2
Review of Literature

Eukaryotic cell evolution is one of the greatest landmarks in the history of life, marking the formation of hyper-structures and ultra-specialized cellular systems, which are probably developed from the earlier simpler forms of organisms, i.e., prokaryotes [Archibald, 2015; Norris and Root-Bernstein, 2009]. These cells perform a variety of cellular tasks with utmost accuracy; and while doing so, cells need a large number of proteins with varying shapes, sizes, subcellular locations, and functions [Ananthakrishnan and Ehrlicher, 2007; Zimmerberg and Kozlov, 2006]. Although cells contain around 20,000 different kinds of protein-coding genes, they still produce and retain only a set of proteins at a time, from all the available sequences, in accordance with the requirements of the cells [Kim *et al.*, 2014; Wilhelm *et al.*, 2014].

Proteins are eminent macromolecules of living cells. Eukaryotic cells, with the help of millions of ribosomes, continuously synthesize new polypeptides in the cytoplasm, and maintain a protein concentration of 50–300 mg/ml, in a crowded cellular environment [Finka and Goloubinoff, 2013; Ingolia *et al.*, 2011; Wolff *et al.*, 2014]. Furthermore, cells need a tight regulation of synthesis and degradation of proteins, without which successful execution of cellular functions is not possible [Gallastegui and Groll, 2010]. Cells comprise a subset of approximately 1400 specialized proteins in their repertoire, which is essentially required to achieve and maintain a functional state of its proteome [Balchin *et al.*, 2016]. A large number of chaperones and their cofactors assist newly synthesized linear polypeptide chains in attaining their functional three-dimensional shapes by intercepting unfruitful inter-domain interactions and protect them against various kinds of cytotoxic stresses, in order to prevent unwanted misfolding events inside the crowded cytoplasmic milieu [Ellis, 1987; Hartl *et al.*, 2011; Hartl and Hayer-Hartl, 2009].

However, defying such kind of tight control, a subset of proteins still remains structurally disordered in the cell, and is further taken care by cellular protein quality control (PQC) machinery [Dunker *et al.*, 2008; Walter and Buchner, 2002]. Interestingly, from the kinetic point of view, some polypeptides can take complex structural forms that exhibit critical features of amyloids [Guijarro *et al.*, 1998]. These represent the lowest-energy states of these polypeptides, and so nascent chains can easily take these forms if left unattended by the cellular folding machinery (the chaperones) [Dobson, 2001].

2.1 A GLIMPSE OF CELLULAR PROTEIN QUALITY CONTROL SYSTEMS

Human cell normally contain billions of proteins, and normal integral function of a protein is dependent on its shape, size, and proper localization in appropriate cellular compartments [Dobson, 2003]. Proteins continuously perform all major work of cells, in order to maintain cellular homeostasis. Millions of ribosomes synthesize nascent polypeptide chains with an approximate rate of six amino acids per second in cells [Duncan and Hershey, 1983; Ingolia *et al.*, 2011].

Disruption in the conformations of three-dimensional structure of a polypeptide chains leads to misfolding, which may result in abnormal toxic aggregation due to the failure of correct assembly or loss of protein quality control mechanism in cells [Bucciantini *et al.*, 2002]. Integrity of a newly synthesized protein is continually at risk of abnormal folding and aggregation due to transcriptional and/or translational failures or exposure of distinct stress conditions at both intracellular and extracellular levels [Chen *et al.*, 2011b]. To survive under such intolerable stress conditions, cells evolved a very efficient interconnected central surveillance protein quality control mechanism that can refold, degrade, and/or separate misfolded proteins with the help of chaperones and two proteolytic system [Tyedmers *et al.*, 2010].

2.1.1 Molecular Chaperones

Eukaryotic cellular system is a highly specialized set of molecular machinery that may function independently or in concerted action with each other to maintain intracellular homeostasis [Alberts, 1998]. A fraction of proteins, called molecular chaperones, assist newly synthesized polypeptide chains to achieve their native three-dimensional conformation and to facilitate their translocation across membranes to reduce the unwanted load of aggregation [Hartl and Hayer-Hartl, 2009; Kim *et al.*, 2013]. Chaperones perform this highly challenging and complex process with an incredible efficiency and extremely rapidly, defying mathematical predictions [Bukau *et al.*, 2006]. They target hydrophobic stretches of unfolded proteins and also recognize misfolded proteins for their further folding or degradation [Hartl and Hayer-Hartl, 2009; Walter and Buchner, 2002]. But, on several occasions, their activities are compromised and they do not suffice to keep the cellular proteins in their native states [Cuervo and Dice, 2000]. Under such conditions, unfolded or misfolded species of proteins start accumulating intracellular [Singh *et al.*, 2015]. Few classes of proteins, which are more prone towards aggregation, starts forming insoluble amyloidogenic aggregates, which underlie as the fundamental mechanism of many systemic and non-systemic diseases [Pawar *et al.*, 2005]. To avoid such unwanted deleterious changes, chaperones may also opt to degrade accumulated toxic proteinaceous burden of the cell, in concerted mechanisms, carried out by cellular proteolytic systems, viz., autophagy and UPS [Arndt *et al.*, 2007; Esser *et al.*, 2004; Kaushik and Cuervo, 2012].

2.1.2 Cellular Proteolytic Machinery: Ubiquitin Proteasome System and Autophagy

Age-related changes and continuous stresses cause a significant decline in efficiency of molecular chaperones, which may result in accumulation of proteinaceous aggregates inside the cells [Tyedmers *et al.*, 2010]. Such conditions may result in progression of various types of cancers and neurodegenerative diseases [Kim *et al.*, 2013; Morimoto, 2008]. Protein degradation machinery of the cell facilitates the degradation of cellular proteins, which have greatly varying half-lives, ranging from few minutes to several hours [Bachmair *et al.*, 1986; Belle *et al.*, 2006; Schwartz and Ciechanover, 2009]. Both of these pathways recognize small ubiquitin molecules attached to cellular proteins (the process is called ubiquitylation), as the tags of death, and initiate the degradation pathways [Ciechanover, 2005]. Ubiquitin is a very small protein of 76 amino acids in length, and approximately 8.5 kDa of molecular weight [Ciehanover *et al.*, 1978; Glickman and Ciechanover, 2002]. Ubiquitylation is a type of post-translational modification of a protein, in which a concerted action of multiple players, lying into a cascade of reactions, attaches a small ubiquitin moiety to a substrate [Hershko *et al.*, 1983; Varshavsky, 1997]. It may lead either to a functional modulation, or it may also be treated as death signals, depending upon the pattern, the ubiquitin molecules are attached [Komander and Rape, 2012; Woelk *et al.*, 2007].

Cells perform different types of ubiquitination, e.g., monoubiquitination, multiple mono-ubiquitination, K48-linked multiubiquitination, K63-linked multiubiquitination and autoubiquitination [Andreassen *et al.*, 2004; Haglund *et al.*, 2003; Komander, 2009; Pickart, 2001; Xu and Jaffrey, 2011]. The way, the number, and the pattern, into which the ubiquitin moieties

get attached to a given protein, provide the code, on which other cellular systems act and decide their diverse fates, as they could be sorted, trafficked, functionally or structurally modulated or degraded [Rajalingam and Dikic, 2016; Rotin and Kumar, 2009]. Apart from elimination of intracellular damaged proteins, the ubiquitin-mediated degradation plays crucial roles in the regulation of transcription, stress response, cell cycle, immune response, DNA repair, internalization of cell surface proteins via endocytic pathway and over all maintenance of cellular quality control events [Ben-Neriah, 2002; d'Azzo *et al.*, 2005; Daulny and Tansey, 2009; Ding *et al.*, 2007; Nakayama and Nakayama, 2006; Zhu *et al.*, 2007].

(a) Ubiquitin Proteasome System

The original discoverers of UPS have identified a series of enzymatic reactions, in which, the formation of a thioester bond between C-terminal glycine residue of ubiquitin and a cysteine residue present on E1 ubiquitin activating enzyme activates the ubiquitin in an ATP-dependent manner [Haas and Rose, 1982]. Another important family of ubiquitin-carrier or conjugating (E2) enzyme retain highly conserved 150–200 amino acid ubiquitin-conjugating (UBC) domain. E1 enzyme transfers the ATP-activated ubiquitin or ubiquitin-like (UBL) proteins to E2 enzyme through a covalent thioester linkage [Burroughs *et al.*, 2008; Hofmann and Pickart, 2001]. E2 enzymes are present in all eukaryotes and Class I E2 enzymes contain only catalytic domain, few have N (Class II) or C (Class III) terminal extensions; in Class IV extensions of both terminals are present [Hofmann and Pickart, 2001; Winn *et al.*, 2004]. Further numerous critical interactions take place and subsequently, ubiquitin-carrier or conjugating enzyme (E2) machinate ubiquitin for conjugation but perhaps, the most important enzyme in this pathway is ubiquitin ligase (E3) because it recognizes specific substrates and completes the transfer of activated ubiquitin onto the substrates [Hershko and Ciechanover, 1998].

In the cells, various types of E3 ubiquitin ligases often adapt different strategies for rapid degradation of specific proteins [Chhangani and Mishra, 2013a; Ravid and Hochstrasser, 2008]. Earlier studies have provided insight that three major classes of E3 ubiquitin ligases exist in cells: Homologous to E6-associated protein C-terminus (HECT) domain; Really interesting new gene (RING) finger domain and U-box domain ligases [Borden and Freemont, 1996; Deshaies and Joazeiro, 2009; Hatakeyama *et al.*, 2001; Huibregtse *et al.*, 1995; Tyers and Willems, 1999]. Recent studies have shown one more class of proteins containing Plant Homeodomain (PHD) domain to have E3 ubiquitin ligase activity [Coscoy and Ganem, 2003].

HECT domain contains near about 350 highly conserved amino acids and approximately 30 HECT-containing E3s have been identified till now in the mammalian cell system, which control numerous cellular and molecular physiological functions [Rotin and Kumar, 2009]. A zinc-binding RING finger domain E3 ligase coordinates through Zn^{2+} and mediates ubiquitin transfer on substrates. RING E3 ubiquitin ligases can functionally exist in three different forms (i) Monomeric RING, (ii) Dimeric RING, (iii) Multi-subunit RING or SCF (Skp1-Cullin-F-box) family members [Joazeiro and Weissman, 2000; Lorick *et al.*, 1999]. Much has been learned about the polyubiquitination mechanism, but still, it remains obscure why cells perform multiple-mono ubiquitination of certain proteins?

What are those E3 ubiquitin ligases that frequently participate and play major roles in such processes? Whether they further cross-talk to chaperones or other components of UPS and autophagy pathway, remains an enigma. How the substrate recognition is a highly selective process in the entire storm of protein degradation events?

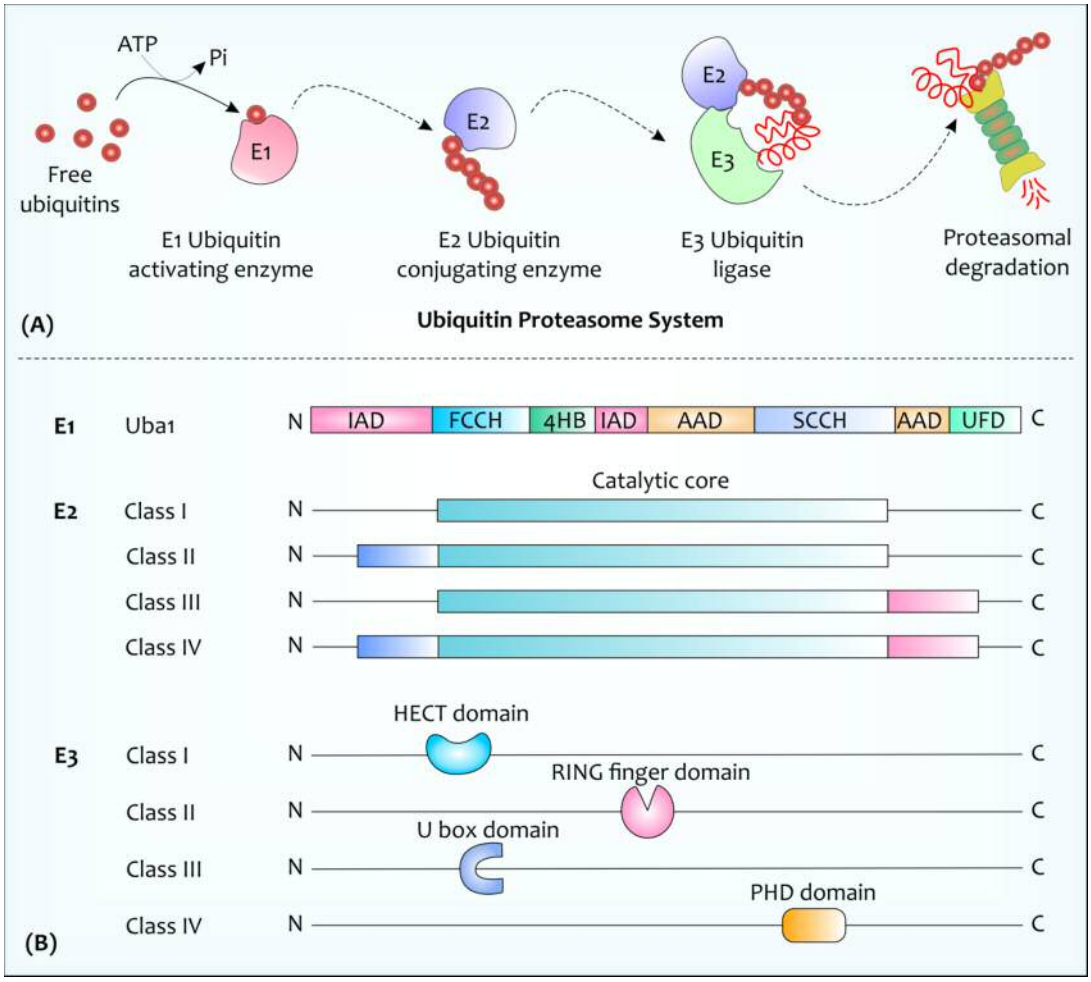

Figure 2.1 : *General representation of ubiquitin proteasome pathway and its enzymes:* (a) Ubiquitin Proteasome System targets many intracellular proteins for their clearance with the help of E1 (ubiquitin activating); E2 (ubiquitin-conjugating) and E3 (ubiquitin ligases) enzymes. (b) Different kinds of ubiquitin-conjugating or carrier enzymes retain highly conserved 150–200 amino acid ubiquitin conjugating (UBC) domain, which provides assistance in the completion of multistep ubiquitination process via E3 ubiquitin ligases. These different classes of E3 ubiquitin ligases overall provide the specificity to ubiquitin proteasome system into crucial substrate recognition process. (Figure published in Upadhyay et al. 2015; Ageing Research Reviews)

Increasing reports suggest that the number of E3 ubiquitin ligases is reaching around thousand, which enables them to provide substrate specificity inside the cells to take control of most of the major and minor cellular pathways [Nakayama and Nakayama, 2006]. These enzymes attach the ubiquitin molecule to the ε-amino group of an internal lysine residue of the substrate protein, or in other cases, to one of the seven lysine residues present on an already attached ubiquitin molecule [Ciechanover, 1994; Hershko *et al.*, 2000]. After finding a suitable degradatory signal, the proteolytic machinery of the cell, the 26S proteasome degrades the substrate into smaller oligopeptides [Finley, 2009]. However, to prevent such degradation signals from being constitutive, cells need to remove these ubiquitin chains immediately from the substrate proteins, once they have been utilized.

To accomplish this task, cells employ another set of approximately hundred different deubiquitinating (DUBs) enzymes, which by shredding off ubiquitin moieties from proteins, contribute largely to their proper recycling [Komander, 2009; Reyes-Turcu *et al.*, 2009]. Therefore, similar to kinases and phosphatases, E3 ubiquitin ligases and DUBs also provide a mechanism of reversible modifications of cellular proteins to alter their functions [D'Andrea and Pellman, 1998; Dikic and Robertson, 2012].

Impaired performance of quality control (QC) E3 ubiquitin ligases compromises elimination of aberrant proteins, which consequently contributes in decline of several cellular functions. Thus, failure of cellular QC dependent E3 ubiquitin ligases impairs proteostasis-associated mechanisms and finally triggers hallmarks of neurodegeneration and ageing [Chhangani *et al.*, 2012]. Interestingly, growing number of findings have recently established a strongly orchestrated link between E3 ubiquitin ligases and sensitive cellular mechanisms at several cellular interfaces. To counter the challenges of protein aggregation and solve the problem of misfolded proteins clearance, cells evolved another alternative mechanism, i.e., chaperone-assisted proteasomal degradation (CAP) which may increase cytoprotective potential of cells against toxic proteins [Kettern *et al.*, 2010]. Overall, the process to degrade all intracellular proteins or misfolded proteins may seem wasteful, but continuous activity of this system serve many crucial physiological processes at both cellular and molecular level [Glickman and Ciechanover, 2002; Nandi *et al.*, 2006]. Therefore, global changes or aberrant function of UPS causes fatal neurodegenerative diseases [Dantuma and Bott, 2014].

(b) Autophagy

Autophagy pathway is involved in the degradation of major random cytoplasmic bulk of cell; recent studies have elaborated the selective mechanism of autophagy pathway with the help of chaperones and E3 ubiquitin ligases in the clearance of non-native or poorly folded proteins [Fimia *et al.*, 2013]. Contrary to UPS, which targets specific proteins for degradation, autophagy pathway circumvents bulk of cellular debris that may also include cytoplasmic inclusions of amyloidic proteins towards lysosomal degradation [Badadani, 2012; Yorimitsu and Klionsky, 2005]. An autophagy pathway also consists of multiple steps involving a number of adapter proteins and multi-protein complexes, and membrane receptors to specifically target the bulk towards lysosomal compartments [Levine and Klionsky, 2004; Mizushima *et al.*, 1998]. Depending upon the specific mechanisms and the molecules involved in the identification and targeting of the target towards degradation, autophagy could be further classified into: microautophagy, macroautophagy and chaperone-mediated autophagy (CMA) [Cuervo, 2010; Dice, 2000].

Macroautopahgy involves formation of a double-membraneous phagosome that later fuses with lysosome; whereas microautophagy consists of ATP-dependent invagination, formation of vesicles inside lysosomal chamber, expansion, and uptake followed by degradation for recycling [Mizushima *et al.*, 2001; Todde *et al.*, 2009]. In living cells, isolation of misfolded proteins, removal of unwanted aggregated proteins, and repair of partially folded or non-native proteins improve interconnected networks of cellular cytoprotective mechanisms. In the process of elimination of damaged proteins, CMA is another very crucial and selective approach that takes help of lysosomes in the removal of specific proteins [Kaushik and Cuervo, 2012]. Another less discussed but important mechanism, chaperone-assisted selective autophagy (CASA) helps in the muscle maintenance via degradation of damaged filamin [Arndt *et al.*, 2010]. Loss of CASA functions leads to muscles weakness and declination of cytoskeleton architecture [Ulbricht *et al.*, 2013].

Induction of the autophagy pathway and the following effects of this upregulation on the overall clearance of accumulated bulk protein inclusions has shown tremendous potential of ameliorating disease pathological symptoms in animal models or patients suffering from Alzheimer's (AD), Parkinson's (PD) and Huntington's disease (HD) [Boland *et al.*, 2008; Moors *et al.*, 2017; Sarkar and Rubinsztein, 2008].

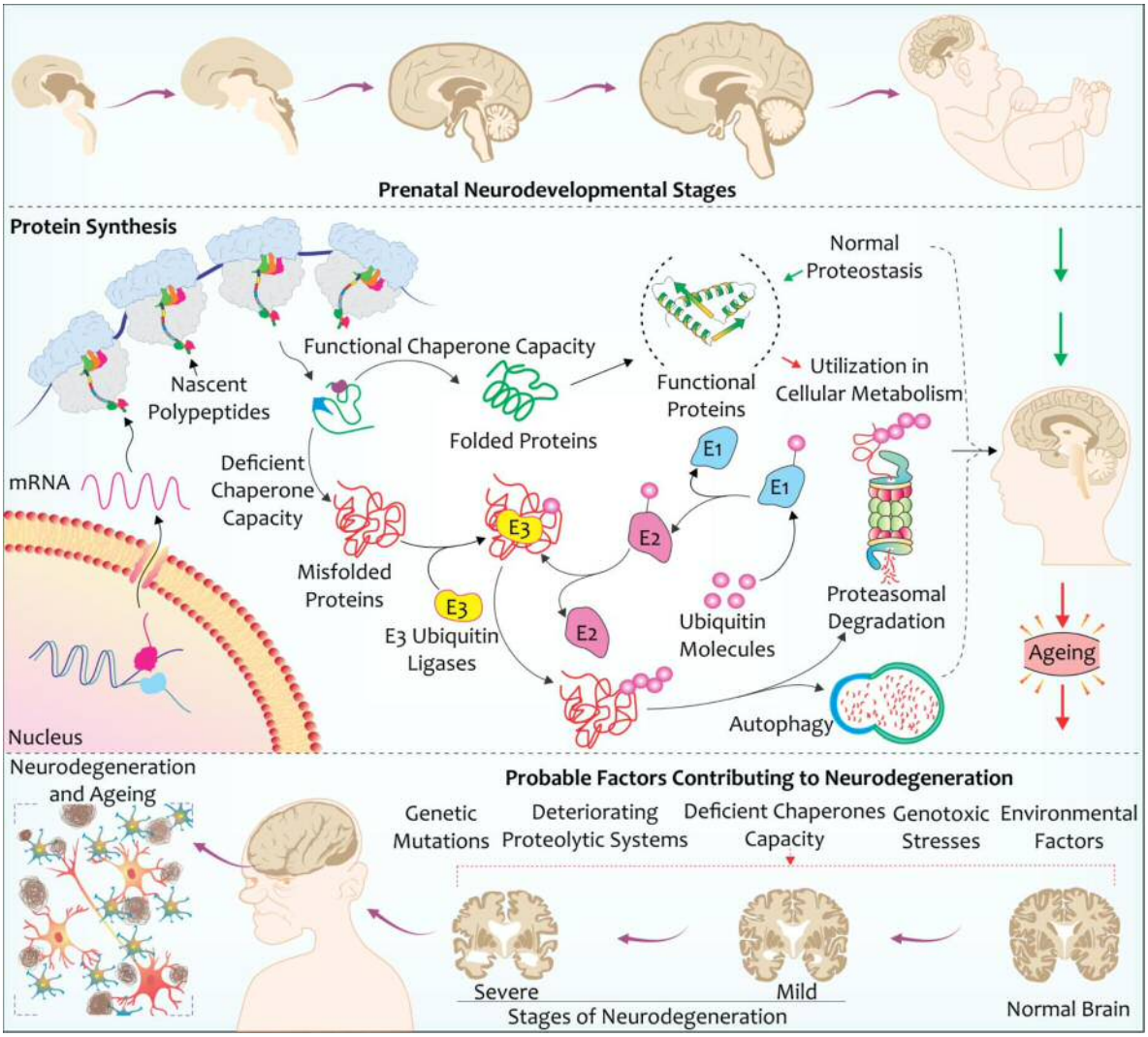

Figure 2.2 : *An illustration of orchestration of factors involved in neurodevelopment and neurodegeneration:* Upper part represents how stepwise modifications occur during prenatal period of brain development. Schematic of cellular translation machinery along with other protein quality control systems has been drawn. A state of proteostasis and active control of PQC system regulate the processes of growth, adolescence and development. However, ageing related metabolic changes, including various kinds of stresses and successive deterioration of quality control systems may lead to accumulation of several proteins, causing formation of inclusion bodies, which results in late-stage neurodegeneration. (Figure published in Upadhyay et al. 2017; Frontiers in Molecular Neuroscience)

2.2 DEFICIENCY OF CELLULAR QUALITY CONTROL AND PATHOLOGICAL CONDITIONS

Genetic mutations in several genes coding for few highly aggregation-prone proteins underlie as a causal mechanism in many neuronal disorders and a compromise in the functioning of PQC machinery may severely add to the pathogenicity of these mutations [Bertram and Tanzi, 2005; Lill and Bertram, 2011]. For example, in the case of different neurodegenerative diseases, a common problem of misfolded protein aggregation and inclusion bodies formation can be easily observed in diseased brains and may serve as clinical hallmarks [Alves-Rodrigues *et al.*, 1998; Heemels, 2016]. Structural modification in certain amyloidogenic or non-amyloidogenic proteins may result in a loss- or gain-of-function that may underlie several diseases or pathologies including Alzheimer's, Parkinson's and Huntington's disease [Bucciantini *et al.*, 2002; Dobson, 2001].

Alzheimer's disease, the most common form of dementia, can be caused by mutations in several genes, e.g. amyloid precursor protein [Goate *et al.*, 1991], tau [Hutton *et al.*, 1998], apolipoprotein E [Saunders *et al.*, 1993], and presenilins [Scheuner *et al.*, 1996]. Similarly, in Parkinson's disease, several mutations have been identified that may directly or indirectly lead to the formation of intracellular amyloidic protein aggregates, which may cause the onset of degeneration of dopaminergic neurons [Antony *et al.*, 2013]. Mutations recognized as putative stimuli for neurodegenerative changes include changes to α–synuclein [Polymeropoulos *et al.*, 1997], parkin [Kitada *et al.*, 1998] and PTEN-induced putative kinase 1 (PINK1) [Valente *et al.*, 2004]. Mutations in TAR DNA-binding protein 43 (TDP-43) [Sreedharan *et al.*, 2008], fused in sarcoma (FUS) [Kwiatkowski *et al.*, 2009], SOD1 [Wong *et al.*, 1995] and several other genes are responsible for the formation of inclusion bodies, which are associated with degeneration of motor neurons in amyotrophic lateral sclerosis [Renton *et al.*, 2014].

Mutation and structural alterations in cellular prion proteins may lead to their conversion into aberrant amyloidic forms that may accumulate in the brain and lead to loss of neuronal cells [Prusiner and DeArmond, 1994]. Huntington's disease has been observed to result from mutations in a single gene, Htt, which contains cytosine-adenine-guanine (CAG) repeats in its exon 1 sequences, that may lead to aggregation of the protein [DiFiglia *et al.*, 1997; La Spada *et al.*, 1991]. Expansion of similar CAG repeats has also been observed to result from sequence mutation of a number of other genes, e.g. ataxin-1, ataxin-3 and ataxin-7, causing multiple types of ataxias [Orr *et al.*, 1993; Paulson, 2009].

In previous studies, it has also been shown that ubiquitin, molecular chaperones, nascent polypeptide chains, transcription factors, and components of UPS and autophagy pathways are associated with aggregates or inclusion bodies [Olzscha *et al.*, 2011]. In fact, higher aggregation of abnormal proteins compromises the proteolytic functions of both UPS and autophagy [Bence *et al.*, 2001; Menzies *et al.*, 2015]. Perturbations in the function of proteolytic machinery generate a major challenge to resolve or find a possible cure against the problem of protein aggregation in neurodegenerative diseases.

2.3 CHARACTERISTICS OF AMYLOIDIC AGGREGATES AND INCLUSION BODIES

Amyloids, in general, are an aligned arrangement of parallel or anti-parallel β-strands of polypeptide segments of a protein, which gives them a characteristic cross-β X-ray diffraction pattern and typical twisted fibrillary structures of 60–120 Å diameter under electron microscope. Their core is made up of β-sheets aligned perpendicularly to the fibril axis [Cohen *et al.*, 1982; Jiménez *et al.*, 1999; Shirahama, 1967]. Pioneering work on poached egg white showing its characteristic X-ray diffraction pattern established the basis of amyloid structure [Astbury *et al.*, 1935].

The amyloid state may be considered to represent a primitive state of protein folding, and perhaps corresponds to protein folding in the very early stages of life [Greenwald and Riek, 2010; True and Lindquist, 2000; Wang *et al.*, 2010]. Multiple lines of evidence, based on nuclear magnetic resonance, X-ray, and electron microscopy data, indicate that most of the protein aggregates are tightly packed cross β-sheets or aligned amyloid fibrils [Harrison *et al.*, 2007]. Hydrogen bonds between successive polypeptides provide the binding forces that give them strength and toughness [Knowles *et al.*, 2007]. Under X-ray imaging, amyloid cross β-sheets give two reflections: one at 9–11 Å and the other at around 4.8 Å. The first reflection is weaker and represents the distance between the β-sheets, whereas the stronger meridional4.8 Å signifies the distance between two strands in a β-sheet [Jahn *et al.*, 2010]. In simpler terms, amyloids are a stacked assembly of equally spaced (4.8Å) β-sheets, which are formed of parallel or anti-parallel β-strands (intersheet spacing 9–11 Å), normal to the fibril axis. The length of the fibril is stabilized by a network of hydrogen bonds at the fibril core [Knowles *et al.*, 2007].

Another important feature is the register arrangement of the strands, i.e. an arrangement in which identical side chains are aligned one above the other. The interdigitation of side chains of two adjacent amyloid fibrils allows a complex stabilized arrangement, called a steric zipper. Complimentary amino acid sequences, interdigitating side chains, hydrogen bonds, and hydrophobic interactions are the major stabilizing forces providing amyloid protofilaments with strength and complexity [Eisenberg and Jucker, 2012]. Young's modulus ranges between a few megapascals and several gigapascals, depending on the size of the protein [Guo and Akhremitchev, 2006; Smith *et al.*, 2006; Xu *et al.*, 2010].

2.3.1 Unique Structural Properties of Amyloids

Several amyloid proteins involved in human disease have been studied in detail. The amyloid forms of most of these proteins have several characteristic features in common, but also differ in shape, size, toxicity, morphology, overall stability, etc. [Breydo and Uversky, 2015]. AD-associated amyloid beta peptide (Aβ42) is one of the best studied. This 42 amino-acid-long peptide exists in multiple structural forms: oligomeric, soluble amyloid fibrils enriched with β-sheets, and annular aggregates [Lashuel *et al.*, 2002; Rochet and Lansbury, 2000]. The protofilaments of Aβ42 are two stacked in-register parallel β-sheets, with a tendency to perpetuate unidirectionally along the fibril axis; toxicity is correlated with the length of the fibril [Luhrs *et al.*, 2005].

Lewy bodies are composed of fibrillar forms of α-synuclein, along with oligomeric and protofibrillar forms [Lashuel *et al.*, 2013]. Sodium dodecyl sulfate (SDS)-resistant dimers of α-synuclein have also been identified, whereas oligomeric forms of α-synuclein can exist in several alternative forms including disordered, α-helical- and β-sheet-rich forms [Breydo and Uversky, 2015; Golts *et al.*, 2002]. Several factors influence the aggregation propensity and toxicity of these proteins, including interacting metal ions, certain small molecules, phosphorylation and many stress conditions [Lashuel *et al.*, 2013; Villar-Pique *et al.*, 2016].

Type II diabetes mellitus-associated human islet amyloid polypeptide (IAPP) monomers form hairpin structures, containing two β-strands each [Bedrood *et al.*, 2012]. These monomers are then stacked into two β-sheets, which are twisted around each other. In-register arrangement of the β-sheets is further stabilized by hydrogen bonds between the two β-strands [Akter *et al.*, 2016]. These IAPP amyloids may cause apoptotic death of pancreatic β-cells through several proposed mechanisms [Janson *et al.*, 1999; Lorenzo *et al.*, 1994]. CAG repeats-containing huntingtin (Htt) protein also shows typical amyloidic features as observed in postmortem brains of Huntington's disease patients as well as in mouse models of the disease [Ghahghaei and Faridi, 2009; Scherzinger *et al.*, 1997].

Exon 1 of the 350 kDa Htt protein contains the CAG repeats, which are primarily involved in fibril formation, with flanking N-terminal α-helical structures providing assistance [Hoop *et al.*, 2014]. Amyloid proteins are usually found in parallel-arranged β-sheets forming fibrils, but several anti-parallel structures for these proteins have also been proposed, e.g. in amyloid structures in Htt protein [Buchanan *et al.*, 2014; Tycko, 2006]. X-ray data revealed slab-like monoclinic lattice structures [Sharma *et al.*, 2005], whereas chemical cross-linking and circular dichroism showed the presence of coiled-coil multimers in overlapping regions between polyglutamine stretches and Q/N-rich domains [Fiumara *et al.*, 2010]. Much experimental evidence supports the formation of nanotube-like cylindrical structures in many amyloidic proteins, e.g. Aβ, Htt, α-synuclein, suppressor protein 35 (Sup35), and superoxide dismutase 1 (SOD1) [Elam *et al.*, 2003; Perutz *et al.*, 2002].

The crystal structure of mutant SOD1 implies an amyloid-like assembly of the dimeric protein, with alternative arrangements in linear or helical patterns, which give rise to hollow nanotube-like structures [Perutz *et al.*, 2002]. Familial amyloid polyneuropathy is a systemic amyloidosis caused by genetic mutations leading to peripheral neuropathy induced by amyloid deposition of transthyretin. It is often considered a model system for studying amyloid formation [Planté-Bordeneuve and Said, 2011]. A head-to-head (or tail-to-tail) anti-parallel alignment of transthyretin subunits helps during assemblage into continuous protofibrils containing β-sheets of indefinite length [Serag *et al.*, 2002]. Several other proteins associated with different disease models showed structural features of amyloidic fibrils. In an experimental rat lentiviral system, major loss of nigral dopaminergic neurons was observed with oligomeric protofibrillar forms of α-synuclein, but not with mature fibrils [Winner *et al.*, 2011]. A higher toxicity of oligomeric species as compared to mature fibrils has also been noted in other model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* [Karpinar *et al.*, 2009].

In a mouse model of Parkinson's disease, newborn α-synuclein transgenic mice were found to have a higher fraction of oligomeric as compared to monomeric or fibrillar α-synuclein, implicating these structural forms in the onset of the disease [Rockenstein *et al.*, 2014]. Under in vitro conditions, oligomeric protofibrils have a higher penetration of lipid membranes than do mature fibrils [Chen *et al.*, 2015]. Although amyloids plaques have long been thought to be the chief factor causing the onset of neurodegeneration, this view has been increasingly challenged by evidence showing that the soluble fraction of many disease-associated amyloidogenic proteins can be more toxic than their fibrillar forms [Dahlgren *et al.*, 2002; Lue *et al.*, 1999; McLean *et al.*, 1999]. Further work is needed to understand the intriguing physiological features and associated toxicities of these different forms of amyloid-forming proteins. It will also be important to address the factors causing intracellular amyloid formation despite the presence of a highly efficient PQC system maintaining cellular proteostasis.

2.4 CELLULAR PROTEIN QUALITY CONTROL PATHWAYS AND A POSSIBLE LINKAGE BETWEEN NEURODEGENERATION AND CANCER

Synthesis of new proteins and simultaneous degradation of old ones from cytosol is required for a balanced healthy proteome in the cell [Amanullah *et al.*, 2017; Chhangani and Mishra, 2013a]. It is also clear that the formation of amyloids inside cells is a regular phenomenon. Therefore, further investigations are needed in order to understand the linkage between amyloid deposition and disease onset. It is possible that cellular changes including an imbalance in proteostasis, caused by stressors or ageing could be the underlying cause of alterations in structural composition of amyloid deposits, which may therefore determine the onset of pathological symptoms [Amanullah *et al.*, 2017].

Brain sections of AD and Down syndrome patients have shown differences in the composition of deposited aggregates, supporting this suggestion [Piccini *et al.*, 2005]. This implies that protein aggregation should be viewed as a normal process of proteostasis inside cells, instead of as a causative agent of various diseases. Cells have evolved a very delicate balance between deposition and resolution of amyloidogenic aggregates [Chen *et al.*, 2011b; Chhangani *et al.*, 2013]. An imbalance between these two tightly bound processes may lead to unwanted accumulation of unfolded or misfolded proteins, interfering with normal cellular processes like signaling and metabolism, and finally resulting in death of neuronal cells. This could worsen the disease condition in many ways, including upregulation of inflammatory reactions and initiation of apoptotic pathways [Amanullah *et al.*, 2017].

An interesting observation is the inverse correlation between amyloidopathy and diseases like cancer. Neurodegeneration and cancer, both considered to be an outcome of post-translational dysregulation of gene expression, are present at two ends of a spectrum. Cancers are a result of uncontrolled proliferation of cells, whereas neurodegeneration occurs because of unwanted neuronal cell death [Du and Pertsemlidis, 2011; Plun-Favreau *et al.*, 2010]. Although a link is yet to be established, accumulating evidence suggests that people suffering from neurodegenerative diseases have a reduced risk of many types of cancer [Bajaj *et al.*, 2010; Ibáñez *et al.*, 2014; Zhang *et al.*, 2015]. Several reports show that many tumour-suppressor genes (e.g. p53, retinoblastoma, p27, cyclin E1, etc.) are upregulated in neurodegenerative disease patients [Absalon *et al.*, 2013; Hooper *et al.*, 2007]. Interestingly, p53 has also been reported to suppress amyloid precursor protein (APP) gene expression, hinting at a possible connection between these two distinct classes of disease [Cuesta *et al.*, 2009].

2.5 MODULATING PROTEIN QUALTIY CONTROL PATHWAYS FOR THERAPEUTIC PURPOSES

Ageing affects many major and minor metabolic pathways, often those crucial for normal functioning of the cells [Stranahan and Mattson, 2012]. Ageing can be seen as evolutionarily conserved deteriorating changes that take place inside cells, due to accumulation of mutations and reduced effectiveness of repair mechanisms [Lopez-Otin *et al.*, 2013]. Ageing interferes with the capacity to produce offspring, and evolution tends to favour characteristics through reproductive success. Natural selection may therefore be less effective in old age than in the healthy reproductive fraction of the population [Hamilton, 1966; Niccoli and Partridge, 2012]. However, id could be assumed that natural selection will not favour traits that make cells or individuals less fit. Interesting questions associated with amyloid formation and ageing can be raised, but there is little evidence available to allow us to reach a conclusion about possible factors that could be favored by evolution.

Instead of being designated as diseases, amyloid-borne diseases could be seen as a class of deteriorating changes, with various malfunctional effects that give rise to several diseases. This view is supported by the fact that these amyloidic diseases are not in the top 12 leading causes of deaths worldwide [WHO, 2014] (Fig. 2.3a). However, many of these causes are related to the formation of amyloids. For example, many types of dementia occur as a consequence of cardiac and vascular diseases [Justin *et al.*, 2013; Launer *et al.*, 2000; Skoog *et al.*, 1996]. Amyloid formation is also accelerated by smoking [Ott *et al.*, 1998] and diabetes [Strachan *et al.*, 2011]. Genetic mutations [Mayeux *et al.*, 1993; St George-Hyslop *et al.*, 1987] and traumatic injuries are also among the factors which can initiate amyloid formation [Van Den Heuvel *et al.*, 2007]. Various stressors [Chen *et al.*, 2011a], increased levels of cellular homocysteine [Kalmijn *et al.*, 1999; Quadri *et al.*, 2004] and apolipoprotein E [Tang *et al.*, 1998], various chemicals and metal exposures [Rondeau *et al.*, 2000], and an unhealthy diet [Kalmijn *et al.*, 1997b] including higher consumption of cholesterol [Simons *et al.*, 2001] may also induce and accelerate amyloidogenesis.

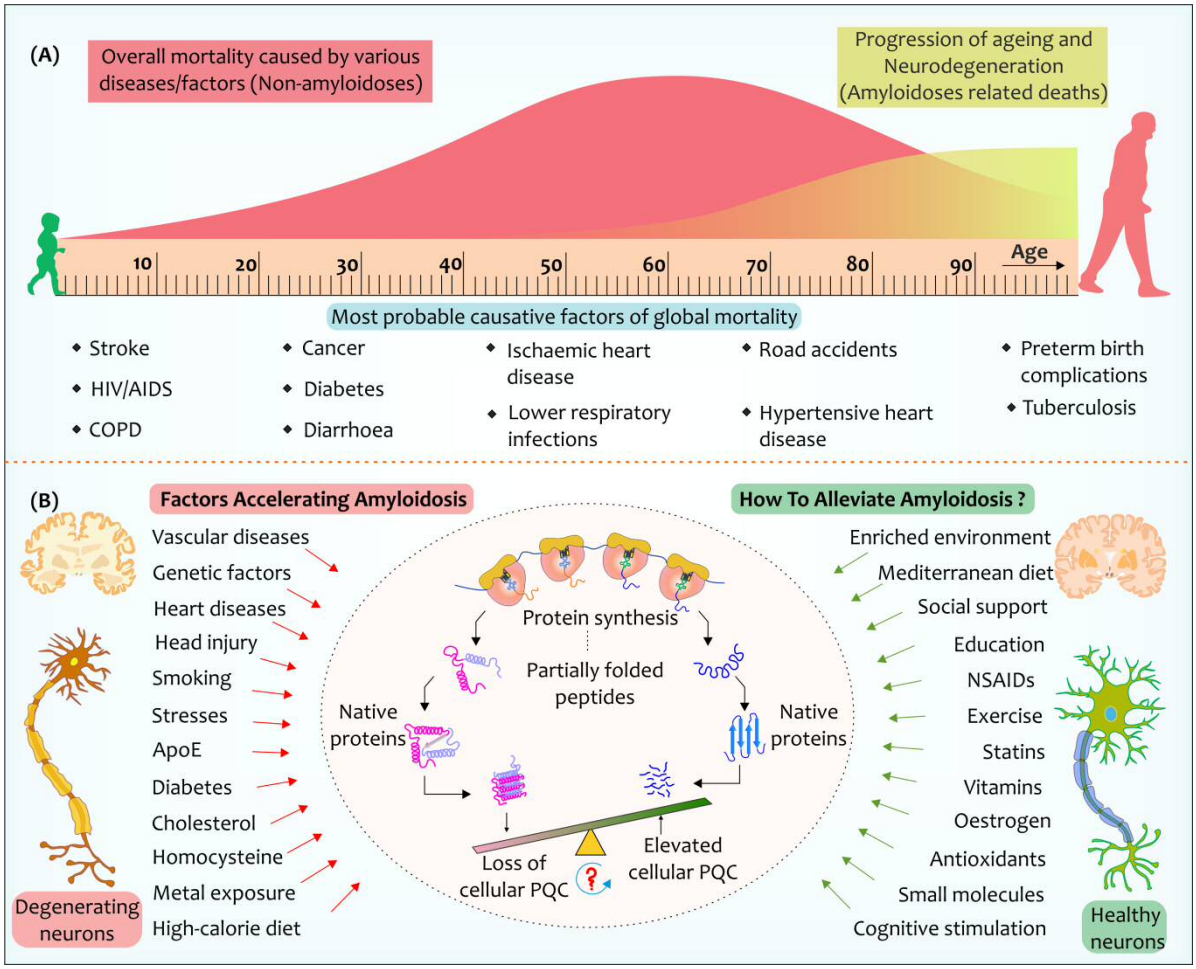

Figure 2.3 : *Overview of current understanding of amyloids*: (a) A schematic overview of global causes of mortality. Although amyloidoses affect a comparatively large proportion of people in old age, whether they directly cause death is still subject to debate. (b) An overview of factors affecting the rate of amyloid formation. Factors accelerating the process of amyloid formation and sequestration of aggregates are listed on the left; factors that reduce the overall rate of amyloid formation are shown on the right. The centre shows a general overview of cellular proteostasis. (Figure published in Upadhyay et al. 2018; Biological Reviews)

2.5.1 Methods to Suppress Amyloid Formation

Scientists are currently working, with some success, on various pathways, diet components, and lifestyles to reduce the risks of these diseases. Many studies have shown that a healthy diet and enriched environment may play a protective role against neurological disorders, and may also enhance memory formation and relieve cognitive impairment [Arendash *et al.*, 2004; Virmani *et al.*, 2013]. Environmental enrichment [Jamal *et al.*, 2016], dietary restriction [Sinclair, 2005] and reduced growth rate, in addition to genetic mutations [Pinkston *et al.*, 2006], have been shown to increase longevity in a variety of animals. An enriched environment [Jankowsky *et al.*, 2005a] and low-calorie Mediterranean diet has been correlated with a reduction in disease-like symptoms [Scarmeas *et al.*, 2006].

Education [Wang *et al.*, 2012] and providing extra care [Yurtsever *et al.*, 2013] to patients suffering from dementia or related disorders may also improve cognitive function. Various physical and cognitive exercises have given positive results in some studies [Gatz, 2005; Teri *et al.*, 2003]. Use of non-steroidal anti-inflammatory drugs (NSAIDs) [in t' Veld *et al.*, 2001; Vlad *et al.*, 2008] and antioxidants, including vitamins are other ways to reduce the likelihood of formation of amyloids [Douaud *et al.*, 2013; Kalmijn *et al.*, 1997a].

Therapies of oestrogen [Brinton, 2004; Janicki and Schupf, 2010] and statins [Jick *et al.*, 2000; McGuinness *et al.*, 2016] have also provided considerable success in fighting the symptoms of cognitive impairment. Similarly, a number of natural and synthetic small-molecule inhibitors of amyloid oligomerization and fibrillation could have possible therapeutic benefits [Necula *et al.*, 2007; Vassallo, 2015]. Inhibition of aspartic proteases, and β- and γ-secretases, is one such proposition now recommended in AD therapeutics [Chang *et al.*, 2004; Dovey *et al.*, 2001; Vassar *et al.*, 1999]. Activation of α-secretase by small molecules has also been investigated for potential to impede the generation of Aβ peptides [Postina *et al.*, 2004].

A separate class of drugs, consisting of galantamine, donepezil, and rivastigmine, that improve cholinergic transmission and thus enhance cognitive abilities of patients by inhibiting acetylcholinesterase, has already been approved for treatment of AD [Colovic *et al.*, 2013; Nordberg and Svensson, 1998]. Memantine represents another major drug class, approved for clinical trials. These molecules obstruct glutamate transmission by binding to N-methyl D-aspartate (NMDA) receptors on neuronal cells, thus reducing overall cell death [Lipton, 2006; Parsons *et al.*, 1999]. Another proposed therapeutic strategy is the prevention of Aβ synthesis by blocking the expression of mutant APP with doxycycline-like compounds [Jankowsky *et al.*, 2005b].

Induction of metal ion chelators is another effective strategy that has been applied to dissolve aggregated amyloid plaques under *in vitro* conditions [Atwood *et al.*, 1998; Huang *et al.*, 1997] in APP transgenic mouse brain [Cherny *et al.*, 2001], as well as in *post-mortem* brain sections of AD patients [Cherny *et al.*, 1999]. Small peptides have also been screened from peptide libraries or experimentally designed with potential to slow down aggregation processes of Aβ and IAPP proteins [Neddenriep *et al.*, 2011; Wang *et al.*, 2011]. β-sheet-breaker peptides belong to a class of molecules that have been tested widely for their amyloid-inhibitory potential against Aβ and prions [Chacon *et al.*, 2004; Soto *et al.*, 2000]. Neuroinflammation is considered to accelerate the overall process of pathogenesis in most neurodegenerative diseases. Hence, chemical compounds or strategies that counter inflammatory changes during neurodegeneration may also be advantageous [Amor *et al.*, 2010; Wyss-Coray and Mucke, 2002].

Receptor for advanced glycation end products (RAGE) is in the immunoglobulin family of receptors [Yan *et al.*, 1996], and could be targeted by small molecules to suppress the inflammatory pathways induced by RAGE in order to reduce neuroinflammation in the AD brain together with reducing levels of circulating Aβ peptides [Deane *et al.*, 2012]. Several other amyloidogenic or misfolded proteins have also been targeted therapeutically, using various inhibitors in several *in vitro* and *in vivo* studies [Aisen *et al.*, 2007; Awasthi *et al.*, 2017; Conway *et al.*, 2001; Heiser *et al.*, 2000; Korth *et al.*, 2001; Miroy *et al.*, 1996; Necula *et al.*, 2005; Zelus *et al.*, 2012]. Studies conducted on model misfolded proteins have helped us to understand the fundamental process of aggregation and disaggregation, with descriptions of the physical forces involved in these mechanisms [Kalhor *et al.*, 2009; Sarkar and Dubey, 2013]. These *in vitro* studies also proposed mechanisms of reducing the aggregation propensity of amyloidogenic proteins [Sarkar *et al.*, 2011c]; for example, using compounds that disrupt or induce random disulfide-bond formation [Sarkar *et al.*, 2011a] or which may alter the surface hydrophobicity of the aggregatory proteins [Sarkar *et al.*, 2011b].

Despite tremendous efforts to understand and solve the complex problem of amyloidogenic diseases, no successful cure has been devised to date. The blood–brain barrier (BBB) is a highly selective permeability barrier, which restricts the passage of numerous drugs and major molecules and essentially, it is one fundamental challenge to deliver drugs across the BBB [Nau *et al.*, 2010; Pardridge, 2011]. In recent years, the scientific community has focused increasingly on the anti-amyloidic properties of natural molecules and plant products in order to minimize potential side effects and to reduce the overall cost. It must be emphasized that

more detailed investigations are necessary to better comprehend the role of amyloidic proteins in the pathogenesis of such diseases. These deleterious effects of amyloid-borne diseases may well be enhanced by a poor lifestyle and improper nutrition. The risk could be minimized by a healthy lifestyle with sufficient exercise and dietary supplements. Much research is still required to establish the possible molecular linkage between amyloids and ageing.

2.5.2 Small Molecules

Natural/plant products and small drug molecules can be classified according to their different targets and modes of action [Re *et al.*, 2010]. Several strategies have been proposed for the treatment of AD, to slow down the progression of Aβ deposition, inhibit the aggregation process or to clear already deposited amyloid plaques [Awasthi *et al.*, 2016; Hawkes *et al.*, 2009; Mangialasche *et al.*, 2010]. Past few years of research have documented several efforts toward the development of therapeutic strategies by exploiting druggability of these E3 ubiquitin ligases, by using many natural and synthetic small molecules, having modulating effects over these ligases. Previous studies on natural molecules like curcumin or diferuloylmethane, extracted from the rhizomes of herb *Curcuma longa* have the abilities to interact with E3 ubiquitin ligase anaphase promoting complex (APC) and induce apoptosis in cancerous cells by inhibiting the Cdc27/APC3 component of APC, which is important for its ubiquitylation function [Lee and Langhans, 2012].

Trehalose, a disaccharide has been reported to increase levels of C-terminus of Hsp70 Interacting Protein (CHIP) and further induce autophagy in disorder of ataxia in fibroblast cells [Casarejos *et al.*, 2014]. MLN4924, an inhibitor of E3 ubiquitin ligase activity of sensitive to apoptosis gene (SAG), has shown a promising effect in sensitizing leukemia cells to effects of retinoic acid [Tan *et al.*, 2011]. It has also been shown that blocking the enzymatic activity of Nedd4, thereby modulating the budding of viruses, filoviruses, arenaviruses and rhabdoviruses, may help in the development of a novel class of antiviral therapy [Han *et al.*, 2014]. Proteasome inhibitor, MG132 has also been shown to increase the level of ubiquitin protein ligase E3 component N-recognin 1 (Ubr1), and this may act as an useful therapeutic strategy in pathologies caused due to reduced amount of Ubr1, like pancreatic dysfunctions and mental abnormalities characteristics of Johansen-Blizzard syndrome [Zenker *et al.*, 2005]. Similar effects of increasing E3 ubiquitin ligase activity have also been demonstrated for Zinc and Ring finger 1 (ZNRF1), in response to treatment of 6-hydroxydopamine [Wakatsuki *et al.*, 2015].

(a) Lanosterol

Lanosterol is an amphipathic terpene molecule that produces cholesterol upon demethylation in animals and ergosterol in yeasts and fungi [Nes, 2011]. In fact, it is involved in synthesis of all the steroids produced in our body, hence plays a very crucial role in overall body metabolism [Risley, 2002]. Lanosterol itself is produced from a multi-step enzymatic reaction that leads to cyclization and of squalene and oxidosqualene, constituting the intermediate steps of cholesterol biosynthesis pathways [Abe *et al.*, 1993]. Lanosterol synthase, a key molecule in lanosterol synthesis has been reported to be mutated in congenital cataract suffering families; and the studies have established that lanosterol treatment reduces the preformed aggregates of crystallins in the animal models of cataractous eyes [Zhao *et al.*, 2015]. Possible anti-tumorigenic effects have also been shown on colon cancercinogenesis in the past [Rao *et al.*, 2002]. Altered sterol biosynthesis in neurodegenerative diseases has dragged tremendous attention in the past [Puglielli *et al.*, 2003]. Identification of decreased lanosterol synthesis in Parkinson's disease mice model and subsequent relief from disease symptoms following exogenously provided lanosterol has provided necessary clues towards its possible neuroprotective properties [Lim *et al.*, 2012]. Also, importance of lanosterol in the cell metabolism and survival of organisms has been exploited in production of anti-fungal drugs [Nigam, 2015].

(b) Ibuprofen

Non-steroidal anti-inflammatory drugs, which are widely used to treat fever, reduce pain and suppress inflammatory reactions by targeting prostaglandin synthase enzymes [Smith *et al.*, 2000]. These enzymes, commonly known as cyclooxygenases (COX1 and COX-2), catalyze the synthesis of prostaglandin H_2 from arachidonic acid [Vane and Botting, 1998]. COX-1 performs few crucial housekeeping functions inside the body, whereas COX-2 is inducible in varying conditions [Hawkey, 2001]. Several important functions are constitutively performed by COX1, which help in maintenance of a healthy gastrointestinal mucosa, renal tract, and platelet functions [Smith *et al.*, 2000]. On the other hand, COX-2 produces few critical prostaglandins that are specifically synthesized inside the body to induce inflammation, fever and pain [Ricciotti and FitzGerald, 2011]. Owing to the wide-spectrum of the downstream effects of these enzymes, regular consumption of the NSAIDs leads to some severe side-effects ranging from gastrointestinal erosions to abnormalities in renal and hepatic functioning and difficulties in platelet aggregation [Suleyman *et al.*, 2007].

In the past few years, enormous reports have suggested that use of NSAIDs, alone or in combination with other drugs, can have enormous chemopreventive potential [Ulrich *et al.*, 2006]. Ibuprofen is a propionic acid derivative affects the activity of both, COX-1 and COX-2, and but has been reported with lesser side-effects as compared to other well-known NSAIDs [Nanau and Neuman, 2010]. It was introduced in 1969 in United Kingdom and soon started to be prescribed for arthritis and other pain-related inflammatory conditions with a suggested dose of 2400 mg per day [Rainsford, 1999]. Ibuprofen is an over the counter drug containing a recemic mixture of S (+) and R(-) enantiomers, in which S(+) ibuprofen is the highly active form. R(-) form is also converted to S(+) form via epimerization and hydrolyzation reactions [Brocks and Jamali, 1999].

In the recent years, a number of studies have been conducted on many NSAIDs, especially aspirin and ibuprofen, showing their anti-tumorigenic effects against various types of cancers [Harris, 2015; Khwaja *et al.*, 2004]. Multiple types of cytotoxic stress inducing properties of NSAIDS, including indomethacin and ibuprofen, leading to increased apoptosis inside the cells, have also been identified [Tomisato *et al.*, 2004; Tsutsumi *et al.*, 2004]. However, further studies are required to investigate the detailed effects of ibuprofen on stress-inducing cellular pathways that help in initiation of pathways leading towards programmed cell death or apoptosis.

2.6 CONCLUDING REMARKS

The need of cost-effective therapies that have minimal side-effects and which can reach up to every individual is not yet successfully fulfilled and thus extensive exploration of natural and synthetic compounds will enable us to devise useful, and effective strategy to prevent and cure such diseases [Collins *et al.*, 2017; Tsukamoto, 2016]. To devise the molecular therapeutics that can be used as potential strategies for modulating the functions and activities of various components of PQC pathways in treating multiple disorders, extensive studies are required, which can characterize these lead molecules for their efficacy, side effects, safety and pharmacological profiles. The above-mentioned small molecules are merely a fraction of research, which could be considered for upcoming strategies to treat fatal neurodegenerative disorders, cancer or other complex diseases, but there still remains a need for more research to be done [Eldridge and O'Brien, 2010; Gurevich and Gurevich, 2014; Skaar *et al.*, 2014].

Targeting specific QC components for the treatment of particular disease is not an easy way to exercise in therapeutic applications, as these components could be involved in multiple pathways simultaneously. Although few drugs, such as Nutlin-3, a Mdm2-p53 interaction inhibitor, is used for treating pathology of cancer, but it is still in clinical trials and has also

shown few side effects in the treatment of retinoblastoma [Secchiero *et al.*, 2011; Vassilev *et al.*, 2004]. Development of ubiquitin variant probes having the abilities to target the ubiquitylation functions of E3 ubiquitin ligase can also prove to be an effective strategy in disease treatment [Zhang *et al.*, 2016].

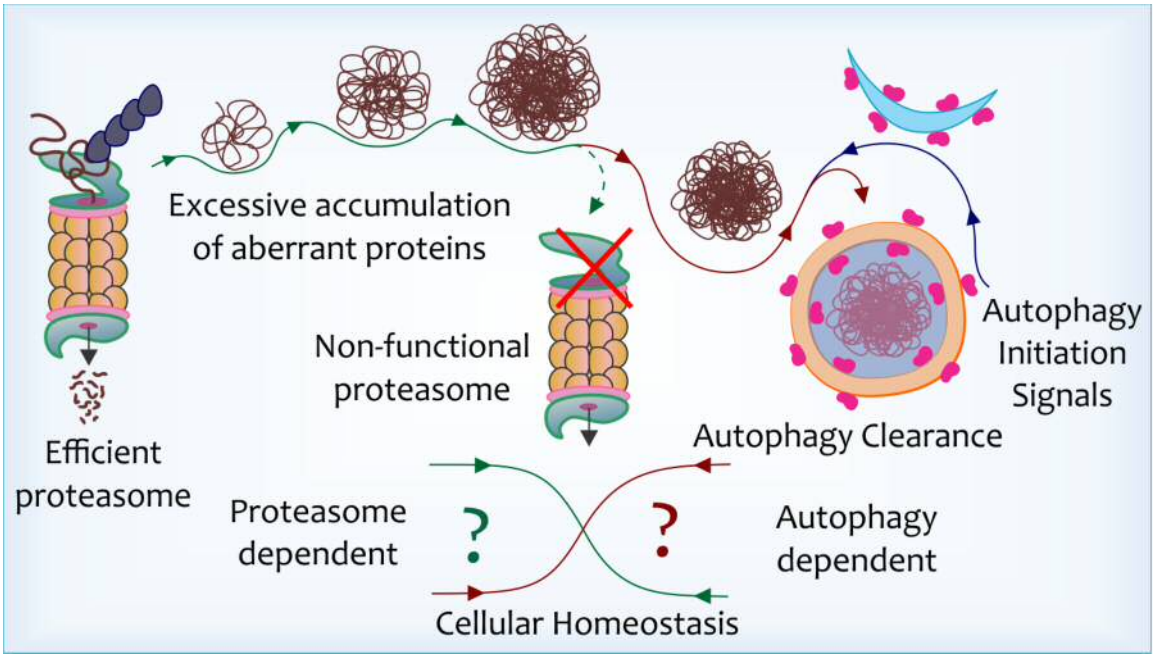

Figure 2.4. *An overview of orchestration of cellular proteolytic machinery to reestablish homeostasis:* Misfolded proteins are targeted towards proteasomal degradation by a concerted action of multiple enzymes of UPS. However, increasing intra- or extracellular stresses tend to accumulate bulk of cellular proteins that may form amyloidic structures and generate inclusion bodies. Under such conditions, autophagy pathway mounts heightened stress response and start degrading and clearing large molecular weight proteinaceous inclusions from the cytoplasm.

Finding out solutions to these problems will significantly help in bringing these drugs or small molecules from basic research to the level of therapeutic applications. Therefore, there remains an immense need of understanding complete functionality and specificity, which these cellular players could provide in order to get the maximum beneficial outcome for future drug development strategies.

...