase with the Luciferase gene and the expression of the gene can be monitored with the help of Luciferase activity. However, in the current study, it is used in different manner. Interestingly, Luciferase can get denatured by heat shock and loses its bioluminescence activity. Heat-denatured Luciferase can be considered as a model misfolded protein. Assaying Luciferase activity leads to quick estimation of active Luciferase form that corresponds to the expression levels of Luciferase. By keeping the transfection conditions and construct amounts as the same, one can achieve almost identical levels of Luciferase protein activity. By changing conditions favourable to misfolded protein degradation, one can observe the effects on the levels of misfolded protein which can be heat denatured Luciferase.

2.7.2 Expanded Polyglutamine Proteins

Polyglutamine protein diseases are also kept under trinucleotide repeat disorders as in such diseases, there exists at least a mutation in gene containing CAG repeats which are expanded to normal coding sequence. As CAG trinucleotides encode for polyglutamine tracts, this leads to protein products with expanded polyglutamine. There are at least nine such disorders that are known to cause neurodegeneration due to expanded CAG repeats. Here in the study, two such expanded CAG repeats containing genes have been used as polyglutamine model misfolded protein encoding constructs. Polyglutamine proteins generally have about 100 glutamine repeats, and it has been seen that increased glutamine repeats correspond to the severity of the disease.

(A) Ataxin 3 Polyglutamine Protein

Throughout the globe, there are more than 60 spinocerebellar ataxia (SCA) diseases; one of them is caused by mutation in ATXN3 gene; this SCA is also named as Machado Joseph Disease (MJD). In this autosomal dominant, rare, yet an incurable neurodegenerative disease, more than 80 CAG repeats have been seen in expanded forms. In human, this gene is encoding a deubiquitinating enzyme which is involved in a vast range of functions, therefore, help maintain proteostasis. Ataxin 3 is involved in the regulation of aggresome formation and misfolded protein degradation [Burnett *et al.,* 2003; Burnett and Pittman, 2005; Zhong and Pittman, 2006].

(B) Huntingtin Polyglutamine Protein

Huntingtin (Htt) protein or Huntington disease (HD) protein is a 347.6 kD polypeptide chain that has been found as essential, as lack of this protein is lethal in mice [Nasir *et al.,* 1995]. This protein has been found interacting with cytoskeleton components such as beta tubulin [Hoffner *et al.,* 2002] and dynein [Pardo *et al.,* 2010]. However, the exact functioning of the protein is still not so clear; it contains polyglutamine coding tract (CAG repeats) in its gene's sequences. Mutations that lead to expansions of these polyglutamine tracts as coding sequences, may cause massive protein aggregation as these expanded polyglutamine proteins (>35

glutamine repeats) eventually become misfolded and make aggregates. Severity of the aggregation has been found proportional to the length of the polyglutamine tract. Huntington disease is an autosomal dominant inherited disease. Various animal models have been generated in order to understand the pathophysiological aspects of the disease [Ramaswamy *et al.,* 2007]. Huntingtin expanded polyglutamine proteins are one of the most popular model misfolded proteins as upon overexpression, it readily makes aggregates and propensity of the aggregation is significant enough to obstruct the normal cellular physiology.

2.8 MAHOGUNIN RING FINGER-1 E3 UBIQUITIN LIGASE

Mahogunin Ring Finger 1 (MGRN1) is highly conserved in eukaryotes. It is also known as mahoganoid as a mutation, with similar to mahogany was observed in C3H/HeJ mice, spontaneously generated in Jackson Laboratory more than half of a century ago. Recently, its plant homolog namely LOG2 has been characterized [Guerra *et al.,* 2013]. During the period various groups of scientists have put efforts in digging the information about the MGRN1 gene, its protein and their pleiotropic effects on the animal physiology and biochemistry. However, various phenotypic and physiological effects of MGRN1 have been studied but still their mechanisms are not fully known which makes MGRN1 worthy to explore.

2.8.1 MGRN1 Gene and its Discovery

MGRN1 gene is cytogenetically located at 16p13.3 in human genome. MGRN1 is also known as RING Finger Protein 156; its mouse homolog is named as KIAA0544. In 1989, Green *et al.,* reported two mouse coat color mutations *mahogany(mg)* and *mahoganoid(md)*. Later, detailed genetic studies were performed by [Miller *et al.,* 1997]. They showed that *mg* and *md* are genetically downstream to *agouti*. They have also found that agouti coat color is an epistatic character probably of an *md* allelic variant. The study shows that *mg* and *md* are genetically upstream to melanocortin 1 receptor (MC1R). MGRN1 was successfully cloned as a newly identified gene by Nagase *et al*. (1998).

2.8.2 MGRN1 Protein and its Biochemical and Physiological Functions

MGRN1 is a really interesting new gene (RING) finger protein, where the RING finger motif possesses the E3 ligase activity of the protein; the RING finger domain is a Zinc metal binding domain. Besides RING finger domain, there exists a PSAP motif in MGRN1 protein. Till date crystal structure of MGRN1 has not been determined. As shown in Figure 2.3, two protein isoforms of MGRN1 are lacking the exon 12 product; they reside in cytoplasm while the rest of the two isoforms can enter the nucleus. The exon 12 product contains the nuclear localizing signal (NLS) sequence.

(A) Mice Coat Color and Pigment Type Switching

There were two pigments described for the mice coat color, eumelanin and pheomelanin. A banding pattern of these pigments in individual hair is said to be agouti. Agouti pattern basically consists of a subapical ticking of yellow band because of pheomelanin pigments in that region of hair. This kind of color pattern is due to a protein called agouti signaling protein (ASP or ASIP). ASP can stimulate the synthesis of pheomelanin from the eumelanin through signaling via melanocortin receptors. This color effect is significantly influenced by the presence of mahogany and mahoganoid. Dominant agouti mice (AA) become obese due to the presence of double mahoganoid mutations (md/md) and recessive agouti with double mahoganoid mutations lead to dark black pigmentation in mice [Miller *et al.,* 1997]. Later, it was explored that mahogany and mahoganoid mutations lead to suppress agouti signaling mediated by melanocortin receptors [He *et al.,* 2003].

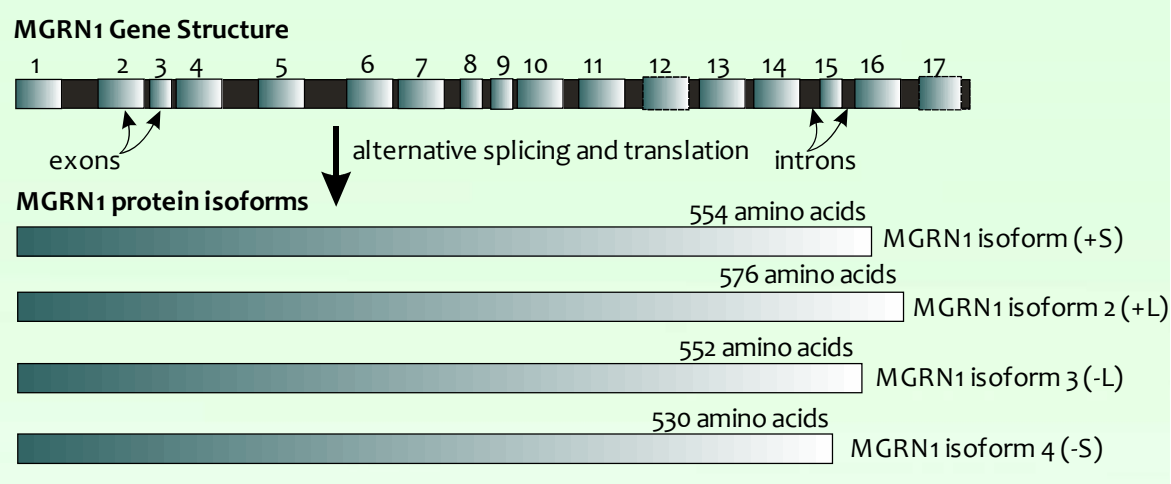

Figure 2.3 : *MGRN1 gene and its isoforms:* MGRN1 gene is present on the chromosome 16 in human. This gene encodes for a RING finger E3 Ubiquitin ligase which has a translational product 17 exons; however, there are a few alterations that lead to four different variants of this protein. In this figure, these forms have been shown by S or L (indicates Short or Long forms of exon 17 respectively) and + or – (indicating the presence or absence of exon 12 respectively).

Mouse coat color regulation mechanism is not yet fully known but in broader aspect, it is regulated through switching from eumelanin to pheomelanin synthesis. Mahogunin along with Attractin, agouti and melanocortin receptor genes has been found involved in regulation of pigmentation in cattle also [Seo *et al.,* 2007]. Pigment type switching has been of interest when we study MGRN1 knockout mice. It has earlier been shown that MGRN1 knockout mice show dark fur phenotype. MGRN1 has been shown to ubiquitinate melanocortin 2 receptor (MC2R). Interestingly, multimonoubiquitination was observed after stimulation of adrenocorticotropic hormone (ACTH), hence, it has been speculated that MGRN1 plays a role in pigment type switching through both the degradation and trafficking of MC2R [Cooray *et al.,* 2011].

Agouti signaling protein (ASP) has been found to be implicated in regulation of pigment type switching in mice hair follicle. ASP acts as paracrine molecule involved in the yellow pelage and obesity in Yellow mouse (A(y)). This A(y) phenotype was described as a resultant of mutations in either Attractin (Atrn) and/or in MGRN1, named as mahogany (mg) and mahoganoid (md) respectively. Overton and Leibel (2011) have shown that Loss-of-function of either MGRN1 or Attractin can suppress the degradation of melanocortin 4 receptor (MC4R) mediated by ASP. Walker and Gunn (2010) have made an attempt to review the complex regulation of pigment type switching phenomenon in mice. In conclusion, mouse coat color is epistatic to MGRN1, where the role of MGRN1 is independent of its ubiquitination activity.

(B) Endosomal Trafficking

Through multimonoubiquitination of tumor susceptibility gene 101 (TSG101), MGRN1 regulates endosomal trafficking. MGRN1 interacts with Ubiquitin E2 variant TSG101 by its PSAP motif and leads to ubiquitylation. As TSG101 is a component of endosomal sorting complexes required for transport 1 (ESCRT1), hence MGRN1 plays a role in the regulation of endosomal trafficking thereby. It is found that Mahogunin depleted cells have enlarged endosomes concentrated towards perinuclear region; where as a clustering of late endosomes (positive for Lamp2) and lysosomes often with vacuole-like structures were observed. It was speculated that MGRN1 may be playing a role in the formation of multi-vesicular bodies

(MVBs). There are a few proteins known to interact with TSG101, but not with MGRN1 like Hrs, Alix/AIP1 and Gag; this indicates that MGRN1 may be competing with these proteins to bind with TSG101 protein [Kim *et al.,* 2007]. Overall, it can be speculated that MGRN1 is one among those proteins that play roles in the endosomal trafficking pathways, however, its role may be through an indirect mechanism, not yet fully explored.

(C) Neurodegeneration

It is known that attractin (Atrn) mutants develop spongiform neurodegeneration; He *et al.* (2003) have speculated the same for mahogunoid and have found that null mutation in mahogunoid causes neuropathology similar to prion diseases in an age-dependent manner yet devoid of protease-resistant prion protein accumulation. Abnormal ubiquitination has been linked with spongiform neurodegeneration in animals with MGRN1 null mutations; this shows that, may be, due to absence of MGRN1 gene, ubiqitination defects are caused which ultimately contributes to neurodegenerative condition [Whatley *et al.,* 2008]. Null mutation of MGRN1 in mice leads to various phenotypic characteristics such as absence of yellow hair pigment, abnormal head shape, decreased viability, defects in LR patterning, mitochondrial dysfunction in cellular physiology and adult onset spongiform neurodegeneration. Most of these phenotypic effects were found varied with different isoforms of MGRN1, for example, expression of I or III leads to normal phenotypes, where as isoform II was found to partially restore these phenotypes to normal, but isoform IV mostly fails to restore normal phenotypic characters [Jiao *et al.,* 2009].

MGRN1 does the multimonoubiquitination of TSG101, a protein which is involved in endosomal-lysosomal trafficking. In mahogunin null mutant mice, spongiform neurodegeneration has been observed which is thought to be because of deregulation in endosomal-lysosomal trafficking and this kind of condition can be devoid of protein aggregation [Kim *et al.,* 2007]. It has also been reported to be sequestered with cytosolically exposed prion proteins. Inactivation of mahogunin was reported to cause prion pathologic conditions [Chakrabarti and Hegde, 2009]. They have shown that MGRN1 interacts with transmembrane form of prion protein ^{ctm}PrP and cytoplasmic aggregates of cyPrP. Interestingly, MGRN1 does not interact with the wild type PrP. The study further elucidated that MGRN1 depletion leads to defects in morphologies of lysosomes. Rocky Mountain Laboratory (RML) Prions inoculation in mice devoid of MGRN1 and/or Attractin was found to be having no effect. MGRN1 overexpression was also not found to be effective. Perez-Oliva *et al. (*2009) have shown the effect of all the four isoforms of MGRN1 in melanocortin receptor signaling. MGRN1 binds with melanocortin receptor and competes for the same with Galpha. This leads to inhibition of the melanocortin signaling which was independent of ubiquitination and internalization of receptors. Aguzzi and Steele (2009) have summarized how MGRN1 interacts with cytosolically exposed prion protein and suggested the possible ways of MGRN1's function hereby. Such results prompted us for exploration of MGRN1's role in protein quality control mechanisms.

(D) Mitochondrial Dysfunction

As MGRN1 was reported to be involved in neurodegeneration, an obvious thought might have made Sun *et al.* (2007) investigate role of the gene in oxidative stress and mitochondrial dysfunction. They have found many of the mitochondrial proteins were depleted in MGRN1 mutants; particularly mitochondrial complex IV was significantly downregulated. They suggested that as mitochondrial dysfunction and enhanced oxidative stress were found to have increased in neuropathic conditions much earlier to disease onset, this approach could serve as common causative mechanism to various neurodegenerative conditions [Sun *et al.,* 2007]. Now, it is evident that impairment in mitochondrial function can be found in degenerative neurons in various neurodegenerative diseases such as Parkinson's, Alzheimer's,

Amyotrophic Lateral Sclerosis (ALS) and Huntington's disease. Parkinson's disease has been shown to be strongly associated with mitochondrial dysfunction as Parkin E3 Ubiquitin ligase and PINK1 have been found implicated in mitochondrial quality control pathway where damaged mitochondria are eliminated; therefore, impairment in Parkin or PINK1 could imply mitochondrial dysfunction at an early stage. Oxidative stresses are also found to be following such conditions in neurodegeneration and are directly related to impairment in mitochondrial functionality.

(E) Left-right Patterning in Mice Embryo

A novel role of MGRN1 in mice embryo development was found by Cota et al (2006). They have reported that MGRN1 mutations affect left-right patterning in early embryonic stage in mice, and this effect is dependent on the ubiquitination activity of MGRN1. Even they stated MGRN1's ubiquitination activity is an essential component in the LR signaling cascade. They have taken into consideration, three genes *lefty1*, *lefty2* and *pitx2* that are involved in left-right patterning in mice embryo and found that the effect of MGRN1 on left right patterning in mice embryo development would be in earlier stages before these set of genes function. MGRN1 E3 Ubiquitin ligase has been recently shown to regulate microtubule stability, recently [Srivastava and Chakrabarti, 2014]. In this report they have shown MGRN1 as an E3 Ubiquitin ligase that mediates ubiquitination of alpha tubulin; interestingly, this ubiquitination was non-canonical. MGRN1 modifies the alpha tubulin protein through K-6 linked polyubiquitination. They have shown that this modulatory effect of MGRN1 confers microtubules' stability and this may be linked with left-right patterning during embryogenesis [Srivastava and Chakrabarti, 2014].

In 2002, Phan *et al.* have explored links of mahoganoid gene with obesity conditions. They have shown that MGRN1 works as an inverse antagonist of alpha MSH, and stimulates hypothalamic neurons through MC3R and MC4R, hence play a role in food intake of the organism. It has been suggested that such mechanism may lead to obesity and diabetes. They speculated that as MGRN1 maps near to loci for type 2 diabetes, coronary heart disease and hypertension there could be a possible role of the E3 ligase in such conditions too.

…