1. Introductory Review

1.1 Neurodegenerative Diseases

Neurodegenerative diseases may be defined as a group of complex neurological disorders comprising of loss of neurons either in the peripheral nervous system or central nervous system. Yet however, finding a precise definition for neurodegeneration is hard to find. Etymologically, the word “neurodegeneration” comprises of the words “neuro” and “degeneration”. “Neuro” refers to nerve cells and “degeneration” may refer to a process in which there is a loss of structure or function. Pathologically it refers to a condition affecting neurons. Hence, neurodegeneration is an umbrella term which pertains to the progressive loss of structure or function of neurons, or may also indicate death of neurons. Neurodegeneration is commonly used to represent a variety of neurological disorders with heterogeneity in their clinical and pathological symptoms. They also affect various specific populations of neurons, in specific anatomical functional regions. The specific cause for neurodegeneration is largely unknown, but once its onset is established it progresses in a largely relentless manner. Pathological conditions which are excluded from the umbrella term of neurodegenerative diseases are neoplasm, edema, hemorrhage, etc. These disorders basically do not involve the primary neuronal regions and hence do not qualify as neurodegenerative diseases. Conditions in which neurons die due to hypoxia, infections or metabolic perturbations are not included under the term neurodegenerative diseases either [1].

Neurodegeneration is a condition which accounts for death of neurons in abundance, due to several reasons. Neurons are post mitotic and terminally differentiated [2], loss of it exacerbates the problems of neurodegeneration debilitating the condition of the brain. Studies on human brain, animal models and cell culture models have revealed the occurrence of several death paradigms in neurodegeneration [3]. It is therefore clear that multiple mechanisms of cell death govern the devastation caused in neurodegeneration. Cross talk between these processes is also an indispensable mode of death of neurons. Cell death mechanisms are induced during development for proper organization of cells with respect to space and content, or during disease condition to eliminate unhealthy dying cells. Different neurodegenerative diseases may be governed by different specific dominant death pathway, however other death paradigms also play minor roles in a death scenario [2]. Different death mechanisms may be acting in varied portions of the same neuron in response to the same stress stimuli [4]. Apoptotic death

of neurons has been extensively studied [5]. Acute and chronic neurodegeneration is associated with both apoptotic and necrotic cell death [6, 7] [8] These death paradigms may be distinctly varied in some situations while in others they may occur in synchronization [9]. The factors that decide the extent of mutualism between the death pathways are the basic energy content of the cell and the strength of the stimulus [9]. Studies showed that the spatial location of neurons governed partly their fate, when subjected to ischemic brain injury the neurons at the periphery underwent apoptosis which was rescued by caspase inhibition, while the core neurons could not be rescued by inhibition of caspases thus indicating that they died through necrosis [10].

When cells are unable to combat stress which is beyond their tolerance level they undergo necrosis, an acute non-apoptotic form of cell death. The mechanism of cell death is conserved from nematodes to humans. Pathological neuronal death may occur by necrosis in the brain in neurodegeneration. Ischemic brain injury inflicts selective neuronal necrosis of highly vulnerable neurons [11]. Necrosis in neurons is triggered by excessive energy depletion [12]. Loss of energy leads to collapse of ionic gradients required for sustenance of neurons which is followed by build up of glutamate at synaptic clefts [13, 14] . Increased accumulation of glutamate at the synaptic clefts causes necrotic death of downstream synaptic target neurons [15, 16]. A common anomaly of neurodegeneration is metabolic perturbation due to which there is excessive release of reactive oxygen species which leads to necrosis [17]. Proteolytic systems also largely influence necrosis [18] [19]. Release of Cathepsin D and E have been noted to cause necrotic death in transient fore brain ischemia [20]. ASP-3 and ASP-4 have been reported to cause necrotic cell death [21]. Spatial localization of cathepsins plays critical roles in neurodegeneration. Young rat brain cortices exhibit lysosomal localization of Cathepsins, while cytosolic localization of Cathepsin is observed in aged rat brain cortices [22]. Accumulating evidence reveals that instead of assuming a chaotic architecture, necrotic cell death may be following a well organized pattern [21, 23, 24]. Extensive studies on the probable molecular mechanism of necrosis brings forth only a limited number of key players and their mechanism that could be involved in the process. Expression of mec(d)4 in caeonorhabditis elegans was shown to cause dysfunction of nerve cells along with several other affected cells. Many neurodegenerative diseases may be caused due to genetic mutations. These mutations may lie in unrelated genes. A common example of this is the Polyglutamine diseases

which occur due to CAG repeats resulting in polyglutamine (polyQ) tract. Perturbations in the intracellular mechanisms may also lead to neurodegenerative diseases. Parkinson's disease and Huntington's disease are both associated with anomaly in the protein degradation pathway. The intrinsic mitochondrial apoptotic pathway accounts for the most common type of neurodegenerative cell death. Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic lateral sclerosis are the four diseases which include strong evidence of mitochondrial dysfunction. Wallerian-like degeneration is triggered due to disruption in the axonal transport system. Whereas; aberrant apoptosis, autophagy and transglutaminases is strongly evident in Alzheimer's disease [25].

1.2 Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease which accounts for about 80% of dementia among the elderly population worldwide. Clinically AD is characterized by the gradual impairment of cognitive functions, which ultimately culminates in death of the individual. Specific regions of the brain are affected in AD. The specific neuropathological sites in AD include the hippocampus, the amygdala, the temporal and frontal cortex. With an increase in life expectancy, as the population ages, there is an increase in the number of patients suffering with dementia. According to the World Alzheimer's Report 2016, 46.8 million people are living with dementia worldwide and this number will increase upto 131.5 million by 2050. The report also provides the information that even in most of the high income countries people with dementia either do not have any access to, or very poor access to appropriate healthcare and awareness. The scenario in these high income countries is such that only 50% of people suffering from dementia receive diagnosis. Whereas in low and middle income countries the number of people being diagnosed drops down to only 10% (World Alzheimer's Report 2016).

1.2.1 Discovery of Alzheimer's Disease

About a 110 years back, in 1906 on the 3rd of November, a German psychiatrist and neuroanatomist, Alois Alzheimer reported "a peculiar severe disease process of the

cerebral cortex" to the 37th Meeting of South-West German psychiatrists in Tubingen [26]. In his report he described the history of a patient, a 50-year old woman, Mrs. Auguste Deter, whom he had followed from her admission with initial symptoms of paranoia until her death five years later.

Figure I.1: Dr. Alois Alzheimer (1864-1915)

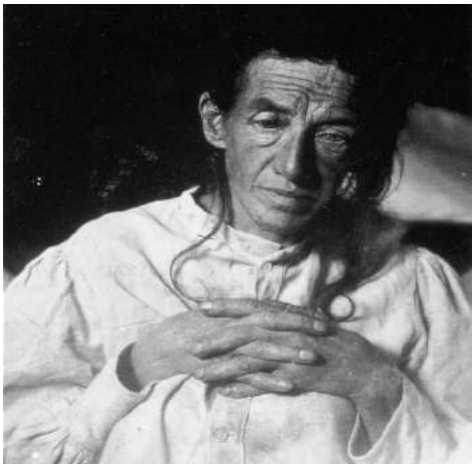

Figure I.2: Auguste Deter (1850 – 1906)

Along with symptoms of paranoia she also suffered with progressive sleep and memory deficits, phrenic malfunctioning, aggression and confusion. On performing autopsy of the brain he observed extensive atrophy of the cerebral cortex. Thin slices of the brain tissue were stained with silver salts which when examined under the microscope revealed distinctive plaques and neurofibrillary tangles around nerve cells. These results of Alois Alzheimer were only able to elicit little interest among others except Emil Kraepilin, a German psychiatrist, who promptly named it "Alzheimer's Disease" and included it in the 8th edition of his text Psychiatrie in 1910. In 1909 Alzheimer further published three different cases and in 1911 he came up with a "plaque-only" variant. Soon after receiving the chair in psychiatry in Breslau Alzheimer died in 1915, aged 51, which was long before AD became a common household disease.

1.2.2 Etiology of Alzheimer's Disease:

Alzheimer's disease being a multifactorial disease is caused by complex interactions between genetic, epigenetic and environmental components. The early onset of autosomal dominant form of AD that occurs at an age earlier than 60 yrs can be caused

by three gene mutations amyloid precursor protein (APP), Presenilin1 (PS1) and Presenilin2 (PS2) [27]. These mutations alter the processing of APP resulting in abnormal production of amyloid β (Aβ) peptides. Late onset AD may be caused due to genetic variations occurring in the promoter sequence of APP leading to enhanced production of APP. The level of APP expression is inversely proportional to the disease progression [28].

1.2.3 Early Onset Alzheimer's Disease

Early onset AD sets in patients of the age group 30 to 60 years of age. This is a rare form of AD. It represents only 5% of the total AD patients worldwide. Usually the cause for early onset Alzheimer's disease is not known, while it has been noticed that such cases are mostly inherited from the previous generations. This type of AD is referred to as FAD. Single gene mutations are mainly the cause for FAD. They may occur in any of the chromosomes 21, 14 and 1. The result of each of these mutations is the formation of abnormal proteins. Abnormal Amyloid Precursor Protein (APP) is a result of mutation in chromosome 21. Mutation in chromosome 14 and chromosome 1 leads to the production of abnormal Presenilin 2 and 1 respectively.

1.2.4 Late onset Alzheimer's Disease

The most prevalent form of AD is the late onset AD which occurs in patients of over 60 years of age. The exact pathological reason behind the occurrence of this late onset AD is still not clear. However, reports suggest a variety of causes behind its occurrence which include genetic, environmental lifestyle factors or a combination of all, these play important roles in the disease-susceptibility of the patients.

As mentioned earlier single gene mutations are responsible for the early onset AD, they do not seem to have any role in the late onset AD. However, a single genetic factor, apolioprotein E, APOE resides on chromosome 19 and is suggested to play a pivotal role in increasing the disease vulnerability among its bearers [29]. The main function of APOE protein is to help in the transport of cholesterol and fats in the bloodstream. In the central nervous system APOE is mainly produced by the astrocytes, these proteins help in the transport of cholesterol to the neurons via the APOE receptors [30]. The three most frequently observed alleles of APOE are APO ε2, APO ε3 and APO ε4. APO

ε2 is less prevalent and may play a role in protection against the disease. Patients that develop AD due to a mutation in APO ε2 do so at a stage later than patients with mutation in APO ε4 would have developed the disease. APO ε3 is said to be a neutral protein, which does not play any role in disease development or progression. APO ε4 mutation is the most predominant form of the mutation found in almost 40% of patients with late-onset AD [31].

1.2.5 Epigenetics

Reports suggest a strong role of epigenetics in development of AD [32]. Studies on human post mortem brain samples, peripheral leucocytes as well as in transgenic mouse models show that epigenetic changes in DNA methylation and histone modifications is observed with aging and AD [33]. Though the presence of epigenetic changes has been validated to be correlated with the disease, however, its ability to be the cause of disease development or progression still remains obscure [33]. Recent studies linking memory and epigenetic changes in the DNA have come to the fore [32]. Day *et al* 2011 [34] showed that pharmacological inhibition of DNA methylation impaired memory in mice. While acetylation of histone improves memory and also leads to enhanced expression of genes associated with learning and memory in aged wild-type mice [35].

1.2.6 Environmental risks

Several environmental risk factors of AD crop up with age. These include hypertension, head sprain, hyperlipidemia, homocysteinemia, obesity, diabetes mellitus and low education levels [36-38]. The most potential environmental risk factor for AD is aging. The association of APO ε4 with the above mentioned risk factors may definitely enhance the risk for late onset AD and the risk for age related dementia.

1.3 Neuropathological Hallmarks of Alzheimer's Disease

The neuropathological hallmarks of AD include Aβ plaques and cerebral Aβ angiopathy, neurofibrillary tangles, and glial responses, neuronal and synaptic loss. The two pathognomonic hallmarks of AD are:

1.3.1 Aβ as a hallmark of Alzheimer's disease

The 4 kDa Aβ peptide, derived from the larger APP, was first isolated as the principal component of Aβ deposits in the brain and cerebrovasculature of AD and Down's Syndrome patients. Extensive research has advanced our knowledge of how the Aβ peptide is produced, and how it is subsequently degraded within the brain, or transported out into the periphery.

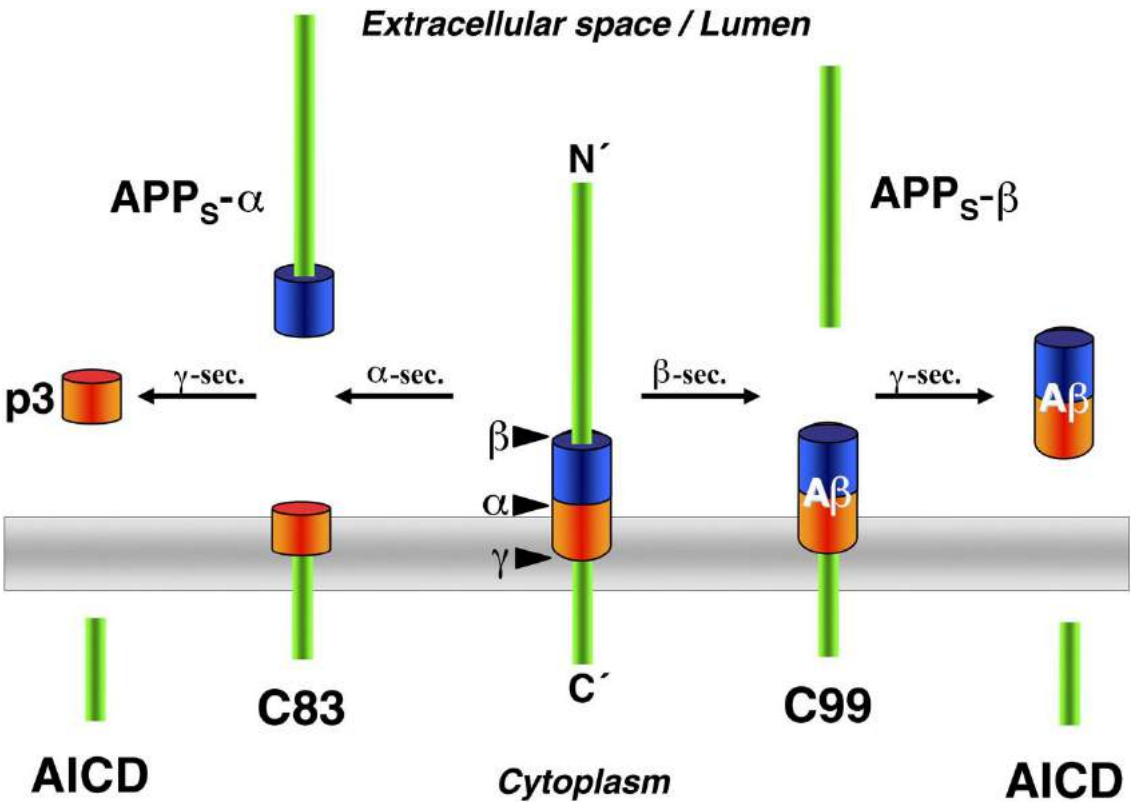

Figure I.3: APP processing pathway
Source: JCB. A lipid boundary separates APP and secretases and limits amyloid β-peptide generation. Christoph Kaether, Christian Haass.
DOI: 10.1083/jcb.200410090. Published December 6, 2004

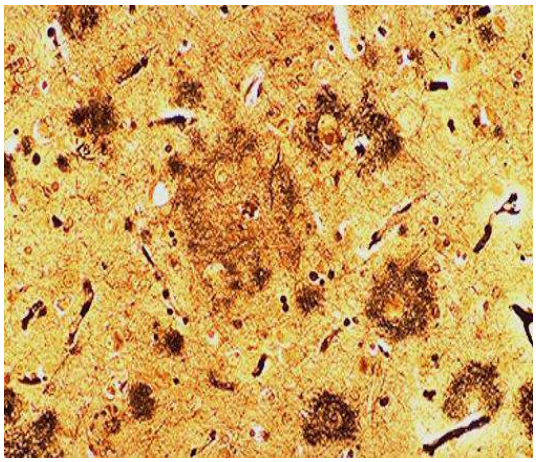

Figure I.4: Amyloid β plaques of varying sizes observed in silver stained brain section of patient with Alzheimer's disease.
Source:https://library.med.utah.edu/WebPath/CNSHTML/CNS090.html

The final amount of Aβ that accumulates as Aβ deposits within the brain is determined by the interplay of these factors. The enzymatic processes responsible for the metabolism of APP to Aβ are now reasonably well understood. APP is sequentially cleaved by two membrane-bound endoprotease activities, β- and γ-secretase. β-secretase first cleaves APP to release a large secreted derivative, sAPPβ. A fragment of 99 amino acids (CTFβ, which begins with the N-terminal aspartyl residue of Aβ) remains membrane bound, and is in turn rapidly cleaved by γ-secretase to generate Aβ. Cleavage by γ-secretase is somewhat imprecise, resulting in C-terminal heterogeneity of the resulting peptide population. Hence, numerous different Aβ species exist, but those ending at position 40 (Aβ40) are the most abundant (~80-90%), followed by 42 (Aβ42, ~5-10%).The slightly longer forms of Aβ, particularly Aβ42, are more hydrophobic and fibrillogenic, and are the principal species deposited in the brain. β-secretase activity is believed to be the rate limiting step in the Amyloidogenic pathway, and processes ~10% of the total cellular APP. The remaining APP, close to 90%, is constitutively cleaved by α-secretase (a collection of metalloprotease enzymes), generating sAPPα and the 83 amino acid CTFα. The subsequent γ-secretase cleavage of CTFα produces the more benign p3 fragment instead of Aβ. β-Secretase activity and protein level are both significantly increased in sporadic AD and although the amount of γ-secretase activity does not appear to increase in AD, alterations in γ-secretase activity leading to the production of longer forms of Aβ are the major genetic cause of early onset, familial AD. Extracellular Aβ plaques formed exert toxicity by acting on specific neurotransmitter

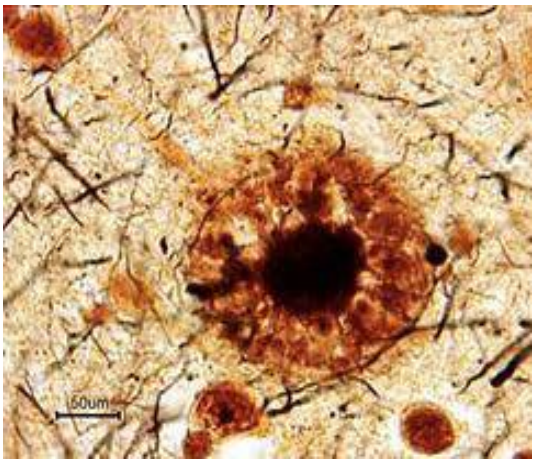

Figure I.5: Magnified view of an Amyloid β plaque.
Source: http://bigthink.com/articles/the-brain-plaques-and-tangles-that-cause-alzheimers-disease

receptors and surface membranes, and by activating pathogenic cellular signaling cascades. Along with the extracellular plaques however several lines of investigation support the notion that the pathogenesis of AD is related to progressive accumulation of Aβ protein inside the cell, which is derived from the proteolysis of Aβ precursor protein [APP] that along with the plasma membrane can take place in the membranes of the Golgi, ER, endosomes, lysosomes and the mitochondria. Abnormal accumulation of Aβ is the result of an imbalance between the levels of Aβ production, aggregation and clearance. Aβ clearance is mediated by proteolytic enzymes such as neprilysin, chaperone molecules such as apoE, lysosomal [e.g. autophagy] and non-lysosomal pathways [e.g. proteasome]. Targeting intracellular Aβ may prove to be a positive therapy for AD [39].

While in familial forms of AD, mutations result in an increased Aβ production or aggregation, in sporadic AD, failure of the clearance mechanisms might play a central role. Progressive accumulation of Aβ results in the formation of Aβ oligomers and fibrils which are the principal components of the plaque. Most evidence supports the notion that the Aβ oligomers rather than the fibrils are responsible for the synapto-toxic effects of Aβ.

1.3.2 Neurofibrillary tangles

The neurofibrillary tangles consist of tau protein which is an important part of the microtubule. In healthy neurons the microtubules remain associated with tau protein. Tau is said to stabilize the microtubules, an essential part of the cell cytoskeleton. Among the major functions of tau are regulating microtubule dynamics, axonal transport and neurite outgrowth. Tau mediates these functions by phosphorylation at specific sites [40, 41]. Disruption in the event of phosphorylation results in dysfunction of tau and acts as a determining factor for

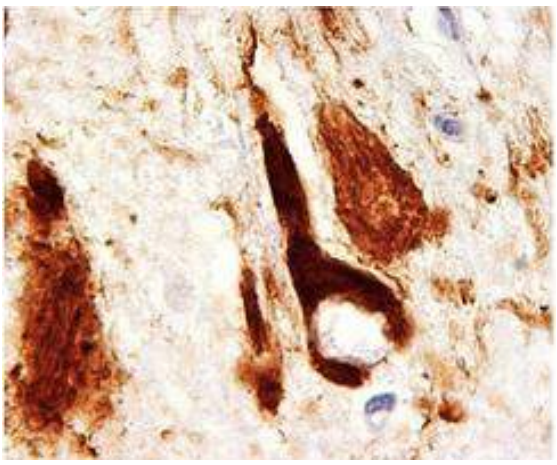

Figure I.6: Magnified view of neurofibrillary tangle.
Source:http://bigt`hink.com/articles/the-brain-plaques-and-tangles-that-cause-alzheimers-disease

AD pathogenesis.

Abnormal hyperphosphorylation of the microtubule-associated protein tau and its simultaneous incorporation into neurofibrillary changes [neurofibrillary tangles (NFTs), dystrophic neurites surrounded by neuritic plaques (NP) and neuropil threads] are major pathological hallmarks of AD pathophysiology [42]. In the CSF, P-tau phosphorylated at threonine 231 (P-tau231P) was studied. However, a near identical phosphorylation of tau, driven by CDKs, also occurs when cells are mitotically active [43] suggesting a close link between cell cycle and tau. Hyperphosphorylated tau containing intracellular neurofibrillary tangles in the brain of an individual with AD are particularly abundant in the entorhinal cortex, hippocampus, amygdala, association cortices of the frontal, temporal and parietal lobes, and certain subcortical nuclei that project to these regions [44, 45].

1.4 Hypotheses of AD Pathogenesis

AD is a complex disease. Hence a single approach is not enough to explain the complexity of the disease. Several different hypotheses have been proposed to explain the pathogenesis of the disease. Among the several hypotheses the earliest proposed was the Cholinergic hypothesis.

1.4.1 Cholinergic hypothesis

According to this hypothesis there occurs a decrease in cholinergic transmission in the disease. This plays a major role in the observed symptoms of abnormalities in the cognitive, functional and behavioural domains of the patients. This hypothesis is based on the fact that cholinergic neurons are specifically vulnerable in this disease scenario [46, 47]. Mandelkow *et al* 1990 show that a specific population of neurons in the nucleus basalis of Meynert in the basal forebrain is specifically degenerated in AD while these neurons are responsible for most of the acetylcholine transferred to the cerebral cortex. However; this hypothesis does not hold strong due to lack of evidence that a cholinergic deficit is implicated in early stages of AD or in patients with mild cognitive impairment

1.4.2 The Amyloid cascade hypothesis

John Hardy and Gerald Higgis in 1992, first proposed the Amyloid cascade hypothesis [48]. Today it is the most accepted hypothesis to explain the etiology and pathogenesis of AD. According to this hypothesis accumulation of Aβ is an event that takes place early in neurodegeneration. Alteration in the processing or expression of APP qualifies as the first event in the pathogenesis of AD. There occurs an imbalance between Aβ clearance and production of it, this leads to the oligomerisation, fibril formation and induced accumulation of Aβ into plaques. Further Aβ accumulation induces microglial activity and astrocytic reactivation, pro-inflammatory response, oxidative stress, followed by formation of NFTs, finally leading to neuronal death.

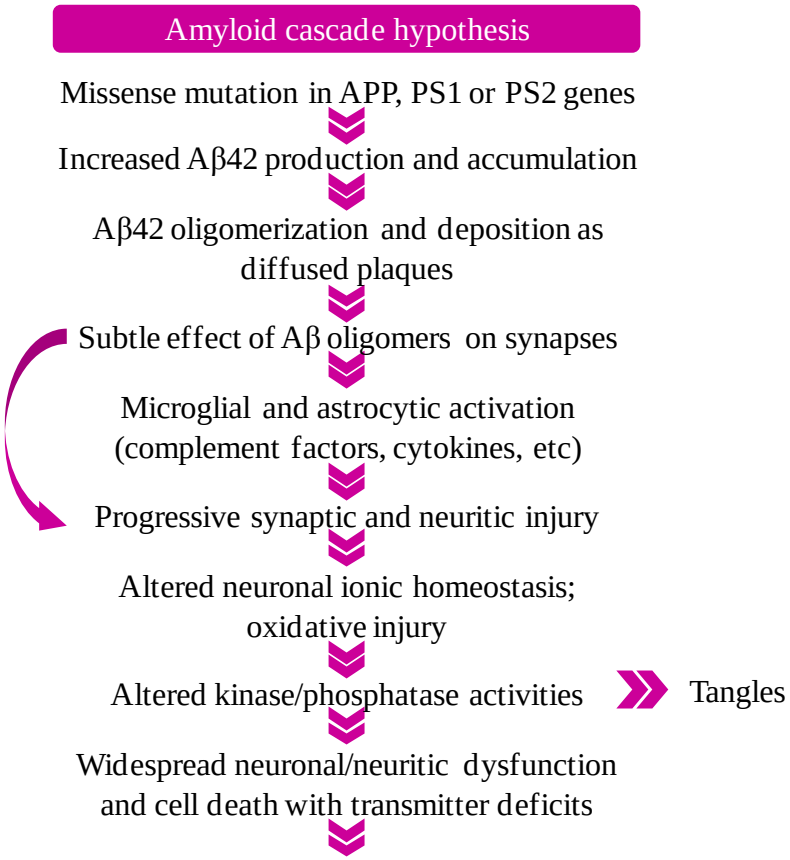

Figure I.7: Amyloid cascade hypothesis
Source: Drug Des Devel Ther (2013). Advances with RNA interference in Alzheimer's disease research. Chen S, *et al;*
DOI: 10.2147/DDDT.S40229

There have been several studies that support the Amyloid cascade hypothesis. The FAD has been characterized by the prevalence of the autosomal dominant mutations in either of the Presenilins or APP genes [49]. This hypothesis has been modified over years as it became clear that the deposition of Aβ in form of plaques is not linearly correlated with cognitive impairment in AD. In contrast, the cognitive deficit shows a prominent relationship with loss of synaptic contacts [50]. Recent studies promotes the 'synaptic Aβ hypothesis', which focuses on the deposition of non fibriller oligomeric Aβ at the synapses. It was said that a decrease in synapses due to oligomeric Aβ leads to subsequent neuron death. This results in cognitive dysfunction and dementia. According to Snyder et al [51] synaptotoxicity by oligomeric Aβ involves nicotin receptor and NMDA receptor leading to reduced glutametargic transmission and impaired synaptic plasticity.

1.4.3 The Tangles hypothesis

In opposition to the Aβ cascade hypothesis, the Tangles hypothesis has been proposed [52]. Due to the hyperphosphorylation of tau, its ability to bind to the microtubules is lost. This results in self aggregation between cytoplasmic free tau, which leads to loss of cytoskeletal function and cell death. Moreover there exists a better correlation between presence of tangles with the status of the disease than with the presence of plaques [53]. Furthermore, tangle formation is said to initiate in those parts of the brain which are specific for memory [54]. That tau can be a pathogenic protein inducing dementia was established by the discovery of tau mutations in families with fronto temporal dementia. Interestingly, it has been observed that tau mutations do not result in plaque deposition while mutation in APP leads to both plaque deposition as well as tau hyperphosphorylation. This explains that amyloid build up precedes hyperphosphorylation of tau in AD.

1.4.4 Other hypotheses

Other hypotheses include **Calcium Hypothesis** which states that alteration in calcium signaling induces both plaque formation and hyperphosphorylation of tau [55]. The **Cholesterol Hypothesis** suggests that the AD pathogenesis develops from anomaly in cholesterol uptake and its metabolism. This results in abnormal trafficking of membrane

proteins which disrupts normal neuronal function and synaptic plasticity [56]. The **APO ε Hypothesis** suggests that APOε lies central to all the pathologies observed in AD [57]. Finally the **Mitochondrial Hypothesis** holds that mitochondria serve as the hub of production of reactive oxidative species and an early target for ROS. Mitochondrial dysfunction poses as a common threat in several neurodegenerative disorders including AD [58].

1.5 Autophagy

Christian De Duve coined the term autophagy (auto phagin from greek which means Self-Eating) in 1963, while he discovered the organelle lysosome in 1955. In 1992, Yoshinori Ohsumi found tremendous similarity between the method of autophagy in yeast and mammals, and hence recommended yeast as a model system for genetic studies on autophagy. mTOR the mammalian target of rapamycin (mTOR) gene was isolated from yeast system in 1993 and from mammals in 1994. In 1995, Alfred Meijer showed that rapamycin, inhibitor of mTOR, induces autophagy. Noboru Mizushima first identified autophagy genes, Atg5 and Atg12 in 1998, and along with Tamotsu Yoshimori in 2000, identified the mammalian homologue of yeast Atg8, MAP1LC3 (also called LC3).

Autophagy is an evolutionarily conserved process in eukaryotes whereby unwanted proteins and shoddy organelles in protoplasm is delivered to lysosomes for degradation by fusion with them. There are 3 major kinds of autophagy in eukaryotes:

- **Chaperone-mediated autophagy**
- **Microautophagy**
- **Macroautophagy**

1.5.1 Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) has been solely characterized in higher eukaryotes. It involves an instantaneous translocation of cytosolic proteins across the lysosomal membrane. Proteins containing a pentapeptide motif (KFERQ or similar sequences) are specifically recognized by HSC70 (Heat Shock Protein Complex 70). The HSC70 facilitates protein unfolding and also delivery of the macromolecule to the CMA receptor lysosome-associated membrane protein type-2A (LAMP2A). LAMP2A functions not only as a receptor but also a pore or a channel facilitating translocation of the proteins across the lysosomal membrane [59, 60].

1.5.2 Microautophagy

Microautophagy, characterized chiefly in yeast, is a process in which an invagination in the vacuole membrane sequesters the unwanted protoplasmic material [61]. It's typically thought that the basal rate of protein degradation in non-stimulated conditions occurs through microautophagy [62, 63]. Microautophagy though the name may suggest it to be trivial, actually possesses the capability to sequester giant structures like entire organelles through both selective and non-selective mechanism.

1.5.3 Macroautophagy

Macroautophagy, hereafter referred to as autophagy, is the best characterized process of autophagy. It had been first studied and characterized in yeast cells by electron microscopic studies [64-66]. This method involves nucleation of a membrane, termed as phagophore or isolation membrane (IM). The IM expands to create a double-membrane sac referred to as the autophagosome. The autophagosome either fuses directly with the lysosome or with endocytic vesicles, generating associate amphisome that eventually fuses with the lysosomal compartment leading to the formation of autolysosome where the sequestered material becomes degraded by lysosomal hydrolases.

1.5.4 Molecular Mechanism of Autophagy

Induction

The molecular mechanism of autophagy involves different Atg proteins. For induction, there should be first repression of mTOR, although mTOR independent path is functional too [67-69]. Stimuli like nutrient deprivation, leads to inhibition of mTOR [70]. This leads to the formation of the phagophore, a step that involves 2 complexes:

1. Category III PI3K/Vps34, Atg6/Beclin1, Atg14 and Vps15.
2. Serine/Threonine enzyme Atg1/ULK1

Elongation and autophagosome formation

The elongation refers to the formation of the characteristic double-membrane sac termed as autophagosome. This step needs 2 ubiquitin-like conjugation pathways, each pathway must be catalysed by Atg7. The first ubiquitin-like system leads to the conjugation of Atg5-Atg12, with Atg16L.

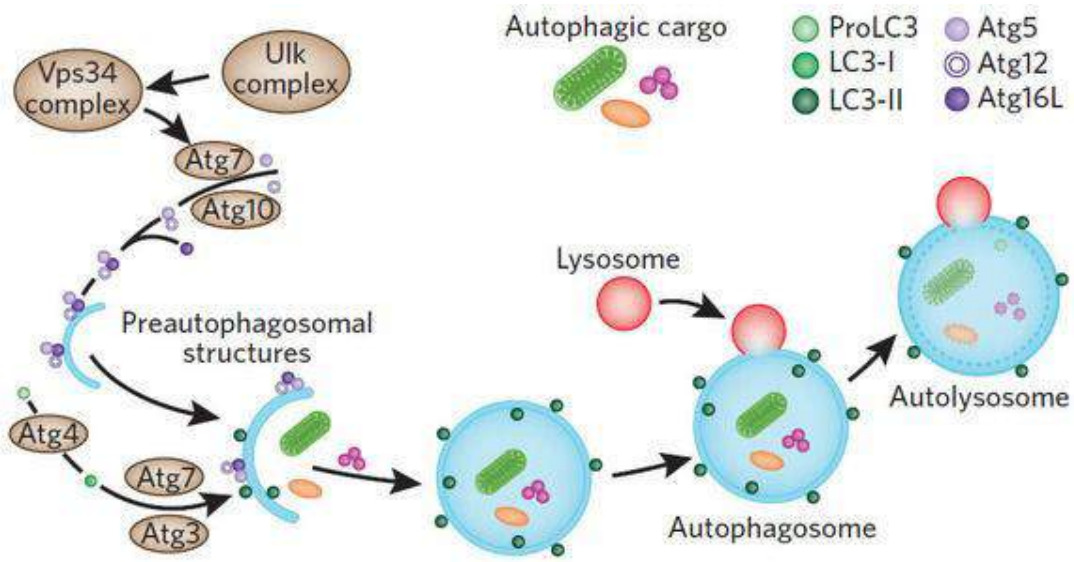

Figure I.8: Schematic representation of autophagy.
Source: Nature Chemical Biology 7, 9–17 (2011). Chemical modulators of autophagy as biological probes and potential therapeutics. Fleming A. *et al;*
DOI:10.1038/nchembio.500

The Atg5-Atg12-Atg16L complex associates with the outer membrane of the extending phagophore [70, 71]. The second system leads to the processing of LC3, encoded by the mammalian homologue of the yeast Atg8. Upon autophagy induction LC3B is

proteolytically cleaved by Atg4 to form LC3B-I. LC3B-I is activated by addition of phospholipid by Atg7 that conjugates it to phosphatidylethanolamine (PE) within the membrane to form processed LC3B-II.

Processed and finished LC3B-II is recruited to the growing phagophore and its integration is dependent on Atg5-Atg12 complex. LC3B-II is found on each of the inner and outer surfaces of the autophagosome, where its presence is needed for the enlargement and completion of the autophagic membrane. When closure of the autophagosomal membrane occurs, the Atg16-Atg5-Atg12 complex dissociates from the vesicle while a little of LC3B-II remains covalently attached to the membrane. Therefore, LC3B-II is used as a marker to observe the extent of autophagosomes formed in the cells, hence the extent of autophagy in the cells [72].

Formation of autolysosome, followed by degradation and recycling

When the autophagosome formation is completed, Atg4 is involved in cleaving the LC3-II still connected to the outer membrane. It is cleaved from phospatidiyl ethanolamine by Atg4 and freed back to the cytoplasm. The fusion between the autophagosome and lysosome leads to the formation of autolysosome and requires lysosomal membrane macromolecule LAMP-2 and also the tiny GTPase Rab7. After fusion, a series of acid hydrolases present in the lysosomes cause degradation of the sequestered substance, simpler molecules resulting from the degradation, importantly amino acids are transported back to the cytoplasm for macromolecule synthesis and maintenance of cellular functions during starvation conditions. p62/sequestosome-1 plays a pivotal role in regulating autophagy flux. p62 being a multifunctional protein contains several protein-protein interacting domains, it is involved in cellular communication protein trafficking, aggregation and degradation. p62 protein can bind to ubiquitinated proteins and regulate their aggregation and degradation via either autophagy or proteasomal degradation pathway. p62 protein has been reported to be seen in association with the intracellular inclusions in primary and secondary tauopathies, α-synucleinopathies and other neurodegenerative brain disorders displaying inclusions with misfolded proteins [73].

1.6 Autophagy in Neurodegenerative Diseases

The autophagic response has been reported in various pathophysiological states, like neurodegeneration, cancer and cardiovascular diseases. Studies have shown that autophagy plays a pivotal role in the removal of misfolded proteins that accumulate within the cell, whereas autophagy is needed for physiological conditioning, non-dividing cells, like neurons, are notably sensitive to changes in autophagic degradation [74]. As autophagy is important for the clearance of aggregate-prone proteins, the thought of autophagy failure as a mechanism predisposing to death has relevance to pathological process in a variety of diseases [75]. Defects in autophagy would lead to accumulation of broken organelles and degradation of durable proteins and aggregates leading to their accumulation within the cell. Formation of intra-cytoplasmic aggregates can be seen in AD, where tau accumulates within the protoplasm and Aβ outside the cell. In Parkinson's disease, α-synuclein is the major part of the aggregates and in Huntington's disease, mutant huntintin is the primary constituent of the aggregates [76]. Under normal conditions, autophagy is a gift at basal levels to maintain proper physiological condition. The demand for cellular internal control through autophagy is especially vital in post-mitotic cells, like neurons and myocytes [77-79] and dysfunctional autophagy has been related to cell death in several neurodegenerative disorders. Thus, enhancing autophagy to eliminate macromolecule aggregates would be a logical therapeutic approach in neurodegenerative diseases. There is growing proof that autophagy exerts a protective role against neurodegeneration, however how autophagy forestalls neurodegeneration isn't clearly understood. Indeed, we all know that autophagy has the capability to selectively eliminate macromolecule aggregates or inclusion bodies, via the special proteins for example p62 and hsc 70 [80]. The key question on whether or not autophagy protects neurons or executes their death in neurodegeneration remains unexplained. Stimulation of autophagy attenuated illness severity in models of Huntington's disease [81] or amyotrophic lateral sclerosis [82] and promoted clearance of α-synuclein in PC12 cells [83]. Huntingtin inclusions induce autophagy and sequester Beclin1 [84]. Mice lacking either Atg5 or Atg7, key elements of the autophagy pathway, were shown to develop ubiquitin positive inclusions, behavior abnormalities and neuronal loss [85]. This knowledge recommends that autophagy is protective against neurodegeneration. On the contrary ultrastructural

analysis have shown the presence of double membrane autophagic vesicles in dystrophic neurites in AD brains, and APP/PS1 transgenic mice [86] and additionally recently, autophagy of mitochondria in AD brains [87, 88] have been reported. Autophagosomes are known as major reservoirs for intracellular Aβ [89]. Continuous autophagy could also be chargeable for cell death [90], or needed for cell death [91], but the interactions between autophagic cell death stay complicated. Together, the information recommends that autophagy plays a crucial role within neurons. So far, no genetic or causative defects within the autophagy pathway are joined to any neurodegenerative illness and therefore the role of autophagy in AD remains unclear. This makes autophagy a very fascinating pathway to review in neurodegenration, its postulated role in Aβ production, degradation of macromolecules and protein aggregates, and its role within the cellular stress response.

1.7 Apoptosis

Apoptosis involves a series of biochemical events resulting in typical morphological changes, together with cell shrinkage, membrane blebbing, plasma membrane disruption, cytoskeletal derangements, chromatin granule condensation, and DNA fragmentation. Apoptotic cells are typically rapidly rounded and degraded by macrophages or neighboring cells before their intracellular contents leak into the extracellular area, thereby avoiding associate inflammatory responses [92]. Apoptosis is mediated by intracellular proteases known as caspases that share the flexibility to cleave their substrates. Caspases are made as inactive zymogens containing a prodomain, a p20 giant fractional unit and a p10 tiny fractional unit. Most of the caspases are activated by cleavage. Caspases are divided into instigator/initator caspases (caspase 2, 8, 9, 10) and effector caspases (caspase 7, 6, 3). Instigator caspases have long prodomains that contain the protein-protein interaction motifs, e.g: DEd (death effector domain) or CARD (caspase accomplishment domain). DEd and CARD are involved in interacting with other proteins. The short prodomain containing effector caspases are usually cleaved and activated by initiator caspases. Active effector caspases are accountable for execution steps of apoptosis by cleaving multiple cellular substrates [93].

1.7.1 Molecular Mechanism of Apoptosis

There are 2 different apoptotic pathways, death receptor (extrinsic) and mitochondrial (intrinsic) pathway that ends up in activation of caspases. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other [94].

1.7.1.1 Extrinsic cell death pathway

The extrinsic cell death pathway involves transmembrane receptor interactions that fuel apoptosis. These death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily. These share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" that plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways Ashkenazi 1998. To date, the best-characterized ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5. Both DR and Fas ligand induce apoptosis in cells by identical signaling pathways [95].

The sequences of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF-α/TNFR1 models. In these models, there is clustering of receptors and binding with the homologous trimeric ligand. Following ligand binding, cytplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP. FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in auto activation of pro-caspase 8. Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP which will bind to FADD and caspase-8, rendering them ineffective.

1.7.1.2 Intrinsic Apoptosis

This pathway involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. In other words, there is the withdrawal of factors, loss of apoptotic suppression, and subsequent activation of apoptosis. Other stimuli that act in a positive fashion include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All of these stimuli cause changes in the inner mitochondrial membrane those results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol. The first group consists of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi. These proteins activate the caspase dependent mitochondrial pathway. Cytochrome *c* binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome" [96]. The clustering of procaspase-9 in this manner leads to caspase-9 activation. The second group of pro-apoptotic proteins, AIF, endonuclease G and CAD (caspases activated DNases) are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell has committed to die. AIF translocates to the nucleus and causes DNA fragmentation into ~50–300 kb pieces and condensation of peripheral nuclear chromatin [97-100]. This early form of nuclear condensation is referred to as "stage I" condensation [101]. Endonuclease G also translocates to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments [102, 103]. AIF and endonuclease G both function in a caspase-independent manner. CAD is subsequently released from the mitochondria and translocates to the nucleus where, after cleavage by caspase-3, it leads to oligonucleosomal DNA fragmentation and a more pronounced and advanced chromatin condensation.

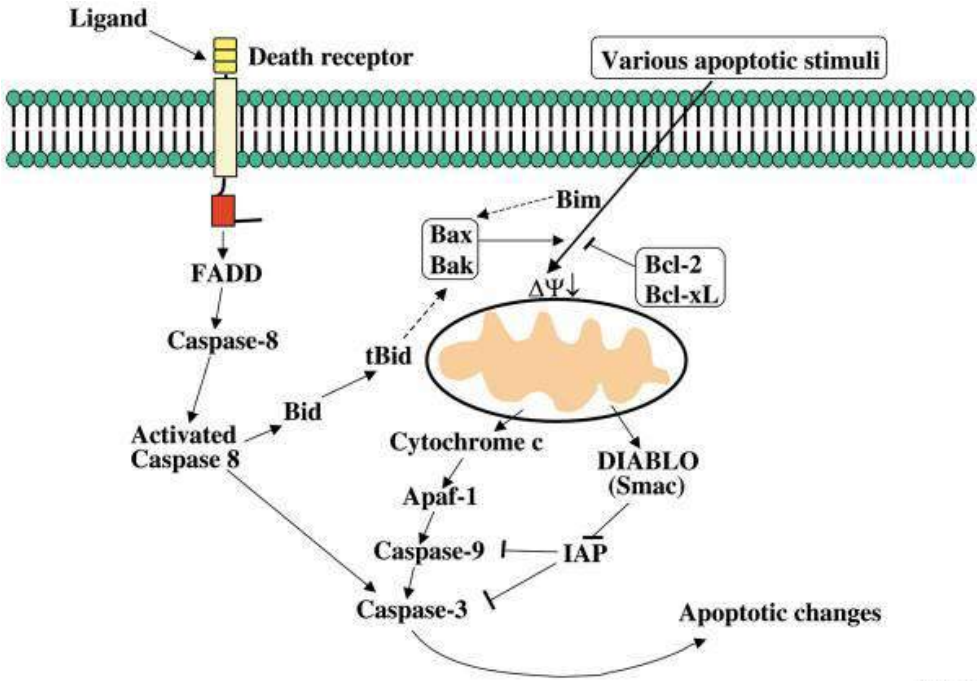

Figure I.9: Intrinsic and extrinsic pathways of apoptosis
Source: Arthritis Research 2002. Signaling for survival and apoptosis in the immune system. Mak T. W; Yeh W. C.
DOI: 10.1186/ar569

1.7.1.3 Execution Pathway

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. It is the activation of the execution caspases that begins this phase of apoptosis. Execution caspases activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases. Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD. In proliferating cells CAD is complexed with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD [104, 105]. CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies.

1.8 Apoptosis in Neurodegenerative Diseases

Apoptosis mediates the precise and programmed natural death of neurons and is a physiologically necessary process in maturation of the central nervous system. However, premature apoptosis /or an impaired apoptosis regulation is involved in pathologic process of neurodegeneration and results in varied chronic illness states, like Alzheimer's disease (AD), Parkinson disease (PD), Huntington's (HD) diseases, Amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and diabetic neurological disorder [106].

Activation of neuronal cytomembrane receptors might end in caspase activation, increased Ca^{2+} levels and generation of reactive oxygen species (ROS) and contribute considerably to programmed cell death. High Ca^{2+} levels and ROS are known to contribute to the mitochondrial pathway of apoptosis Mitochondrial pathway of apoptosis is initiated by a range of signals, as well as Bcl-2 proteins, high Ca^{2+} levels and ROS [107]. These signals accumulate at the mitochondria, leading to the discharge of many pro-death molecules into the cytoplasm [108]. Mitochondria, additionally to their role in energy production, contribute to cell death by releasing pro-death molecules into the neuronal cytoplasm [109]. One pro-death molecule with specific implications in neurodegenerative diseases is cyt c that exists unremarkably within the electron transport chain on the mitochondrial membrane [108]. Upon getting into the cytoplasm, cyt c associates with apoptosome complex with a procaspase, nucleotide and Apaf-1 [110]. This complex then works as a cell death signal by promoting proteinase activation.

1.9 Cross Talk between Autophagy and Apoptosis

The relationship between autophagy and apoptosis is quite complicated. In some instances autophagy proves to be a protective mechanism while in other cases it seems to induce apoptosis and lead to death of cells. This differential functionality of autophagy seems to be dependent on circumstances. However past literature continues to support the idea of cross talk between these two processes to maintain cell homeostasis, which is the ultimate aim of cell during survival. This also indicates how

intricately these are connected to decide the fate of cell [111-115]. Autophagy activation due to any kind of stress, may lead to inhibition of apoptosis, also autophagy inhibition has been reported to activate both caspase dependent and independent cell death pathways. Conversely it has also been shown that Autophagy selectively degrades caspase-8 and inhibits TRAIL induced apoptosis. Further Atg7 has been shown to form complex with caspase-9 and hence keeps a check on its pro-apoptotic activity [116, 117].

The interplay between Bcl-2 family proteins regulates both apoptosis and autophagy. Bax, a pro-apoptotic protein was shown to decrease autophagy induction by mediating cleavage of Beclin-1. Similarly, Bim inhibits Beclin-1 mediated autophagy, independent of its pro-apoptotic functions. Also Beclin-1 has been shown to enhance cisplatin-induced apoptosis by enhancing caspase-9 activity. Autophagy adaptor protein p62 has also been found to play a role in the effective activation of caspase-8 during extrinsic apoptotic pathway [118, 119]. Wirawan et al. (2010) reported that the N-terminal (Beclin-1-N, aa 1–133) and C-terminal (Beclin-1-C, aa 150–450) cleavage products of Beclin-1 failed to induce Autophagy. More importantly, he proved that Beclin-1, once cleaved by caspases, can acquire a new apoptosis-promoting function. Indeed, Beclin-1-C translocated from the cytosol to mitochondria and amplified apoptosis induced by interleukin-3 deprivation [120, 121].

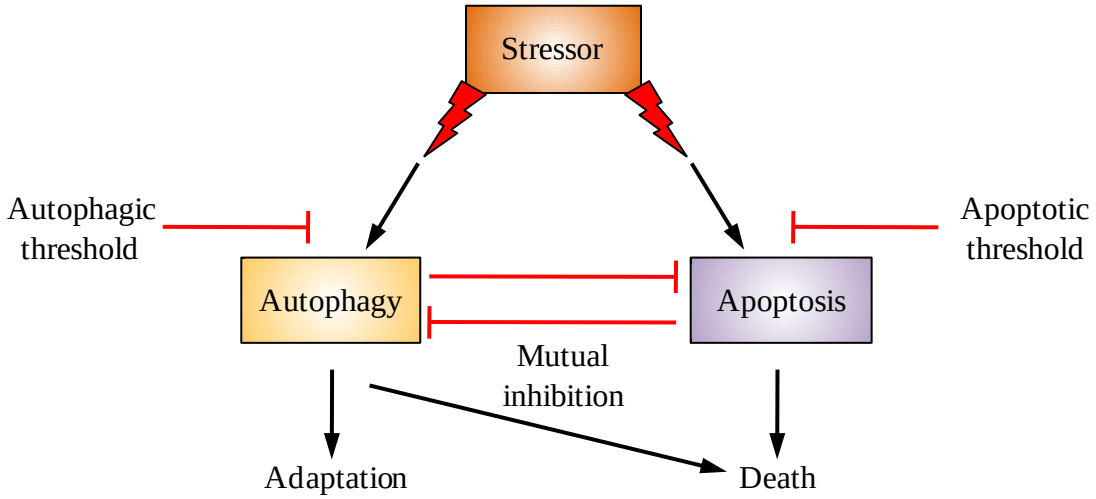

Figure I.10: Inter-relation between Autophagy and Apoptosis
Source: Nature Reviews Molecular Cell Biology 8, 741-752 (September 2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. Maiuri M. C. *et al*.
DOI: 10.1038/nrm2239

Autophagy and apoptosis are the two highly organized cellular processes that play vital role in embryonic development and tissue homeostasis. Dysregulation of these two processes have been associated with a number of pathological conditions, such as neurodegenerative diseases, autoimmune diseases and cancer. Various studies have very well established that autophagy and apoptosis extensively communicate with each other and the final outcome decides the fate of the cell during physiological and pathological conditions. Under stress conditions, cells initiate autophagy as a pro-survival mechanism to combat apoptosis. However, as stress increases towards a point of no return or threshold, cells block autophagy and initiates apoptotic cell death. The decision when to switch off this prosurvival autophagic process may mainly depend on the level of stress and is regulated by various proteins involved in autophagy and apoptosis. Autophagy antagonizes the effect of apoptosis and the extent may vary with the stimulus, cell type, and stress level. Inhibition of autophagy has been reported to enhance sensitivity toward apoptosis. Indeed, inhibition of autophagy has been shown to enhance both caspase dependent and independent cell death. At the same time, for effective onset of cell death, autophagy is inhibited by degradation of Atg proteins by apoptotic proteins such as caspases and calpains. Conversely some studies also highlight the importance of autophagy in activation of initiator caspase 8 without the involvement of receptor related signal from outside the cells. Puma and Bax induced autophagy have been demonstrated to contribute to the apoptosis in response to mitochondrial stress. Another study concluded that poly ADP ribose polymerase (PARP) and receptor interacting protein 1 are required for effective induction of autophagy, which enhances the apoptotic cell death. Luo and Rubinsztein have shown that the interplay between Bcl-2 family proteins not only regulates apoptosis but autophagy as well. Bcl-2 binds to both the pro-apoptotic proteins (e.g. Bax) and pro-autophagic proteins (e.g. Beclin 1). Furthermore, mitochondria localized Bcl-2 inhibits activating molecule in Beclin1 regulated autophagy (AMBRA1) induced autophagy but ER localized Bcl-2 inhibits Beclin 1 induced autophagy. Therefore, it is not surprising that the molecular crosstalk between these two pathways is very complex and sometimes contradictory as well.

Besides the ability of Bcl-2 family proteins to regulate autophagy through the interaction with Beclin-1, caspases have been shown to inhibit autophagy via a mechanism mediated by the cleavage of autophagy-related proteins, such as Beclin1, PI3K, ATG5 and ATG4D. Their cleavage facilitates their localization to mitochondria

where they serve new functions such as promoting mitochondria-mediated apoptosis. Cell death orchestrates life during development and also delivers the essence of life. Cell death via apoptosis can be of two main types, caspase dependent programmed cell death or apoptosis and caspase independent programmed cell death or necroptosis (Kinally et al 2011, Smith and Yellon 2011). These may be interconnected by caspases which could activate non-caspase proteases, while theses proteases could in turn activate the caspases. P53 which was initially thought to be a transformation-associated protein, was later found to be involved in several other dimensions as in cell cycle control, DNA repair, cell differentiation, apoptosis and autophagy [122-128]. Profound study has been done in understanding the role of p53 in apoptosis. Irradiated mouse thymocytes were the first group of cells where p53 mediated apoptosis was first discovered [129, 130].

1.9.1 Autophagy and Apoptosis in Alzheimer's Disease

Autophagy is a lysosomal degradative process by which cellular constituents are recycled for new synthesis and energy, in response to a number of different cellular stresses or damaged organelles, and proteins eliminated by selective targeting to lysosomes [131-135]. There are at least three subtypes of autophagy: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy [136]. While the mechanisms for lysosomal delivery and the specific cargo for each of the three autophagic processes differs, the final step, lysosomal degradation, is common to all forms of autophagy. Macroautophagy, hereafter referred to as autophagy, is initiated by the inhibition of mTOR kinase (mammalian target of Rapamycin) or activation of AMP activated protein kinase (AMPK). Sequestration of cytoplasm within a double membrane limited vacuole, the autophagosome, is coordinated by the sequential involvement of multiple signaling and ubiquitination complexes.

Autophagosomes mature by fusing with endosomes and ultimately with lysosomes to form an autolysosome. Autophagy is completed upon digestion of the autophagosome and its contents and the release of amino acids and other metabolic products. In many of the diseases associated with autophagy anomalies, it is the final stage of autophagy-lysosomal degradation that is disrupted. In several disorders, including AD, defective

lysosomal acidification contributes to this proteolytic failure. The robust pathological effects of autophagic disruption in Alzheimer's disease have been well characterized [137-139]. Autophagic vacoules accumulate progressively in affected neurons and are the predominant organelles within grossly swollen dystrophic neurites, another hallmark of AD neuropathology. These autophagic lesions likely reflect the selectively impaired axonal transport of autophagy/lysosomal related compartments, a known pathological consequence of lysosomal proteolysis inhibition [140]. Current evidence indicates that autophagy is principally defective at the stage of autolysosomal proteolysis in AD. The selective accumulation of autophagic vacoules and especially autolysosomes in dystrophic neurites [86, 141] implies that autophagosomes can form and fuse with lysosomes, but that elimination of substrates from these autolysosomes is defective. Apoptosis is a self-destructive process with carefully choreographed steps, a series of intracellular events that come into play to decommission the unwanted and dangerous cells. Mechanically, two major apoptotic signaling pathways, including the extrinsic and intrinsic signals, converge onto these caspases to initiate cell death. The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily [142]. Members of the TNF receptor family share similar cyteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" [143]. This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. Other stimuli that act in a positive fashion include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All of these stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol [144]. The first group consists of cytochrome *c*, Smac/DIABLO, and the serine

protease HtrA2/Omi [145-147]. These proteins activate the caspase dependent mitochondrial pathway.

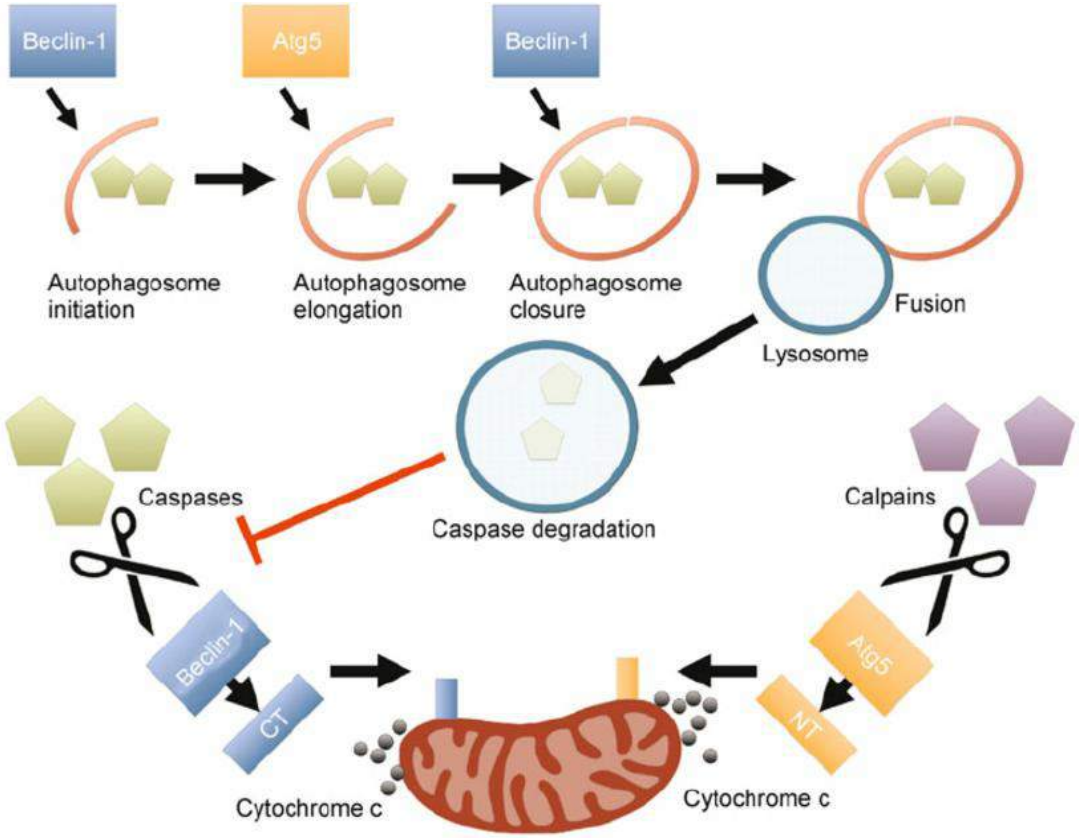

Figure I.11: Molecular mechanism of crosstalk between Autophagy and Apoptosis
Source: Protein Cell (2012). The crosstalk between autophagy and apoptosis: where does this lead? Claire Gordy, You-Wen He.
DOI: 10.1007/s13238-011-1127

Several studies presently indicate that apoptosis might occur in and contribute to AD onset and progression. Stimuli for apoptosis in AD include increased oxidative stress, dysregulation of ion homeostasis, growth factor deprivation, accumulation of Aβ, metabolic impairment, reduced clearance of toxin, mitochondrial dysfunction, DNA damage, protein aggregation. Nevertheless, while the role of apoptosis in in-vitro models and transgenic animal models of neurodegeneration has been largely documented, its occurrence and role in human postmortem AD brain is controversial. Caspases seem to have a direct role in Aβ precursor protein (APP) processing and in the biogenesis of Aβ peptide species. Particularly, the C31 C-terminal peptide obtained by caspase-3 mediated APP cleavage seems to mediate apoptosis by transcriptional regulation of some genes. Caspase-3 mediated APP cleavage also stabilizes BACE, the

β-secretase enzyme initiating the APP cleavage to produce Aβ peptide which accumulates in endosomes, where increases Aβ production. APP-derived toxic peptides may not only originate by apoptosis activation but may also be responsible of it in viable neurons. APP-derived Aβ peptides can activate caspases through the extrinsic pathway, implicating binding of extracellular Aβ to cell sites, while other studies suggest that the intrinsic pathway may be more relevant. Intracellular accumulation of Aβ in endosplasmic reticulum or endosomes may activate apoptotic mechanism(s) through the unfolded protein response (URP) or endoplasmatic reticulum stress. Alternatively, intracellular Aβ may bind to alcohol dehydrogenase within mitochondria and activates apoptosis causing mitochondrial stress. Pharmacological or molecular inhibition of particular members of the caspases family, such as caspase 2, 3, 8 and 12 has been reported to offer partial or complete protection against Aβ-induced apoptotic cell death in vitro. Studies from cellular and animal models indicate that caspases have also been implicated in mechanisms of tau-mediated neurodegeneration in AD. According to this hypothesis, Aβ peptide promotes neuronal pathological tau filament assembly by triggering caspases activation leading to tau cleavage. This event, in turn, generates a proteolytic product that assembles more rapidly and extensively into tau pathological filaments. Colocalization of hyperphosphorylated tau and active caspase-3 and 6 has been recently detected in brainstem of young and old AD patients. Caspase-9 activation and caspase-cleaved tau forms have been documented in AD hippocampal brain sections .

1.10 Tribbles family of proteins

Trib3 my molecule of interest, first was described as the neuronal putative cell death inducible kinase NIPK. Trib3/TRB3 is the mammalian homolog of the Tribbles gene of Drosophila Melanogaster. Trib3 acts to negatively regulate Cdc25 or String gene in Drosophila, where it blocks mitosis in the early phase of mesoderm formation. Tribbles regulates oogenesis in the fruit fly by degrading Slbo which is the ortholog of mammalian transcription factor C/EBP. Tribbles may function as a part of the E3 Ligase complex to accomplish the degradation of C/EBP. During oogenesis in the fruit fly, Tribbles subjects String to proteasomal degradation, while Tribbles mutant embryos have been found to undergo an extra round of cell division [148]. Tribbles also plays an

important role during wing development of the fruit fly whereby it causes proteasomal degradation of String as well as Twine, another ortholog for cdc25, By doing so it leads to the development of few large cells, a special requirement for wing development in a fruit fly Tribbles therefore regulates cell division by proteasomal degradation of String , Twine and Slbo [149].

Mammalian Tribbles family of proteins are pseudokinases, in that they lack the specific catalytic residue required for kinase activity. Tribbles family of proteins are encoded by three genes, namely Trib1 (C8FW, SKIP1), Trib2 (C5FW, SKIP1, GS3955) and Trib3 (NIPK, SKIP3) [150-153]. The gene products of all these three genes are invariably associated with human malignancies [154-158]. The specific expression of the three tribbles proteins is also different. It has been reported that Trib1 is located on chromosome 8 at q24.13, while Trib1 mRNA is expressed in high concentrations in muscles, thyroid gland, pancreas, peripheral blood leukocytes and bone marrow [149, 159]. Reports suggest Trib1 protein contains a proline rich residue at its N-terminal which is essential for its nuclear localization [149]. The Trib1 protein binds to an enzyme 12-Lypoxigenase (12-Lox), which is involved in metabolism of arachidonic acid. Apart from this Trib1 can also interact with MEK1, MKK4 and SEK1. It has been shown that these interactions not only modulate MAPK activity but also regulate their own levels probably in a positive feedback loop mechanism [149, 150].

Trib2 gene is located on chromosome 2 at p24.3. Trib2 mRNA is found in great abundance in the peripheral blood leukocytes. Trib2 has been found to be highly expressed in metastatic prostate cancer cells [149]. Oncogenic protein Notch1 regulates Trib2 transcriptionally [154, 155]. Trib2 can bind to COP1 E3 ligase and hence cause degradation of C/EBP-α, thus upregulating conditions of leukemia [153-155]. An inverse expression level exists between Trib2 and Bim, in melanoma and non melanoma cells [154]. Trib2 has been reported to interact with Akt, whereby it is able to block the phosphorylation caused by IGF1 (Insulin like Growth factor1) at both the phosphorylation sites S 473 and Thr308 [160].

The Trib3 gene is located on chromosome 20 at p13-p12.2 in the mammals and also displays splice variants [149, 161]. The human Trib3 gene consists of four exons which are interspersed by three introns. The mRNA isoform 1A of Trib3 is detectable in low doses in unstressed HepG2 cells, while treatment with toxins that induce ER stress like

thapsigargin and arsenite treatment increase the expression of the Trib3 mRNA by 2 to 4 fold. Trib3 promoter activity increases by about 500 fold upon thapsigargin treatment as checked by increased luciferase activity of the Trib3 promoter [161, 162].

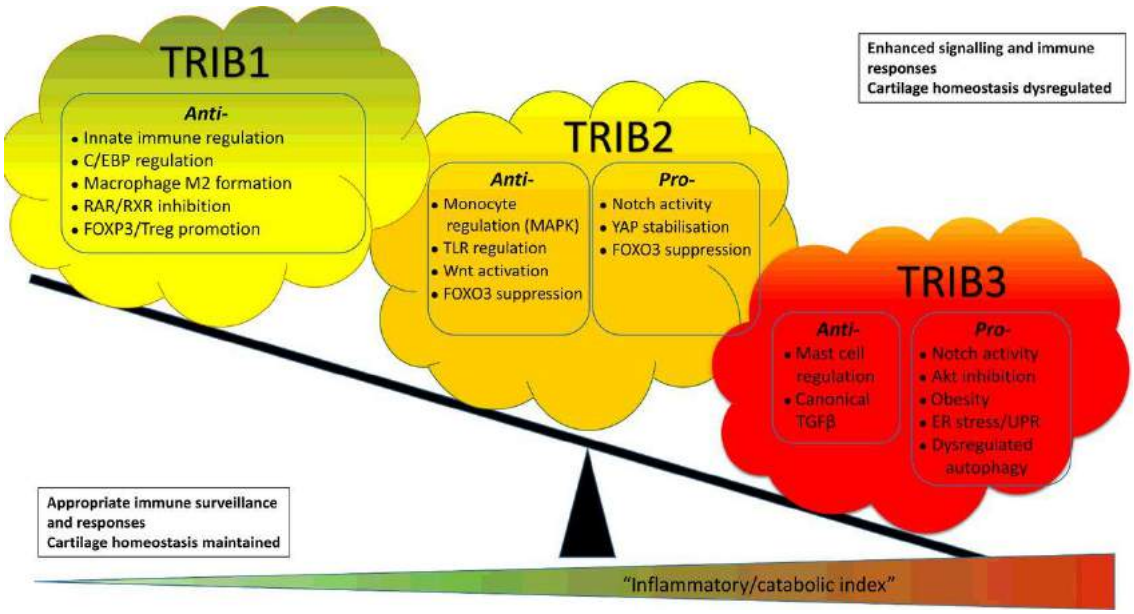

Figure I.12: Different roles of the Tribbles family of proteins
Source: Biochemical Society Transactions (2015). Tribbles and arthritis: what are the links? Andrew D. Rowan, Gary J. Litherland.
DOI: 10.1042/BST20150076

1.10.1 The TRB3/Trib3 Protein

Trib3 protein is a pseudokinase as its ATP binding domain lacks the catalytic residues as confirmed by biochemical assays [156, 159, 160]. Reports suggest Trib3 is able to regulate several proteins by actually binding to them; it therefore has a wide range of functions and binding partners.

Trib3 interacts with the transcription factors ATF4 and CHOP [156, 161, 163]. CHOP is a transcription factor belonging to the C/EBP family of proteins. CHOP is known to cause apoptosis during DNA damage, cell cycle arrest and ER stress [164]. ATF4 belongs to the activating transcription factor family of proteins and it functions by heterodimerizing to CHOP and further regulating genes downstream leading to apoptosis of cells [165]. ATF4-CHOP heterodimer binds to the promoter region of Trib3 inducing the mRNA levels upon treatment with toxins that induce ER stress and apoptotic cell death [161, 163]. A self regulatory network has been reported to act between Trib3-ATF4-CHOP, whereby Trib3 inhibits the transcriptional activities of the

ATF4-CHOP complex as seen in HepG2 cells [163]. There occurs differential regulation of Trib3 during stress conditions, ER stress leads to an upregulation of Trib3, whereas genotoxic stress downregulates Trib3 via p53 [166]. It has been shown that there is upregulation of Trib3 and PUMA upon ER stress induced by tunicamycin in neuronal PC12 cells. This may occur via the inactivation of Akt by tunicamycin, activation of FoxO and hence upregulation of PUMA [167].

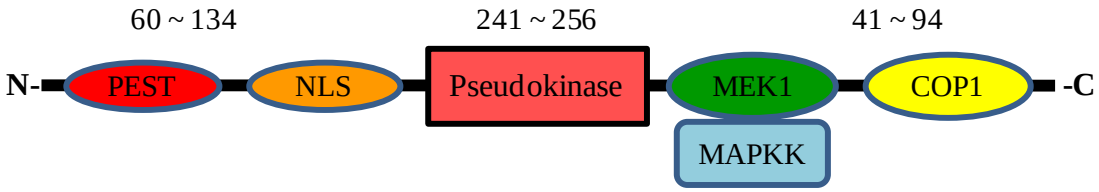

Figure I.13: Structure of Trib3 protein.
Source: Biochimie (2016). Tripping on TRIB3 at the junction of health, metabolic dysfunction and cancer. Mondal D. *et al*.
DOI: 10.1016/j.biochi.2016.02.005

Trib3 also affects NF-κB signaling. NF-κB belongs to a group of transcription factors that are involved in immunoregulation. They are responsible for regulating several cytokines, chemokines and adhesion molecules. NF-κB comprises of two subunits, a DNA binding subunit p50 and a transactivator subunit p65. It has been reported that Trib3 binds to p65, inhibiting its phosphorylation and thus regulating NF-κB dependent transcription in a negative manner [168].

Trib3 also plays an essential role in several lung, esophageal, colon carcinomas and myeloid leukemia [156, 169, 170]. Studies reveal that Trib3 mRNA levels increase when human prostate carcinoma cells PC6 are subjected to nutrient starvation [171]. Trib3 is also reported to induce SMAD3 expression, whereby they are involved in a positive feedback loop. Activated SMAD3 translocates to the nucleus and in turn upregulates the expression of Trib3 [172].

Trib3 gene also regulates the MAP kinases MEKK1 and MKK7. It has been shown to enhance JNK and ERK activation at low doses, while no such effect has been observed at high doses. MKK overexpression has been shown to upregulate Trib3 protein [159]. Trib3 transcripts and proteins have been shown to possess short half lives. MAPKKs interact with Trib3 and stabilize its protein level. Studies show that E3 ligase SIAH1 (seven in absentia homolog) bind to Trib3 protein and lead to its ubiquitination and proteasomal degradation [173]. Trib3 is known to induce lipolysis in fasting condition,

by binding to E3 ligase COP1 and promoting degradation of ACC (Acetyl-Coenzyme-A Carboxylase) [174]. ACC is known to be involved in long chain fatty acid synthesis.

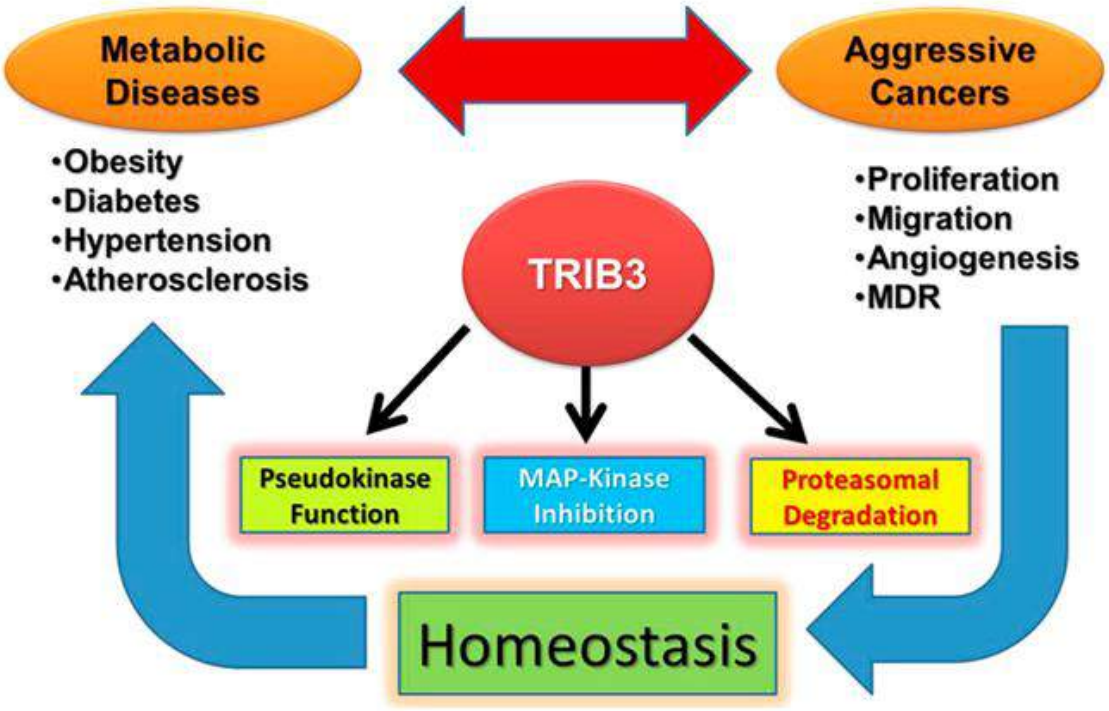

Figure I.14: Functions of the Tribbles3 protein
Source: Biochimie (2016). Tripping on TRIB3 at the junction of health, metabolic dysfunction and cancer. Mondal D *et al*.
DOI: 10.1016/j.biochi.2016.02.005

Most interestingly Trib3 has been known to regulate Akt130, [175-178]. That Trib3 is a binding partner of Akt1 was first revealed in a yeast two-hybrid screen system [160]. Studies in HepG2 cells show endogenous Trib3-Akt interaction [160]. Detailed study revealed that a splice variant of Trib3 lacking the residues 239-265 only weakly bound to Akt [160]. IFG1 induced phosphorylation of Akt at Ser-473 and Thr-308 were blocked by overexpression of Trib3. The study also revealed that Trib3 can bind to both phosphorylated and unphosphorylated Akt, yet the binding between unphosphorylated Akt and Trib3 is of higher affinity [160]. Detailed study revealed that residues 240-315 were necessary for interaction of Akt with Trib3 [160]. Trib3 binds to Akt in non-neuronal cells during insulin signaling. Studies in mice revealed that Trib3 was induced 4-5 fold by starvation, and it was found to be in association with Akt [160]. Trib3 is also upregulated in diabetic obese mice. Here increased Trib3 levels blocked Akt

activity, which led to several conditions like insulin resistance, hyperglycemia and non-alcoholic fatty liver disease (NAFLD) [175].

Further studies on Trib3 also revealed its potent activity to acts as an inducer of neuron death. Subjecting PC12 cells and sympathetic neurons to NGF deprivation led to an upregulation of Trib3 cDNA levels for upto 24 h [179]. It was reported by the same group that in response to Ca^{2+} ionophore excitotoxicity there is an upregulation of Trib3 in cortical neurons [179]. In corroboration to these findings another group reported that there was an induction in Trib3 mRNA levels in developing rat sympathetic neurons when subjected to NGF deprivation for 18 h [180]. Furthermore Trib3 was upregulated in neuronal PC12 cells which were treated with 6OHDA (6-Hydroxy Dopamine). 6OHDA is a neurotoxin that enters and kills dopaminergic neurons via dopamine transporters, hence used to create Parkinsonism in the laboratory [181].

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