# Implications of polyglutamine aggregates on autophagy

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## MASTER OF SCIENCE

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by

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# DECLARATION

I hereby declare that the thesis entitled **"Implications of polyglutamine aggregates on autophagy"** is a result of investigations carried out by myself under the guidance of **Dr. Ravi Manjithaya** at Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the norm of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

April, 2018                                                                                   Swati Keshri

## CERTIFICATE

This is to certify that work described in this thesis titled **"Implications of polyglutamine aggregates on autophagy"** is the result of investigations carried out by Ms. Swati Keshri in Autophagy lab, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India, under my guidance.

Dr. Ravi Manjithaya

(Research Supervisor)

Date:

# Acknowledgements

# List of Abbreviations

| | |
|---|---|
| UPS | Ubiquitin proteasome system |
| CMA | Chaperone-mediated autophagy |
| ERAD | Endoplasmic reticulum associated degradation |
| UPR | Unfolded protein response |
| mHTT | Mutant huntingtin |
| NDs | Neurodegenerative disorders/diseases |
| ALS | Amyotrophic lateral sclerosis |
| HD | Huntington's disease |
| PD | Parkinson's disease |
| AD | Alzheimer's disease |
| GFP | Green fluorescent protein |
| LC3 | Microtubule-associated proteins 1A/1B light chain |
| FTLD | Frontotemporal lobar degeneration |
| SNARE | Soluble NSF attachment protein receptor |
| PSEN1 | Presenilin-1 |
| HAP1 | Huntingtin-associated protein 1 |
| TRE | Tetracycline response element |
| ºC | Degree celcius |
| kDa | Kilo Dalton |
| μl | Micro litre |
| μM | Micro molar |
| μg | Micro gram |
| PBS | Phosphate-buffered saline |
| DMSO | Dimethyl sulfoxide |
| BafA1 | Bafilomycin A1 |

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# SYNOPSIS

Cells employ several quality control mechanisms to maintain cellular homeostasis by clearing dysfunctional or potentially toxic cellular components such as misfolded proteins and damaged organelles. Mutations and certain post-translation modifications in proteins can give rise to misfolded aggregate-prone proteins. Failure of quality control mechanisms also results in accumulation of unfolded or misfolded proteins in the cell which eventually organize into higher order structures such as protein oligomers and aggregates. These aggregates are toxic in nature and ultimately lead to cell death. Most neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's disease manifest this pathology i.e. aggregation of misfolded proteins in neurons. While most eukaryotic cells can dilute toxic protein aggregates via cell division, this does not occur in mitotically incompetent neurons. In order to degrade these misfolded proteins, cells have two major pathways - the ubiquitin-proteasome system (UPS) and the autophagy related pathways. UPS is a highly regulated mechanism of intracellular protein degradation and turnover. Proteins destined for degradation via UPS are tagged with ubiquitin and directed towards a barrel shaped 26S macromolecular assembly, the proteasome whose core enzymatic machinery has proteolytic activity. During pathogenic accumulation of large toxic aggregates inside the cell, it is believed that such overwhelming load of protein oligomers and complexes render UPS to be non-functional. The second major machinery that contributes to intracellular turnover is (macro) autophagy. The aggregate-prone proteins and protein aggregates are known to be substrates for autophagy and hence can be degraded by the same. Autophagy is a catabolic process which leads to lysosomal degradation of damaged organelles and misfolded proteins with the help of concerted actions of various

autophagy related proteins. In this process cargo destined for degradation is captured in a double membrane vesicle known as autophagosome which is trafficked to lysosome for fusion and form autolysosome. Here in autolysosome, captured cargoes are degraded by the action of lysosomal proteolytic enzymes and basic building blocks are pumped back to cytoplasm for further usage.

Along with these two major components of proteostatic machinery processes like microautophagy, chaperone-mediated autophagy (CMA) and unfolded protein response (UPR) or endoplasmic-reticulum-associated protein degradation (ERAD) contribute towards maintaining proteostasis.

In case of neurodegenerative diseases, due to overload of misfolded proteins, most of the components of proteostatic machinery fail to degrade such aggregate-prone species and aggregates as their large size limit their clearance. Although autophagy is known to be beneficial in clearing the same, but basal level of autophagy becomes inefficient. Therefore, autophagy upregulation by genetic or pharmacological approaches can overcome the blockade in the machinery to facilitate enhanced capture and clearance of these aggregates. The selective clearance of aggregates by autophagy is known as aggrephagy.

Autophagy flux is impaired at various steps in neurodegenerative diseases ranging from defects at autophagosome biogenesis, cargo recognition, trafficking of autophagosome to lysosome and degradation of cargo inside autolysosome. Thus, it is important to understand specific shortcomings in autophagy flux in such disease contexts to find therapeutic interventions.

In our studies we have chosen mutant huntingtin (mHTT) protein as a cargo to study aggrephagy, where an expansion of the polyglutamine (polyQ) tract in its N-terminus leads to aggregation and is a cause of Huntington's disease (HD). It is a unique cargo to study aggrephagy as all the cases of HD are attributed to the mutation or polyQ expansion in the single gene coding for HTT protein. Since cargo (mHTT) recognition failure has been reported in HD, it becomes intriguing to understand the incompetency of autophagy adaptors to recognize and capture mutant huntingtin (mHTT) aggregates which culminates in ineffective clearance of the same.

In an attempt to address some of the above mentioned questions, we established a cellular model of HD wherein we studied aggregation of mHTT, its subcellular localization and its effects on basal autophagy.

We also studied the role of autophagy induction on clearance of mHTT aggregates by boosting autophagy with a known small molecule autophagy inducer Torin1. This proof-of-principle study reiterates the importance of autophagy induction on increased recognition, capture and subsequent degradation of mHTT. In addition, towards this objective, we are developing a cell-based temporal model for aggrephagy to study the dynamics of mutant huntingtin aggregate formation and its interaction with the autophagy machinery. Future directions of this work would be to get more insights into the aggrephagy mechanism.

**Chapter 1**

**Introduction**

**1.1 <u>Proteostasis</u>**

A protein is synthesized as a linear chain of amino acids on ribosomes. In order to attain its biological function, it folds in a unique three dimensional conformation with the help of protein folding machineries in the cell (Alberts, Johnson et al. 2002). Misfolding of proteins is a common phenomenon observed during this process but a cell with its army of molecular chaperones takes care of such misfolded species and guides them along a productive folding pathway. If a misfolded protein species is irreparable then it is necessary to remove them from cell to maintain the integrity of intracellular protein homeostasis (proteostasis) (Araki and Nagata 2011). Maintenance of proteostasis becomes even more important in case of neurons as they are terminally differentiated cells and are susceptible to the accumulation of damaged proteins and organelles (Bence, Sampat et al. 2001). Thus, neuronal survival is highly dependent on maintaining protein quality control by efficient degradation mechanism. The synergetic action of proteostatic machineries maintains homeostasis of intracellular pool of functional and healthy proteins. On the contrary, the failure of proteostatic network can result in accumulation of aggregate-prone misfolded proteins which tend to form toxic protein oligomers and aggregates. Furthermore, the capacity of the proteostatic machineries in the cell declines with aging which predispose exclusively non-dividing neurons at a risk of accumulating damaged cell organelles and misfolded proteins (Duennwald and Lindquist 2008).

The two key components of proteostatic machinery are – the ubiquitin proteasome system (UPS) and the autophagy-related pathways.
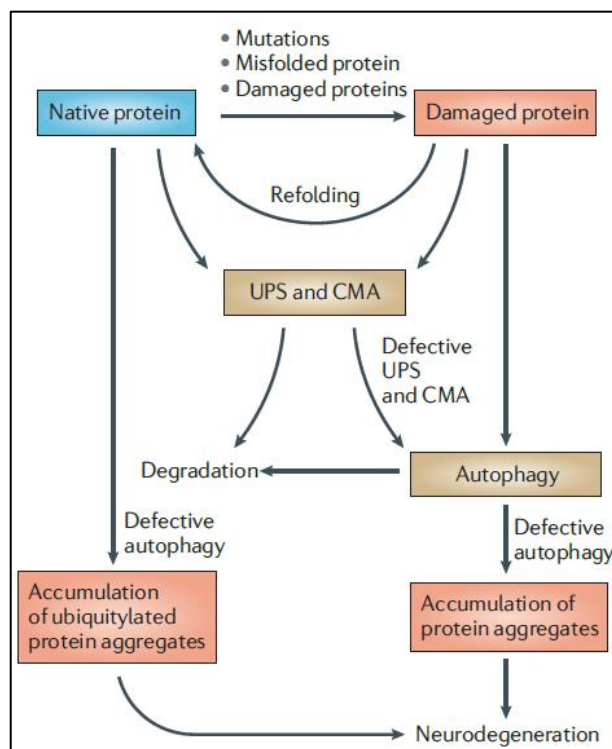


**Figure 1: Proteostatic network in the cell.**

**{With permission from Bedford, Lynn, et al. *Nature reviews Drug discovery* (2011)}**

### 1.1.1 The Ubiquitin Proteasome System (UPS)

The UPS is one of the quality control mechanisms which is involved in protein catabolism. It is a central pathway for many cellular processes like antigen processing, apoptosis, response to stress and regulation of protein turnover. The substrates intended for degradation by UPS are tagged with multiple ubiquitin molecules through covalent attachment by the action of E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin ligase) enzymes. Such tagged species are recognized by proteasome followed by deubiquitination and unfolding. The unfolded protein species are then translocated into the

hollow core of proteasome and subsequently degraded. In mammals, 26S proteasome consists of one 20S subunit which forms catalytic core and two 19S regulatory subunits (Hartl, Bracher et al. 2011). As mentioned previously UPS is involved in variety of cellular processes and defects in its activity can lead to several pathogenic conditions.

Dysfunctional UPS has been reported in case of several neurodegenerative disorders (NDs) like Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) (Heinemeyer, Ramos et al. 2004). The common pathology observed in NDs is accumulation of misfolded aggregate-prone proteins and their relentless build up resulting in inclusion bodies. These inclusion bodies are rich in ubiquitinated proteins suggesting impaired proteasome activity. Several studies have shown implications of UPS in clearing misfolded proteins from the cell as in one report inhibition of proteasome by lactacystin in PC12 cells show increased accumulation and aggregation of alpha-synuclein which is generally mutated and tends to aggregate in Parkinson's disease (Hipp, Park et al. 2014).


Endoplasmic Reticulum Associated Degradation (ERAD)

Endoplasmic reticulum (ER) is the primary site for protein quality control which aids in protein folding and their post-translational modifications. Impairment of ER function due to certain pathophysiological conditions can lead to ER-stress resulting in accumulation of misfolded proteins in the ER lumen (Labbadia and Morimoto 2015). ER-stress activates unfolded protein response (UPR) which shuts down global protein synthesis by phosphorylation of α-subunit of eukaryotic translation initiation factor (eIF2α) and accelerates degradation of misfolded proteins through ERAD pathway (Olzmann, Kopito

et al. 2013). Transient eIF2α phosphorylation is beneficial to cells which are overloaded with misfolded proteins, since it reduces protein synthesis and increases the availability of chaperones thereby promoting refolding of misfolded proteins. However persistent high levels of p-eIF2α levels are detrimental and exacerbate neurotoxicity as shown in one of the studies. Several lines of evidence implicate abnormalities in ERAD with NDs. UPR is activated in striatum of transgenic mouse model of HD and ER-stress sensors e.g. phospho-eIF2α were found to be upregulated in postmortem brain samples of HD patients (Leitman, Barak et al. 2014). In mammalian cells the homologue of Cdc48 complex is Np14-Ufd1-p97 complex which helps in translocating misfolded ER resident proteins to cytosol for UPS mediated degradation. Entrapment of these essential ERAD proteins by polyQ-expanded HTT fragments have been observed in PC12 cells which render ERAD pathway to be non-functional. In line with this, one report shows overexpression of Np14 and Ufd1 in both yeast and PC12 cells ameliorates polyQ toxicity (Rideout, Larsen et al. 2001).

It has been reported that accumulation of misfolded proteins in the cell during pathogenic conditions cause excessive load of higher order structures such as large protein oligomers and complexes which cannot be cleared by UPS as their size restricts their clearance, but autophagy-related pathways have been shown to provide an alternative route for degradation of the same (Verhoef, Lindsten et al. 2002).
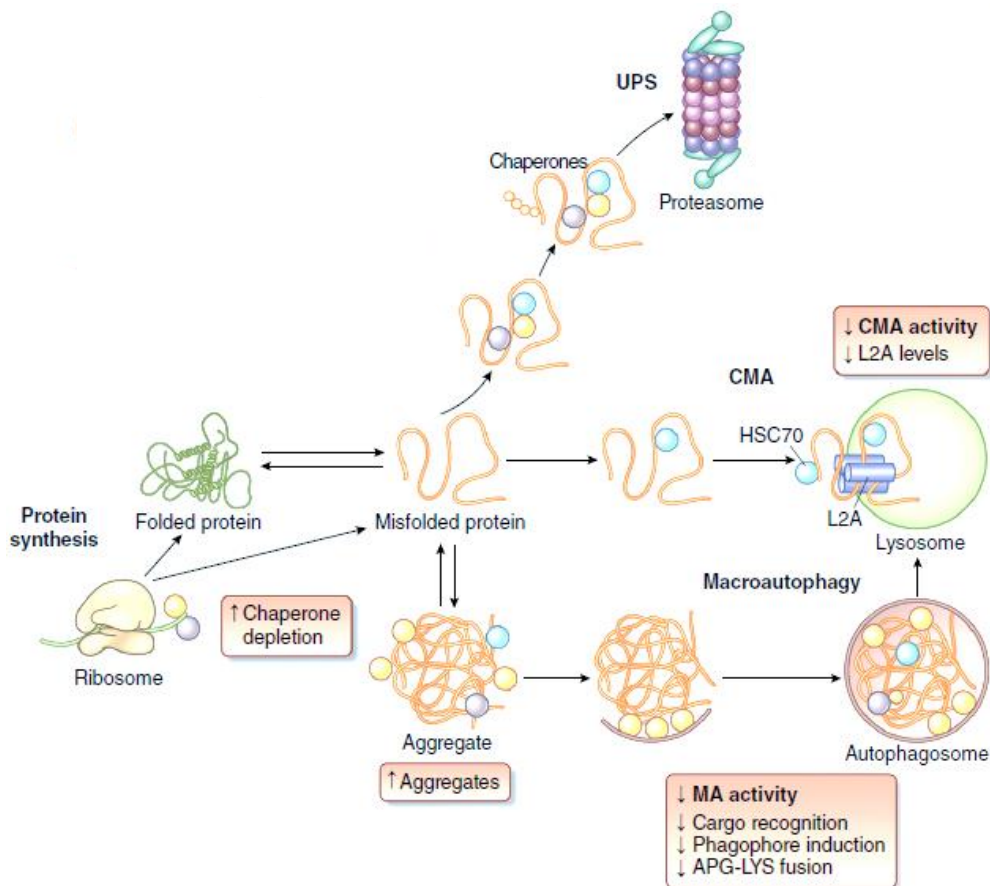
**Figure 2: Proteostatic machineries in the cell.** Changes in intracellular proteostasis system occur in pathological conditions. Chaperones and two proteolytic systems, the UPS and autophagy maintain proteostasis in the cell. Chaperones (blue, yellow and gray circles) assist protein synthesis and folding. Unfolded or misfolded proteins are targeted for degradation by the proteasome or autophagy. Single soluble proteins can reach the lysosomal lumen through a membrane transporter in chaperone-mediated autophagy (CMA). Once misfolded proteins organize into oligomers or insoluble aggregates, they are cleared by macroautophagy (MA). Boxes indicate changes which occur in different steps or components of intracellular proteostasis networks in case of NDs. APG-LYS, autophagosome-lysosome; HSC70, heat-shock cognate protein of 70kDa; L2A, lysosomal-associated membrane protein type 2A.**{With permission from Kaushik, Susmita, and Ana Maria Cuervo. *Nature medicine* (2015)}**

## 1.1.2 Autophagy and its related pathways

Autophagy is a catabolic process that results in lysosomal degradation of various cellular components ranging from damaged cell organelles to abnormal proteins. The term 'Autophagy' was coined by Christian de Duve has been derived from Greek words 'auto' and 'phagy' which means self-eating (Tooze and Yoshimori 2010). It is an evolutionarily conserved process and vital for maintaining cellular homeostasis. Basal level of autophagy occurs in all cell types under normal growth conditions and helps in housekeeping functions (Mizushima, Yamamoto et al. 2004, Mizushima 2007). The mechanism by which cargo gets sequestered autophagy can be divided into three types: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy.

Microautophagy

During microautophagy, the cytosolic components destined for the degradation are directly taken up by the lysosome through invagination of lysosomal membrane and then get degraded (Kaushik, Singh et al. 2010).

Chaperone-mediated autophagy (CMA)

In CMA, the cargo bears a recognition mark in the form of a pentapeptide motif (KFERQ) which is recognized by a cytosolic chaperone, heat shock cognate protein Hsc70 which targets the substrate and bind to lysosome-associated membrane protein type 2A (LAMP-2A) on the lysosome surface which unfolds and transfers the cargo across lysosomal membrane for degradation (Kaushik, Singh et al. 2010). CMA is an integral part of the proteostatic network of the cell and its dysfunction has been linked to several pathogenic conditions. The downregulation and remunerative up-regulation of various components of

CMA such as LAMP2A and Hsc70 have been associated with neurodegeneration (Koga and Cuervo 2011).

α-synuclein is a substrate for CMA, and its accumulation leads to formation of inclusion bodies known as 'Lewy bodies', is a characteristic hallmark of Parkinson's disease and a few tauopathies. It was found that LAMP2A and Hsc70 were downregulated in the substantia nigra of PD brain as compared to age-matched control brain samples (Alvarez-Erviti, Rodriguez-Oroz et al. 2010). On the contrary, CMA is known to get upregulated in HD but becomes inefficient in clearance of poly-Q expanded HTT fragments as compared to the full-length protein. A successful study in R6/2 mouse model of HD has shown selective targeting of poly-Q expanded HTT fragments for degradation via CMA resulting in amelioration of HD phenotypes (Bauer, Goswami et al. 2010).

Macroautophagy

Macroautophagy (hereafter, autophagy) is the major form of autophagy responsible for bulk degradation of cargoes. It starts with the formation of an isolation membrane which expands and captures cargoes in cytoplasm. Further expansion in the membrane results into a double membrane bound closed compartment known as autophagosome which is trafficked to the lysosome for fusion, forming autolysosome. Lysosome has various proteolytic enzymes which degrade the captured cargoes and the basic building blocks are recycled back to the cytoplasm for anabolic or other processes (Mizushima 2007).

## 1.2 <u>Molecular mechanism of autophagy</u>

Autophagy is a complex pathway and involves a concerted action of several autophagy related proteins to accomplish the degradation of cargoes. This process can be divided into various steps; it starts with induction of autophagy and vesicle nucleation, followed by vesicle elongation and concomitant cargo loading on autophagosome, which then fuse with lysosome culminating in degradation of the cargo.

### 1.2.1 Autophagosome biogenesis and vesicle elongation

Nutrient starvation is the most commonly studied stimuli for autophagy induction. Various pathways involved in sensing nutrient status of the cell coalesce at Mammalian Target Of Rapamycin (mTOR) which is a key regulator of cell growth and autophagy in response to nutritional status, growth factor and stress signals (Wullschleger, Loewith et al. 2006). mTOR phosphorylates unc-51-like autophagy activating kinase 1 (ULK1) at Ser757 and inhibits its activation thereby negatively regulates autophagy (Mizushima 2010).

AMP-activated protein kinase (AMPK) is a sensor of AMP/ATP ratio in the cell and positively regulates autophagy by phosphorylating ULK1 at Ser317 and Ser777 (Kim, Kundu et al. 2011).

Formation of autophagosome is initiated by the assembly and activation of multiprotein initiation complex constituted of ATG13, ATG101, FIP200 and ULK1 which is then targeted to isolation membrane (Itakura and Mizushima 2010).

In yeast autophagosome synthesis initiates at a single site known as 'pre-autophagosomal structure (PAS)', but there is no evidence of such structure in mammals (Mari, Tooze et al. 2011, Lamb, Yoshimori et al. 2013). The primary seeding membrane can be derived from endoplasmic reticulum (ER), recycling endosomes, and mitochondria. Recently ER-

mitochondria contact sites and plasma membrane also have been reported to be a source of membrane for autophagosome formation (Ravikumar, Moreau et al. 2010, Hamasaki, Furuta et al. 2013). Early events in autophagosome biogenesis are followed by the elongation of PAS or isolation membrane by incorporation of phospholipids from various sources which allows the recruitment of a multiprotein complex with class III phosphatidylinositol 3-kinase (PI3K) activity, comprises of several other members like beclin 1 (BECN1), phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3; known as VPS34), VPS15 and ATG14L which is a sensor of membrane curvature. Upon activation, VPS34 generates phosphatidyl 3-phosphate (PI3P) which helps in further expansion of autophagosomal membrane (Burman and Ktistakis 2010, Jaber and Zong 2013). Class III PI3K complex engages other PI3P-binding proteins such as WD repeat domain phosphoinositide-interacting protein 2 (WIPI2), and Double FYVE domain containing protein 1 (DFCP1) which are also involved in elongation of the isolation membrane (Pattingre, Tassa et al. 2005). ATG9 is a transmembrane protein which brings lipids to the expanding phagophore in the form of small vesicles and help in further expansion of autophagosomal membrane (Yamamoto, Kakuta et al. 2012).

As neurons are terminally differentiated post-mitotic cells, they substantially depend on basal autophagy to clear cellular insults like misfolded proteins and damaged cell organelle considering they lack the aid of cell division to dilute out such insults.

Neuronal autophagy is distinctly regulated as compared to non-neuronal cells. A study using GFP-LC3 transgenic mouse model showed disparity in the level of induction of autophagy upon starvation in brain and liver, wherein autophagy was highly upregulated in liver but not in the brain (Mizushima, Yamamoto et al. 2004). The impediment in inducing autophagy in brain has been indicated in various studies.

Autophagy induction also declines with aging as shown by reduced expression of Beclin-1 in aged brain samples. In Alzheimer's disease downregulation of Beclin-1 has been observed which contributes to beta amyloid accumulation and neurodegeneration (Pickford, Masliah et al. 2008).

Furthermore, in case NDs like Huntington disease, mTOR signaling is known to be altered. In one study PolyQ-HTT fragments have been shown to form a ternary complex with Rheb (GTPase) and mTOR near perinuclear region in striatal cell line which keeps mTORC1 complex active resulting in decreased autophagy (Pryor, Biagioli et al. 2014).

Parkinson's disease is a NDs characterized by degeneration of dopaminergic neurons in substantia nigra of the brain. In this disease, alpha-synuclein (SNCA) protein forms aggregates in neurons known as 'Lewy bodies'. These inclusion bodies have been shown to inhibit Rab1A which is a small GTPase, helps in Atg9 translocation to isolation membrane (Winslow and Rubinsztein 2011).

Canonical autophagy depends upon two ubiquitin-like conjugation systems for vesicle elongation step. First one involves ATG7 and ATG10 which are responsible for the formation of a complex comprised of ATG5, ATG12 and ATG16L (Glick, Barth et al. 2010). The second one involves ATG3, ATG4 and ATG7 which promotes cleavage of full length cytosolic LC3 and its conjugation to phosphatidylethanolamine (PE). This lipidated LC3 also known as LC3-II is recruited to expanding autophagosome membrane to function as receptors for autophagy substrates (Tanida, Ueno et al. 2004).

The deletion of core autophagy genes involved in autophagosome biogenesis such as ATG5 or ATG7 in the mouse central nervous system results in manifestation of progressive

deficits in motor function that are accompanied by accumulation of cytoplasmic inclusion bodies in neurons (Hara, Nakamura et al. 2006, Komatsu, Waguri et al. 2006).

## 1.2.2 Cargo loading onto autophagosomes

In the last decade it has become evident that autophagy is not just a non-selective bulk degradation pathway instead the discovery and characterization of several autophagy adaptors like p62 (SQSTM1), NBR1 (neighbor of BRCA1), Optineurin (OPTN), NDP-52 (nuclear dot protein 52 kDa) etc. which target specific cargoes to lysosome for degradation, has accelerated our understanding of selective autophagy. Selective autophagy can be of different types depending upon the type of cargoes: mitochondria (mitophagy), peroxisomes (pexophagy), intracellular pathogens (xenophagy) and protein aggregates (aggrephagy). Hence selective autophagy plays a crucial role in maintaining the integrity of cellular homeostasis (Johansen and Lamark 2011).

This selective nature of autophagy is attributed to the recognition of distinct cargoes by specific autophagy adaptors which have unique structure and biochemical activity. There are two common structural features which are found in these autophagy adaptors: ubiquitin binding domain (UBA) and LC3 interacting regions (LIR) (Wild, McEwan et al. 2014).

In mammals, a substrate destined for degradation is tagged with ubiquitin which acts as a signal for degradation. Ubiquitination can be mono- or polyubiquitination depending on the number of ubiquitin tags attached to the substrate and its site determines preferred pathway for degradation, e.g. Lysine 48 (K48) ubiquitination is a signal for proteasomal degradation while ubiquitination at K63 assist autophagy mediated degradation of substrates. Autophagy adaptors recognize ubiquitinated substrates on one hand and interact

with LC3 through LIRs on the other hand resulting in loading of cargo onto autophagosome (Tan, Wong et al. 2008).

p62 is one of the most studied autophagy adaptors helps in clearance of protein aggregates, pathogens and itself is an autophagy substrate. It undergoes phosphorylation at S403 by Tank-binding kinase 1 (TBK1) and ULK1, which enhances the affinity of p62 for ubiquitinated cargo (Matsumoto, Wada et al. 2011, Lim, Lachenmayer et al. 2015).

OPTN is known to mediate clearance of intracellular pathogens such as *Salmonella,* protein aggregates, and mitochondria. Phosphorylation of OPTN at S177 by TBK1 induces xenophagy while phosphorylation at S473 is associated with mitophagy (Wild, Farhan et al. 2011).

Several mutations in autophagy adaptors have been linked to NDs. In ALS and FTLD, mutations in OPTN is associated with its loss of function and subsequent accumulation of protein aggregates (Cirulli, Lasseigne et al. 2015, Freischmidt, Wieland et al. 2015). OPTN has been implicated in HD also, under normal conditions it interacts with HTT and Rab8 to facilitate post-Golgi trafficking but this function is impeded in disease condition (Del Toro, Alberch et al. 2009).

At least 40 mutations in p62 have been reported in sporadic and familial cases of ALS/FTLD. Although studying all these mutation in the context of disease is difficult but certain studies have indicated that they cause loss of function of p62 in selective autophagy and thus exacerbating the disease (Rea, Majcher et al. 2014).

In HD, mHTT known to enhance phosphorylation of p62 at S405 (by TBK1) and at S409 (by ULK1) which increase the affinity of p62 for ubiquitin chains (Matsumoto, Wada et al. 2011). Furthermore, studies have shown improper cargo recognition and loading of

mHTT on autophagosomes which led to the concept of 'empty autophagosomes' (Martinez-Vicente, Talloczy et al. 2010). On the contrary, p62 knockdown in HD mouse model resulted in increased lifespan, suggesting a complex role of p62 in HD pathology (Kurosawa, Matsumoto et al. 2014).

A recent study reported a new autophagy adaptor which has implications in clearance of mHTT, Cue5 (CUE domain targeting adaptor protein, CUET) in yeast whose mammalian homolog is Tollip (Lu, Psakhye et al. 2014).


### 1.2.3 Fusion of autophagosome to lysosome and degradation of cargo

Upon completion of autophagosome formation, it needs to be transported to lysosomes for fusion to form autolysosome. The molecular machinery involved in fusion is composed of myriad of proteins; one of them is GTPase RAB7A which is required for autophagosome maturation as well as for autophagosome-lysosome fusion (Gutierrez, Munafó et al. 2004, Eskelinen 2005). The tethering complex HOPS is a bona fide factor involved in autophagosome-lysosome fusion which interacts with autophagosomal SNARE, syntaxin17 (STX17) (Jiang, Nishimura et al. 2014).

Autophagy process culminates with the degradation of cargo by the action of hydrolytic enzymes such as cathepsin B and D present in the lysosomes (Eskelinen 2005).

In case of AD, accumulation of autophagic vesicles (AVs) in axon terminal was shown in human AD brain samples and are a cause of excitotoxicity (Sanchez-Varo, Trujillo-Estrada et al. 2012). This increased number of AVs in the axon terminal was due to impairment in retrograde trafficking to soma. Additionally defective lysosomal proteolysis has been linked to PSEN1 mutation which leads to defect in autolysosome acidification and

cathepsin activation. PSEN1 binds to v-ATPase and helps in its N-glycosylation which is essential for its efficient delivery to lysosome, but mutated PSEN1 results in failure in targeting of v-ATPase subunits to lysosome which leads to inefficient clearance of amyloid precursor protein (APP) causing AD pathology (Wolfe, Lee et al. 2013).
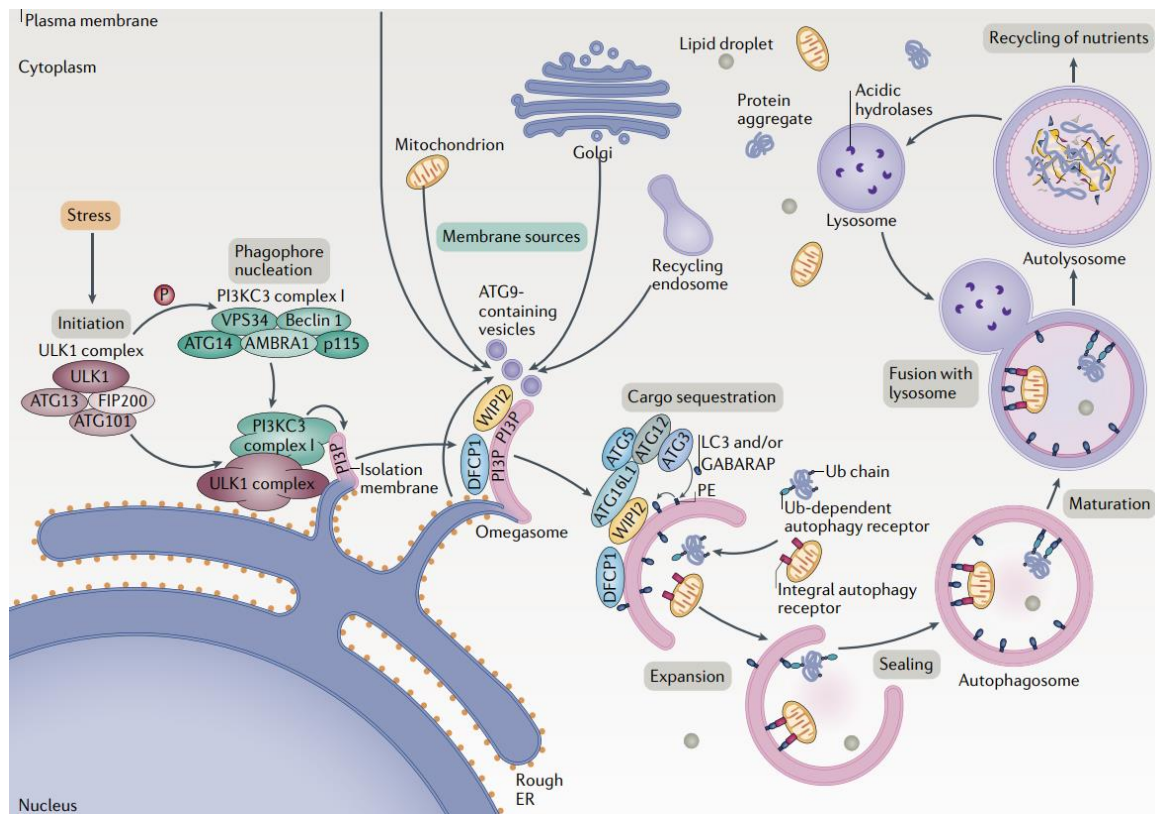


**Figure 3: Overview of autophagy process.** Signals that activate the autophagic process (initiation) typically originate from various conditions of stress, such as starvation, hypoxia, oxidative stress, protein aggregation, endoplasmic reticulum (ER) stress and others. The common target of these signaling pathways is (ULK1) complex which then triggers nucleation of the phagophore by phosphorylating components of the class III PI3K (PI3KC3) complex I, which in turn activates local phosphatidylinositol-3-phosphate (PI3P) production at a characteristic ER structure called the omegasome. PI3P then recruits the

PI3P effector proteins WD repeat domain phosphoinositide-interacting proteins (WIPIs; here WIPI2) and zinc-finger FYVE domain-containing protein 1 (DFCP1) to the omegasome via interaction with their PI3P-binding domains. WIPI2 recruits the ATG12~ATG5–ATG16L1 complex that enhances the ATG3-mediated conjugation of ATG8 family proteins (ATG8s), including microtubule- associated protein light chain 3 (LC3) proteins and γ-aminobutyric acid receptor-associated proteins (GABARAPs) to membrane-resident phosphatidylethanolamine (PE), thus forming the membrane-bound, lipidated forms; for example, in this conjugation reaction, LC3-I is converted into LC3-II - the characteristic signature of autophagic membranes. Several cellular membranes, including the plasma membrane, mitochondria, recycling endosomes and Golgi complex, contribute to the elongation of the autophagosomal membrane by donating membrane material (part of these lipid bilayers is delivered by ATG9 containing vesicles, but the origin of the rest of the lipid bilayer is currently unknown). Sealing of the autophagosomal membrane gives rise to a double-layered vesicle called the  autophagosome, which matures n(including stripping of the ATG proteins) and finally fuses with the lysosome. Acidic hydrolases in the lysosome degrade the autophagic cargo, and salvaged nutrients are released back to the cytoplasm to be used again by the cell. Ub, ubiquitin.

**{With permission from Dikic Ivan et al *Nature Reviews Molecular Cell Biology* (2018)}**

## 1.3 Therapeutic potential of autophagy in neurodegeneration

In most of the NDs aggregate-prone misfolded proteins are known to give rise to an array of toxic higher order structures which contribute to pathology of the disease. Therefore reducing the amount of these species in the cells could be a viable approach to alleviate toxicity in NDs. Since autophagy has the potential to degrade such aggregate-prone species, upregulating autophagy could be helpful in clearance of the same.

In order to enhance autophagy several small molecules modulator of autophagy have been employed. These autophagy inducers can be of two types depending on their activity: mTOR-dependent or mTOR-independent. mTOR inhibition leads to activation of autophagy. mTOR inhibitors also can be classified as ATP-competitive inhibitors e.g. Torin 1 or non-ATP-competitive inhibitors e.g. rapamycin and its analogs (Kim and Guan 2015).

Rapamycin and rapalogs such as CCI-779 have been shown to have beneficial effects as autophagy inducers in animal models of AD, PD and HD (Ravikumar, Vacher et al. 2004). The mTOR-independent autophagy enhancers such as Metformin and Trehalose work via AMPK, has shown therapeutic effects in HD and AD mouse models (Tanaka, Machida et al. 2004). In addition to these other autophagy enhancers such as rilmenidine, clonidine and verapamil which are modulator of cyclic AMP (cAMP) have been reported to be beneficial in case of HD (Rose, Menzies et al. 2010).

Furthermore, non-small molecule approaches for autophagy enhancement are also becoming popular. For example in case of HD, knockdown of calpain (RNAi) in *Drosophila* resulted in upregulation of autophagy with concomitant reduction in mHTT aggregates and increase in cell survival (Menzies, Garcia-Arencibia et al. 2015).

## 1.4 <u>Huntington's disease (HD)</u>

HD is an autosomal dominant ND characterized by involuntary motor movement, cognitive decline and psychiatric illness. HD is one of the nine polyglutamine disorders in which a trinucleotide CAG repeat expansion in exon 1 of HTT gene codes for expanded polyQ tract in N-terminal of the protein making it aggregate-prone (MacDonald, Ambrose et al. 1993). These mHTT aggregtaes cause a selective degeneration of the striatal and cortical neurons that project to the striatum in brain of HD patients. The length of the polyQ tract is correlated with the severity of disease. An expansion of the polyQ tract in HTT to greater than 36Q causes disease, and the greater the number of glutamine residues, the younger is the age of onset of disease. The mutation confers a toxic gain-of-function to HTT protein resulting in severe neurodegeneration in striatum (DiFiglia, Sapp et al. 1997). Mutant HTT (mHTT) is a misfolded protein which has higher propensity to form proteinaceous aggregates. These aggregates are found in the nucleus and cytoplasm as intraneuronal inclusion bodies (Sapp, Schwarz et al. 1997). It is important to understand physiological function of normal HTT in order to understand the toxicity imparted by polyQ expansion. The complete knockout of HTT in mice is embryonically lethal and its role in mouse embryonic brain development has been demonstrated (Cattaneo, Rigamonti et al. 2001). Moreover, it is crucial for survival of adult neurons in the forebrain. It is suggested that the pathology in case of HD is a summation of loss-of-function of the wild type form of HTT and a toxic gain-of-function because of polyQ expansion in mHTT. Although the mechanism by which toxic gain-of-function affects neurons is poorly understood, but transcriptional dysregulation, mitochondrial and autophagy dysfunction are some of the consistent features observed in HD patients (Kegel, Kim et al. 2000).
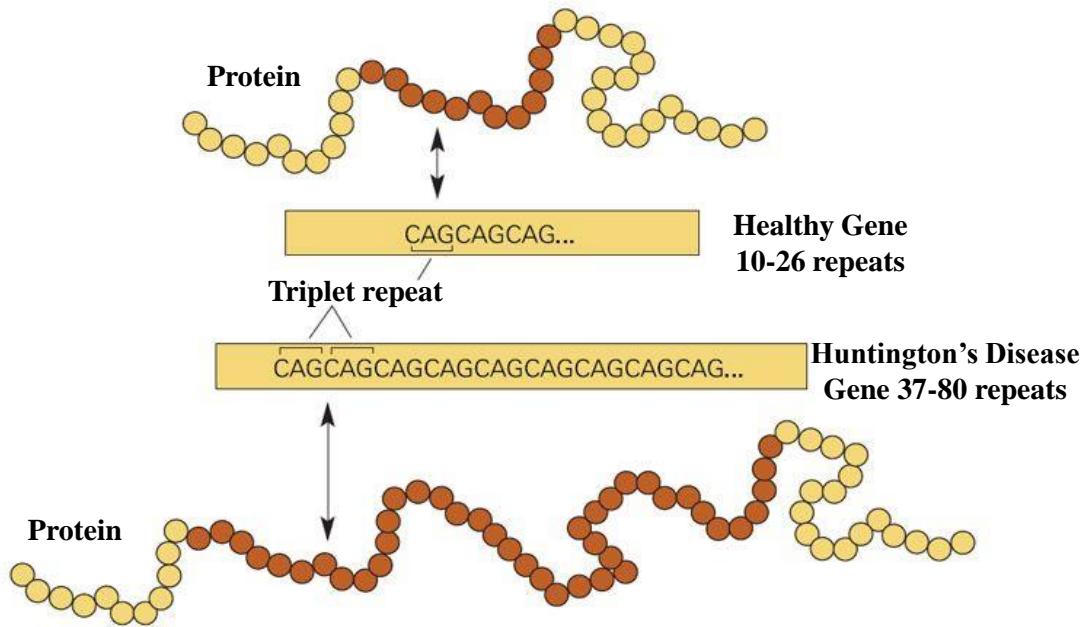
**Figure 4: Mutation in huntingtin protein.** A trinucleotide CAG repeat expansion in

huntingtin gene codes for a Poly-Q tract in protein.

**{https://www.nist.gov/public_affairs/tech-beat/tb20110412.cfm}**

## 1.5 Role of Huntingtin protein in autophagy

Huntingtin is a large protein of 350 kDa which is expressed ubiquitously in all tissue types

with highest expression observed in brain and testes. HTT contains HEAT (Huntingtin,

Elongation factor 3, PR65/A regulatory subunit of PP2A, and Tor1) repeats which mediate

protein-protein interaction (Tartari and Gissi 2008). The normal polyQ tract following 18

amino acids of exon 1 forms a polar zipper which helps in interaction with other factors

bearing polar residues. The C-terminus of HTT has an active nuclear export signal (NES).

Additionally the first 18 amino acid sequence has been shown to interact with TPR, a

nuclear pore protein that mediates nuclear translocation. Point mutation in this particular

region of HTT results in its accumulation in the nucleus and cellular toxicity (Cornett, Cao et al. 2005).

The wild type HTT promotes autophagosome formation since it has an autophagy-inducing domain spanning amino acids 553-586. This is regulated by a post-translational modification (PTM) i.e. myristoylation at G553 which promotes membrane binding. Myristoylation of HTT has been linked to increased autophagy flux by demonstrating elevated levels of LC3-II (Martin, Heit et al. 2014). mHTT undergoes decreased myristoylation as compared to wild type suggesting decreased membrane association and altered autophagy induction.

A bioinformatics analysis has predicted that HTT has 11 LIRs (LC3 interacting repeats), of which only one is present in the first 600 amino acid residues and most of them are towards C-terminus of the protein which has homology with yeast autophagy proteins Vac8 and ATG11. This observation could suggest the reason behind toxicity associated with shorter fragments owing to decreased association with LC3 (Kalvari, Tsompanis et al. 2014).

HTT also play a role in promoting autophagosome trafficking. In neurons autophagosomes are generated near distal axons and are trafficked to soma by retrograde transport. HTT along with HTT-associated protein 1 (HAP1), facilitates retrograde transport of autophagosomes (Block-Galarza, Chase et al. 1997).

**1.6 <u>Dysfunctional Autophagy in HD</u>**

An altered or dysfunctional autophagy has been reported in case of HD. An increased number of autophagic vesicles along with an increase in autophagy markers like p62 and LC3-II has been shown in striatum of HD mouse models indicating decreased autophagic

flux. On the contrary, mHTT aggregates sequesters mTOR thus inhibiting its activity and inducing autophagy (Ravikumar, Vacher et al. 2004, Kim and Guan 2015).

There is one more explanation for decreased autophagy-mediated degradation of mHTT i.e. cargo recognition failure which leads to formation of empty autophagosomes. It has been suggested that it is the aberrant p62-mHTT interaction which causes this inefficient loading of cargo onto autophagosomes. This deficient autophagic cargo recognition has detrimental effects on autophagy turnover of cellular organelle as demonstrated by accumulation of defective mitochondria in a cell model of HD (Martinez-Vicente, Talloczy et al. 2010).

In neurons autophagosome biogenesis happens at axonal tip and they have to be trafficked via retrograde transport to cell body for autophagosome lysosome fusion. As mentioned above HTT along with HAP1 regulate the motor activity and processivity of microtubule motor proteins such as dynein, dynactin and kinesin to facilitate retrograde transport of autophagosomes (Block-Galarza, Chase et al. 1997). In HD, mHTT interaction with HAP1 is altered resulting in bidirectional or stationary dynamics of autophagosomes in HD neurons. This dysregulation of motors disrupts the retrograde transport of autophagosomes thereby leading to inefficient degradation of cargo.

Although toxic gain-of-function by mHTT has been implicated to cause HD pathology, the loss-of-function of wild type HTT can also contribute to the same.

## 1.7 <u>Tetracycline inducible expression system</u>

The unregulated overexpression of gene of interest might have unwanted physiological or toxic effects. Therefore the development of inducible expression system becomes important to study gene function as inducible promoters can provide control over regulation of gene expression. This system is invincible if a protein of interest is toxic in nature and only controlled expression is required.

One such system is Tetracycline inducible expression system. This system is tightly regulated and highly responsive system that provides with robust inducible expression of gene of interest.

Expression through Tetracycline inducible promoter is driven by conjugation of two components i.e. and a) pTRE-tight inducible promoter and b) Transactivator.

### a) pTRE-tight inducible promoter

pTRE-tight inducible promoter is a synthetic promoter which has modified Tet-response element (TRE) which contains seven direct repeats of a 19 nucleotide tetracycline operator sequence (TetO) followed by a minimal CMV promoter.

### b) Transactivator

The transactivator or tetracycline-controlled transactivator (tTA), is a hybrid transcription factor resulting from the fusion of the prokaryotic Tet repressor (TetR) with a eukaryotic transcriptional transactivation domain of virion protein 16 of herpes simplex virus (HSV). The TetR moiety confers sequence specific DNA binding and sensitivity to tetracycline. The binding of tetracycline alters affinity of tTA to their cognate binding sites, TetO.

Based on transactivator, Tet system can be two types:-

## 1) Tet-off system

This was the first system to be developed where in the presence of tetracycline, expression from a Tet-inducible promoter is reduced. In the absence of tetracycline, the tetR portion of tTA will bind these tetO sequences and the activation domain promotes expression. Whereas when tetracycline is present, it goes and binds to tetR. This hinders tTA binding to the tetO sequences and thereby resulting in reduced gene expression
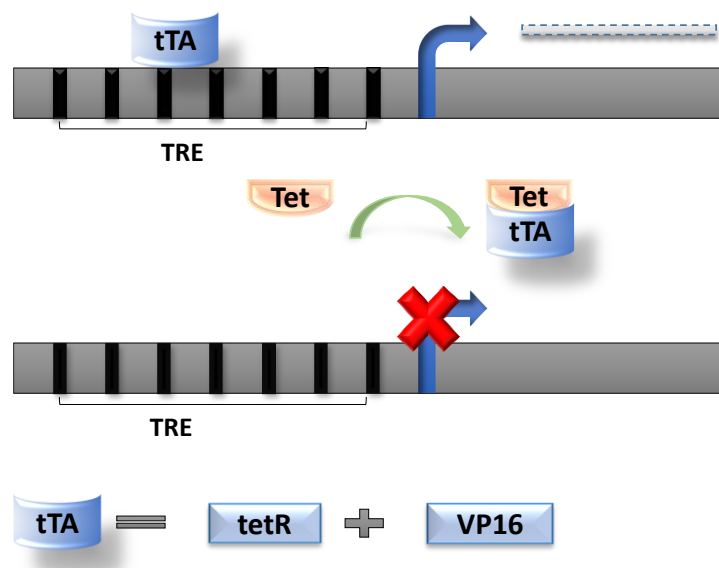


**Figure 5: Tet-off system.** tTA binds to TRE and drives the expression of gene in absence of tetracycline. While in the presence of tetracycline tTA preferentially binds to it and can no longer drive the expression of gene of interest.

## 2) Tet-on system

In 1995, Gossen et al used random mutagenesis to identify the amino acid residues of TetR, which are responsible for tetracycline dependent repression. Identification and subsequent mutation in these residues led to the development of a reverse Tet repressor, or rTetR

(reverse tetracycline-controlled transactivator). This mutated version had opposite phenotype and facilitated expression only in the presence of tetracycline. rtTA was generated upon fusion of this rTetR with VP16 of HSV. rtTA requires tetracycline for binding to tetO, thus drives expression of gene of interest only in the presence of tetracycline.
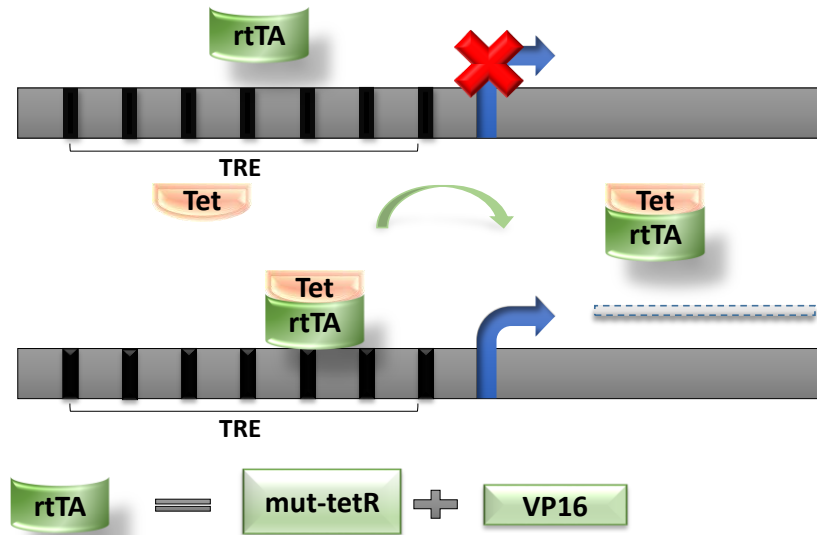


**Figure 6: Tet-on system.** rtTA can only bind to TRE when it is bound to tetracycline and facilitate expression of gene of interest.

Advantages of using tetracycline inducible system is that it provides extremely tight regulation for expression of gene of interest also the bipartite nature of this system makes it highly specific.

**Chapter-2 Materials and Methods**

**2.1 Cell culture**

Cells were maintained in growth medium comprised of Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, D5648) supplemented with 3.7g/L sodium bicarbonate plus 10% fetal bovine serum (FBS; Pan, 3302-P121508) and 100 units/ml of penicillin and streptomycin (Sigma-Aldrich, P4333) at 80% relative humidity, 5% $CO_2$ and 37ºC.

**2.2 Transfection**

**Transfection for microscopy:**

Cells were grown on coverslips in 35mm dish until 60-70% confluency. Growth medium was removed and fresh growth medium (1 ml) was added. The dish was kept in 37ºC incubator for few minutes to allow pre-warming of the medium. Cells were transfected with different plasmids such as EGFP-HTT-Q23 (Addgene plasmid #40261), EGFP-HTT-Q74 (Addgene plasmid #40262) and RFP-LC3 (Addgene plasmid #21075) etc. Transfection mix was prepared by first diluting 1.25μg of DNA and 2.5μl of Lipofectamine 2000 (11668-019, Invitrogen) in OPTI-MEM (31985-070, Invitrogen) in 50μl separately, followed by incubation for 5 minutes. The DNA and Lipofectamine dilutions were then mixed and incubated for 20 minutes at room temperature. Then the DNA-lipid complex mixture was added to the cells. Transfection medium was removed after 6 hours and fresh growth medium (2ml) was added. Cells were incubated at 37ºC for next 48 hours followed by treatments.

**Transfection for Western blot:**

Cells were plated in 6-well cell culture plates until 60-70% confluency. Growth medium was removed and fresh growth medium (1ml) was added in each well which was supposed to be transfected. The plate was kept in 37ºC incubator for few minutes to allow pre-warming of the medium. Cells were transfected with different plasmids such as EGFP-HTT-Q23 (Addgene plasmid #40261) and EGFP-HTT-Q74 (Addgene plasmid #40262). Transfection was performed as mentioned above by adding 1.25µg DNA in each well and mixed with appropriate amount of Lipofectamine i.e. in 1:2 ratio of DNA:Lipofectamine. Transfection medium was removed after 6 hours and fresh growth medium (2ml) was added to each well. Cells were incubated at 37ºC for next 48 hours followed by treatments.

## 2.3 Cell culture treatments

**Microscopy and Western blotting:**

For microscopy in HeLa cells, 48 hours post-transfection cells were either left untreated or treatment was given with Earle's balanced salt solution (EBSS) or Torin1 (1µM) (Tocris Bioscience, 4247) in growth medium for two hours to induce autophagy. After treatment cells were processed for immunofluorescence.

For Western blotting, 48 hours post-transfection HeLa cells were given treatment with EBSS (2 hours) and Torin 1 (2-8 hours) to induce autophagy. Lysates were collected in 1X SDS buffer post-treatment and processed further for western blotting.

**Cell culture treatments for rtTA expressing cell line:**

For microscopy in rtTA HeLa cell line, 48 hours post-transfection cells were treated with Doxycycline hyclate (Sigma-Aldrich, D9891) in order to induce gene expression. Doxycycline hyclate was used at a concentration of 1µg per ml across various time points ranging from 6-24 hours. Similar Doxycycline treatments were done for western blotting experiment also.

## 2.4 <u>Immunofluorescence</u>

Upon appropriate transfections and treatments on cells plated on coverslips, cells were washed with PBS three times and fixed in 4% paraformaldehyde followed by three washes in PBS again. Permeabilization was done using 0.25% Triton X-100 (Himedia, MB031) for 15 minutes. Then the coverslips were incubated with anti-SQSTM1/p62 (anti-mouse, Abcam, ab56416) at 4ºC overnight. After incubation PBS washes were given to remove excess antibody and coverslips were then incubated with secondary antibody (anti-mouse Atto-633, Sigma-Aldrich, 78102) for 2 hours. The coverslips were mounted with VECTASHIELD antifade reagent (Vector laboratories, H-1000/H-1200). The transfected cells which were not followed by antibody staining were directly mounted after fixation step. Imaging for cells was carried out using Delta Vision microscope [(API, GE, USA, 29065728), (Olympus 60X/1.42, Plan ApoN, excitation and emission filter Cy5, TRITC, FITC and DAPI, polychroic Quad)].

## 2.5 <u>Western blotting</u>

Upon appropriate transfections and treatments on cells plated on 6-well culture dishes, cells were washed with ice cold PBS (Sigma-Aldrich, D5773). Cells were then lysed by adding 100 μl of 1X SDS buffer (10% [wt:vol] SDS, 10 mM DTT, 20% [vol:vol] glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% [wt:vol] bromophenol blue) and the lysates were collected by using a rubber cell scraper followed by boiling at 99ºC for 15 min.

The cell lysates were electrophoresed on different percentages of SDS-PAGE based on the size of the protein to be analyzed and transferred onto PVDF membrane at constant current of 0.15 Ampere for 2 hours (Biorad). After transfer, blocking was done by incubating blots in 5% skimmed milk for an hour at room temperature. Upon blocking blots were kept for overnight incubation with the appropriate primary antibodies such as anti-EGFP (Roche, 11814460001); anti-MAP1LC3B [Sigma-Aldrich, L7543]; anti-phospho-p70S6K (Thr389) [Cell signaling Technology (CST), 9205]; anti-p70S6K (CST, 9202); anti-β-actin (CST, 4970) and anti-β-tubulin (E7 DSHB-C1-377) made in 0.05% BSA (Sigma-Aldrich, A7906) and PBS at 1:5000 (except anti-EGFP, 1:8,000) dilution. Secondary antibodies conjugated to HRP, goat anti-rabbit (Bio-Rad, 172-1019) and goat anti-mouse (Bio-Rad, 172-1011) were used at 1:10,000 dilution. Blots were developed using ECL substrate (Bio-Rad, 170-5061) and image were captured using an auto capture program in Syngene G-Box, UK. ImageJ software (NIH) was used for quantification of band intensities.

## 2.6 <u>Kill curve for standardizing puromycin antibiotic concentration on HeLa cells</u>

50,000 HeLa cells were plated in each well of a 24-well cell culture plate. Cells were allowed to grow until they reached 50-60% confluency. Growth medium was replaced by medium containing increasing concentration of puromycin dihydrocholride (Sigma-

Aldrich, P8833).The range of antibiotic concentration tested was from 250ng - 6μg per ml of medium. Antibiotic was replenished every third day up to a week. MTT assay was performed after 7 days.

Cells were given PBS washes and fresh growth medium was added in each well. 100μl of 12 mM (5mg in 1 ml) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Amresco, 0793) was then added to each of these wells and incubated for 2 to 4 hours until purple precipitates became visible. Medium was removed and purple crystals so obtained were dissolved in DMSO (Sigma-Aldrich, D8418) by mixing thoroughly with pipette. Absorbance was read at 570nm using Varioskan Flash, Thermo Scientific, USA.

## 2.7 Cloning

Subcloning of EGFP-HTT-Q23 (Addgene plasmid #40261) and EGFP-HTT-Q74 (Addgene plasmid #40262) insert was done into pTRE-tight vector (Clontech). Compatible restriction sites (HindIII and NotI) were generated through PCR in the inserts. Upon PCR, restriction digestion was performed followed by ligation (T4 DNA Ligase) and transformation. Clones so obtained were confirmed by PCR and restriction digestion. Subsequent validation of expression was done using fluorescence microscopy and Western blotting.

## Primer details

| Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|
| TTGCGGCCGCATGGTGAGCAAGGG | CCCAAGCTTTTATCTAGATCCGGTG |

# Chapter-3

## Polyglutamine aggregates and autophagy

### 3.1 Background information

Cellular proteostasis is maintained by components of proteostatic network in the cell which ensures continuous clearance of dysfunctional or potentially toxic cellular components such as misfolded proteins and damaged cell organelles. If these machineries do not function properly, it leads to accumulation of unfolded or misfolded proteins which have a higher propensity to form aggregates. In case of various NDs, such as Alzheimer's, Parkinson's and Huntington's disease aggregation of misfolded proteins in neurons is observed. In order to bring about degradation of such misfolded proteins, cells have two key pathways - the ubiquitin-proteasome system (UPS) and autophagy related pathways. Due to large size of these aggregates, they cannot be cleared by UPS but are a bonafide substrate for autophagy. This selective clearance of aggregates by autophagy is termed as aggrephagy. In NDs due to overload of misfolded proteins, basal autophagy becomes inefficient. On the other hand, if autophagy is upregulated it leads to clearance of aggregate-prone proteins or aggregates thus reducing the load of such proteinaceous species in the cell. In this study we employed poly glutamine aggregates as a cargo to study aggrephagy.

Huntington disease (HD) is one of the nine polyglutamine disorders wherein a trinucleotide repeat (CAG) expansion in the gene translates to a polyglutamine tract in the protein which makes it highly hydrophobic and aggregate-prone. Mutant huntingtin (mHTT) protein having 74 such glutamine residues was used in the current study to understand its aggregation, subcellular localization and its effect on basal autophagy. Furthermore,

consequences of autophagy induction were studied as a function of mHTT clearance. These efforts were directed towards employing PolyQ aggregates as a cellular model system to study aggrephagy.

## 3.2 Results

### 3.2.1 Establishing a cell-based model to study polyglutamine aggregates

In order to generate a cell-based model to study the aggregation of polyglutamine protein, huntingtin constructs were transfected and overexpressed in HeLa cells. These constructs (**Fig. 1**) have N-terminal EGFP tagged exon 1 of huntingtin protein (first 14 amino acids) followed by 23 glutamine (control) or 74 (mutant) glutamine residues. Upon overexpression in HeLa cells, as shown in the microscopic images (**Fig. 2**), EGFP-HTT-Q23 showed a diffused pattern of expression but EGFP-HTT-Q74 formed aggregates. In accordance with other studies (Kazantsev, Preisinger et al. 1999), only the expanded PolyQ tracts showed accumulation of GFP positive aggregates/structures, suggesting that N-terminal truncated fragments of huntingtin with expanded glutamine repeats can aggregate in cells.
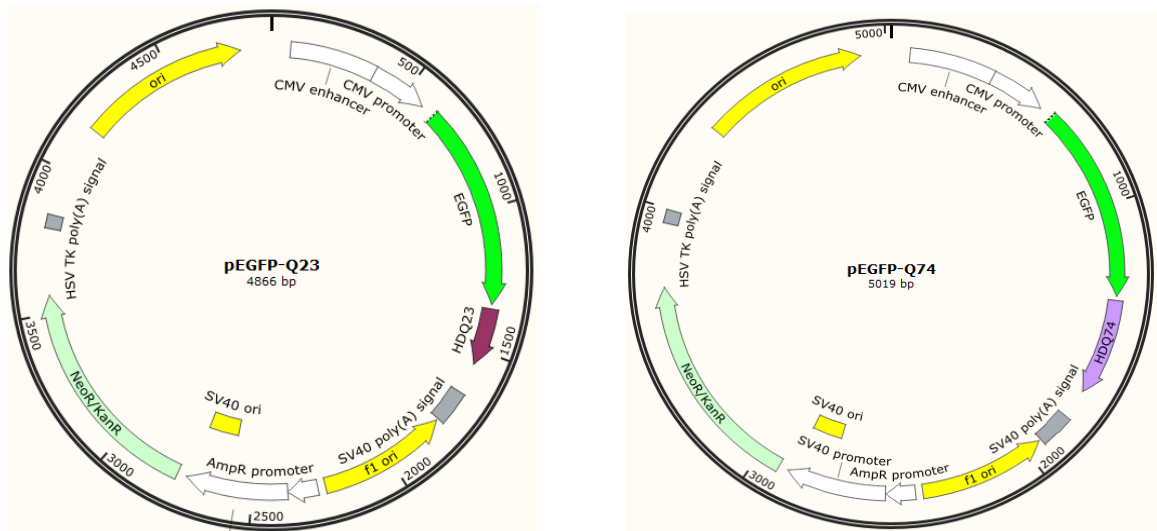
**B)**

**Figure 1: Plasmid maps of huntingtin constructs.** A detailed map of **A)** EGFP-HTT-Q23 construct and **B)** EGFP-HTT-Q74 construct. These plasmids have constitutive CMV promoter for driving expression of N-terminal EGFP tagged exon 1 of huntingtin having 23 (A) or 74 (B) glutamine residues.

| EGFP | DAPI | MERGE |
|------|------|-------|
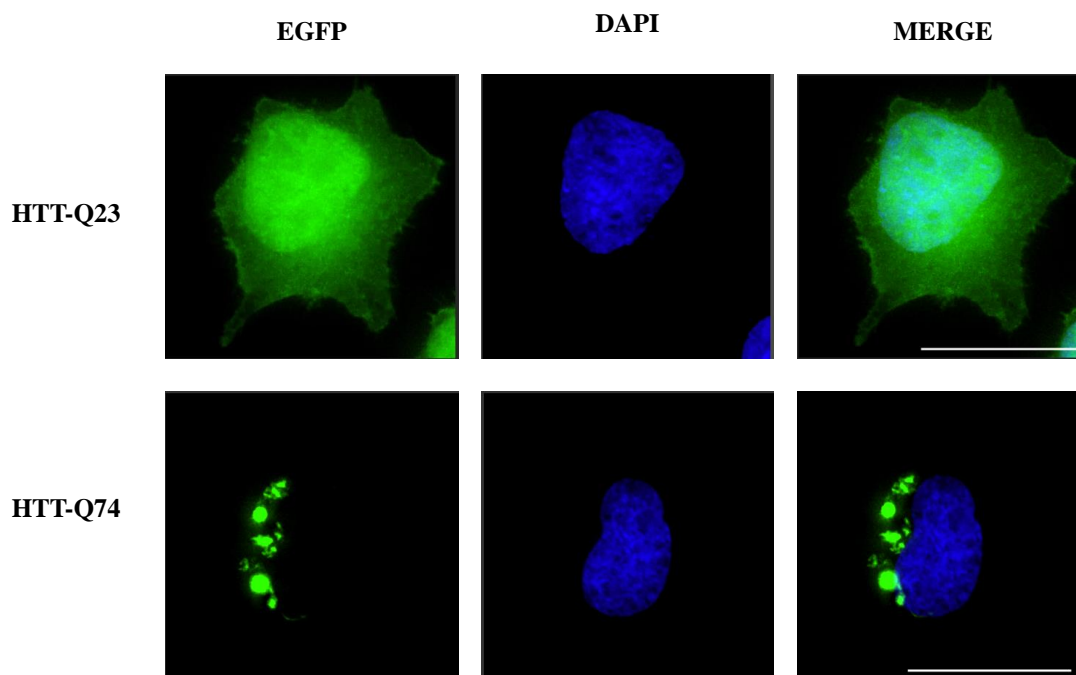


**HTT-Q23**

**HTT-Q74**

**<u>Figure 2: Overexpression of huntingtin constructs in HeLa cells.</u>** Hela cells transfected with EGFP-HTT-Q23 (top) and EGFP-HTT-Q74 (bottom). EGFP-HTT-Q23 showed a diffused expression whereas EGFP-HTT-Q74 is showed aggregates in the perinuclear region. Scale bar-15 μm.

Next, the distribution of these aggregates in the cell was studied (**Fig. 3**). Based on their subcellular localization, the aggregates were classified in three categories, 1) perinuclear, 2) nuclear and 3) both perinuclear and nuclear. Upon quantitation of the number of cells showing aggregates in these three locations it was observed that these aggregates are primarily found in perinuclear region. This could be a result of interaction of huntingtin with β-tubulin and γ-tubulin of microtubule-organizing center (MTOC).
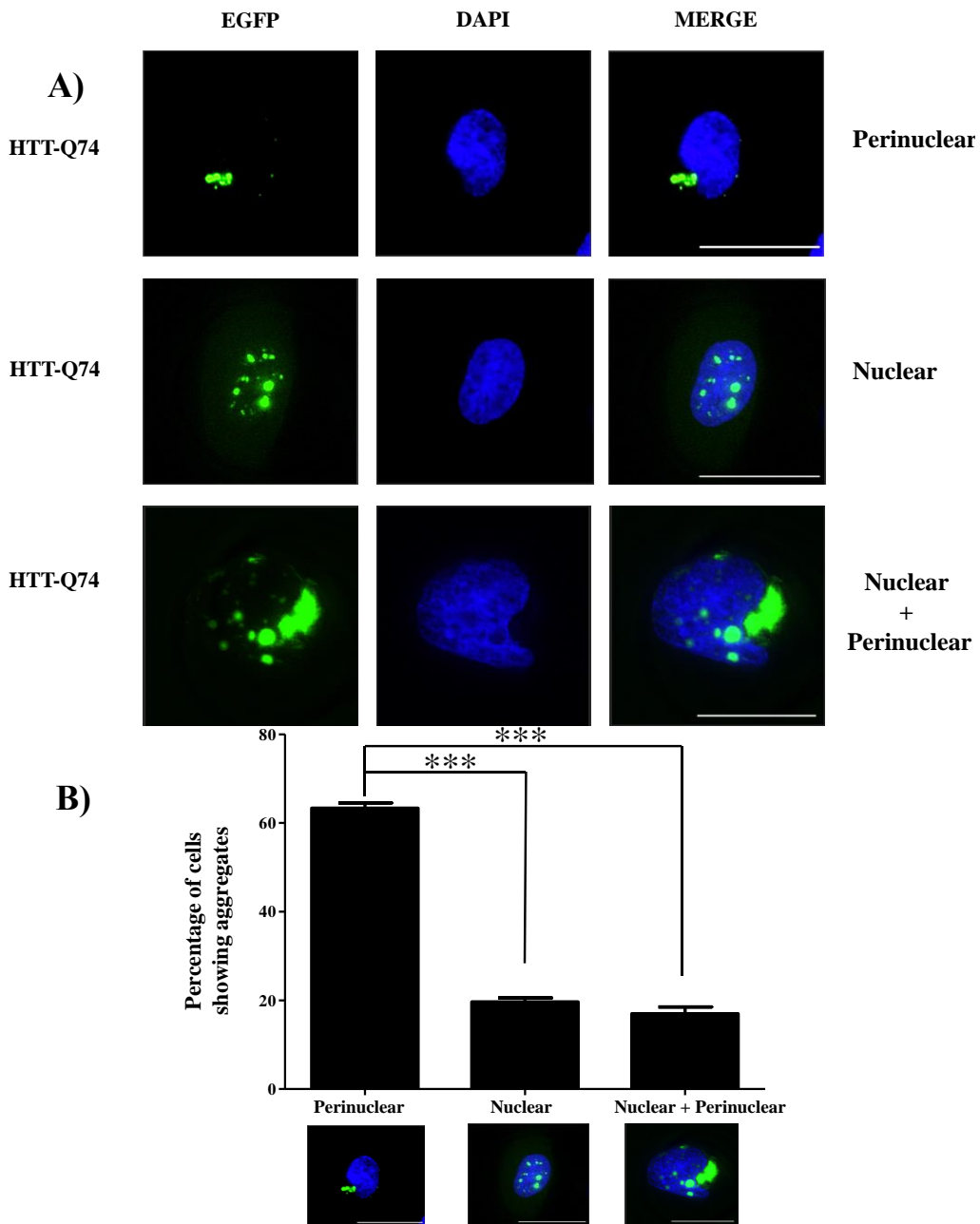
**Figure 3: Distribution of huntingtin aggregates.** **A)** HeLa cells transfected with EGFP-HTT-Q74 showed distribution of aggregates primarily in three regions in the cell, only perinuclear, only nuclear, both nuclear and perinuclear. Scale bar- 15 μm. **B)** Quantitation of percentage of cells showing aggregate in different regions of the cell. As depicted in graph, aggregates were predominantly found in perinuclear region. N=3, n=100 cells. 1way ANOVA, *** p ≤ 0.001.

### 3.2.2 Mutant huntingtin aggregates and autophagy

**Number of autophagic vesicles increase upon mHTT overexpression**

HeLa cells were co-transfected with huntingtin construct and RFP-LC3 (autophagosomal marker) for 48 hours followed by processing for fluorescence microscopy. Number of LC3 puncta were counted in wild type, and upon overexpressing EGFP-HTT-Q23 orEGFP-HTT-Q74. As shown in **Fig. 4**, number of LC3 puncta increased significantly in case of EGFP-HTT-Q74 overexpression whereas the difference between wild type and HTT-Q23 was not significant.

This increase in number of LC3 puncta upon EGFP-HTT-Q74 overexpression indicates some alteration in autophagy flux. This corroborates with autophagy defect found in striatal knock in Q111/111 cell line model of HD, where an increase in the number of LC3 puncta was observed in basal as well as induced autophagy conditions (Martinez-Vicente, Talloczy et al. 2010). This increase can be due to enhanced autophagosome biogenesis or an impairment at autophagosome-lysosome fusion step.

Further studies have to be carried out in this regard to comment upon the status of autophagy in cells expressing EGFP-HTT-Q74.
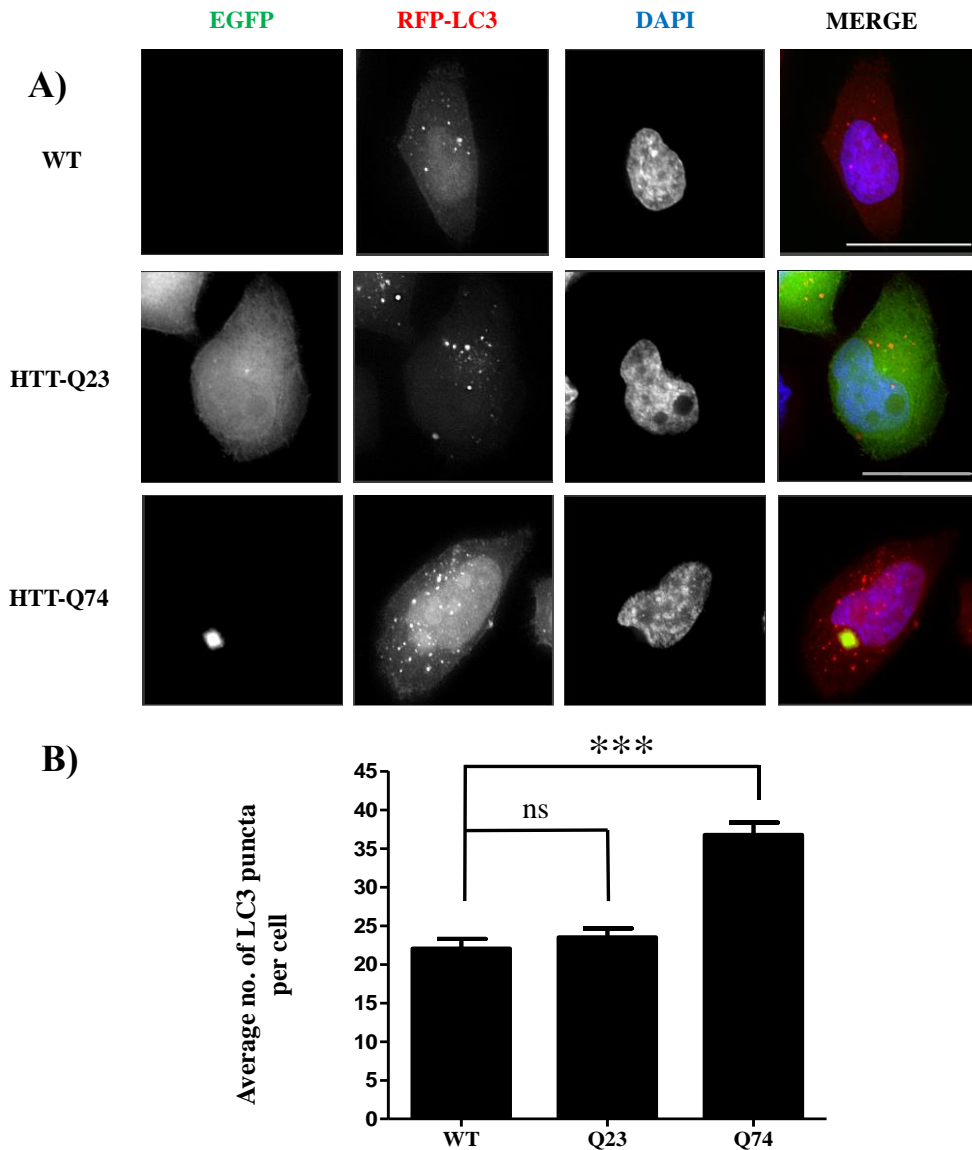
**Figure 4: Average number of LC3 puncta upon overexpression of huntingtin. A)**
Microscopic images showing number of LC3 puncta (RFP-LC3) in wild type condition
and upon overexpressing EGFP-HTT-Q23 or EGFP-HTT-Q74. Scale bar-15 μm. **B)** Graph
representing quantitation of the average number of LC3 puncta across all three conditions.
The average number of LC3 puncta were comparable between WT and EGFP-HTT-Q23
with no significant difference but they were significantly increased in case of EGFP-HTT-
Q74. WT-wild type, Q23- EGFP-HTT-Q23, Q74- EGFP-HTT-Q74, ns- non significant.
Unpaired t-test, *** $p \leq 0.001$, N=3, n=20 cells.

**Cargo capture in autophagosomes is reduced upon EGFP-HTT-Q74 overexpression**

One of the hallmarks of aggregates capture in aggrephagy involves ubiquitination of these aggregates followed by recruitment of autophagy adaptors such as p62, NBR1 and optineurin. The autophagy adaptors bind to ubiquitin moieties on the cargo via ubiquitin binding domain (UBD) as well as to LC3 (autophagosomal marker), since they have LC3-interacting region (LIR). One of the well-studied adaptor is p62 which captures cargo destined to be degraded by autophagy.

Therefore, to discern the status of interaction between autophagy cargo adaptor p62 and LC3, HeLa cells were co-transfected with EGFP-HTT-Q74 and RFP-LC3 constructs followed by immunostaining with p62 antibody (**Fig. 5**). It was found that although there was an increase in number of LC3 puncta upon overexpressing EGFP-HTT-Q74 but the percentage co-localization between p62 and LC3 was reduced. While upon overexpressing EGFP-HTT-Q23, the co-localization was found to be comparable to that of wild type condition.

This result suggested that even though the number of autophagosomes increase upon EGFP-HTT-Q74 overexpression, there is reduced co-localization between the adaptor p62 and LC3 which could lead to inefficient cargo recognition. One study in mouse model of HD has shown the defect at cargo recognition level during autophagy. In electron microscopy images, autophagic vacuoles were found to be empty indicating a failure in trapping cytosolic cargo resulting in accumulation of aggregates in the cell (Martinez-Vicente, Talloczy et al. 2010).
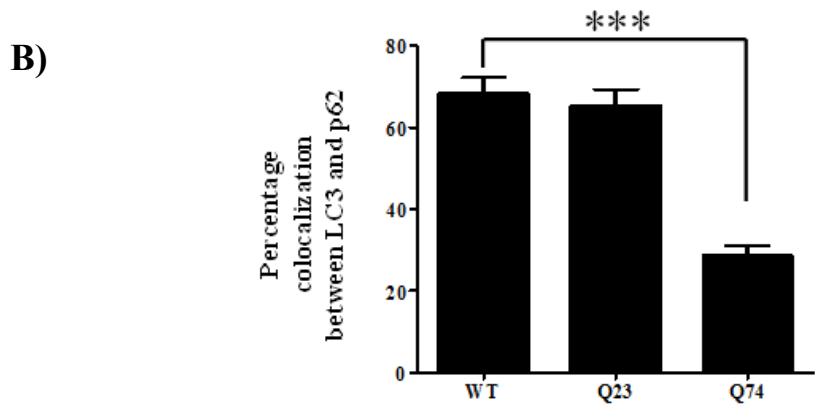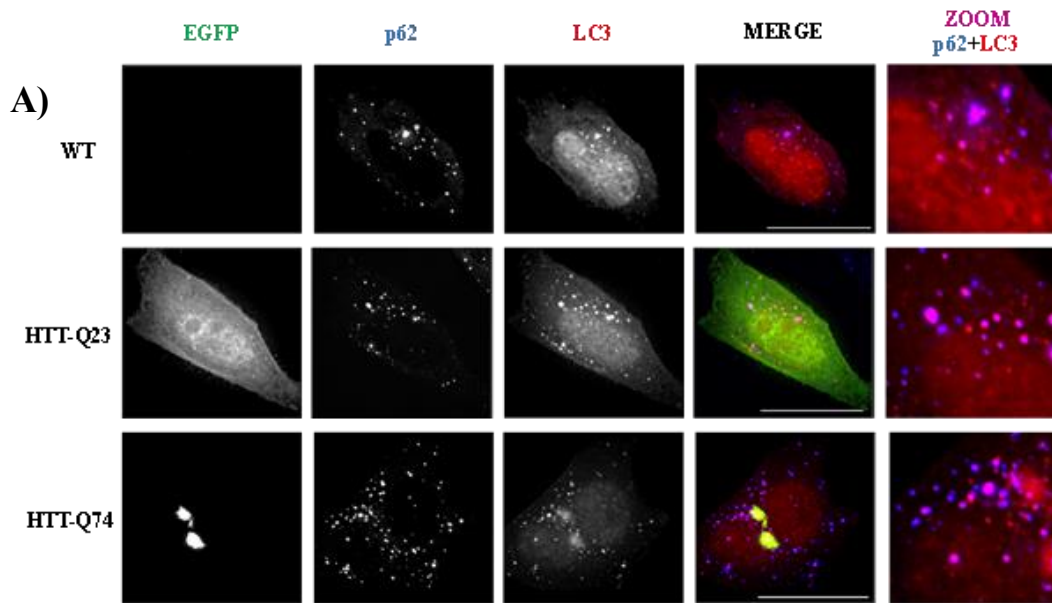
**Figure 5: Co-localization of LC3 and p62 upon overexpression of huntingtin** A) Microscopic images showing LC3 and p62 signal in wild type condition and upon overexpressing EGFP-HTT-Q23 or EGFP-HTT-Q74. Zoom panel shows the co-localization between LC3 and p62. Scale bar-15μm. **B)** Graph representing quantitation of percentage co-localization between LC3 and p62. In wild type and EGFP-HTT-Q23 the percentage co-localization was 60-70% but it was found to be lesser (20-40%) in case of EGFP-HTT-Q74. WT-wild type, Q23- EGFP-HTT-Q23, Q74-EGFP- HTT-Q74, Unpaired t-test, *** $p \leq 0.001$, N=3, n=20 cells.

### 3.2.3 Chemical modulation of autophagy using a well-known autophagy inducer Torin1 to study aggrephagy

To check whether upregulation of autophagy has a role to play in efficient capture and subsequent clearance of aggregates, autophagy was induced by either nutrient deprivation (starvation) or by using a well-known autophagy inducer Torin1. Torin1 is a potent and selective ATP-competitive inhibitor of mTOR which is a key regulator of cell growth in response to starvation, growth factors and stress signals (Liu, Chang et al. 2010). In nutrient-rich conditions, mTOR inhibits autophagy.

To check the effect of Torin1 on autophagy, HeLa cells were treated with 1μM Torin1 for 2 hours in growth medium. Cells were also incubated for 2 hours in nutrient deprived condition i.e. EBSS (Earle's Balanced Salt Solution) to induce starvation mediated autophagy. Cell lysates were processed for immunoblotting and phosphorylation status of one of the mTOR substrates i.e. P70S6K was checked as a readout for mTOR activity (**Fig.6**). As expected, in growth medium mTOR was found to be active as displayed by phosphorylation of P70S6K (lane 1 in Western blot picture), while no phosphorylation was of P70S6K was observed either in starvation or upon Torin1 treatment (lane 2 and 3 respectively). Autophagy status was checked by probing for LC3-I to LC3-II conversion. Enhanced LC3-II accumulation was observed in starvation induced (EBSS) and Torin1 treatment conditions.
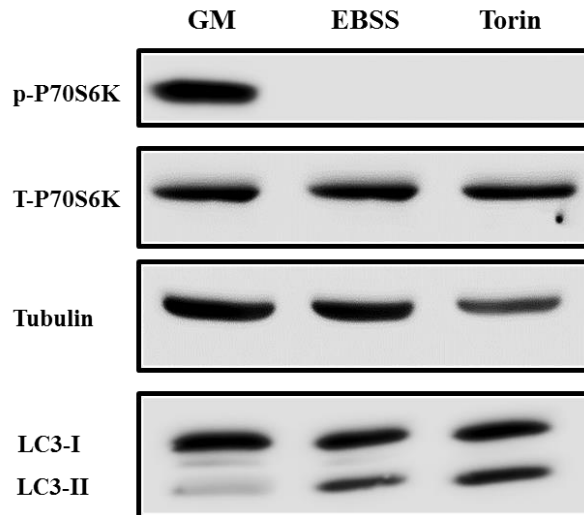
**Figure 6: Torin is an autophagy inducer.** HeLa cells were incubated with EBSS (starvation medium) or treated with 1μM Torin1 in growth medium to induce autophagy. Starvation and Torin treatment both inhibit mTOR, the activity of mTOR was assessed by probing for phosphorylation of one of its substrates i.e. P70S6K. Inactivity of mTOR leads to autophagy induction hence no phosphorylation of P70S6K was observed in autophagy induced condition. Immunoblotting was also done for LC3 to observe LC3 conversion for checking autophagy status. Higher level of LC3-II was observed in starvation and upon Torin1 treatment. In the figure, phosho-P70S6K is represented as p-P70S6K-, while total-P70S6K T-P70S6K-, LC3- Microtubule-associated proteins 1A/1B light chain 3B, EBSS-Earle's Balanced Salt Solution, GM- growth medium.

Although an increase in LC3-II level was observed upon Torin1 treatment but to conclude it as autophagy induction further experiments were done using BafA1, a known inhibitor of autophagosome-lysosome fusion.

HeLa cells were pre-treated with 100 nM BafA1 followed by Torin1 treatment and lysates were processed for western blotting. As expected, under autophagy induced condition (EBSS and Torin1 treatment) an over and above accumulation of LC3-II was observed as compared to growth medium. This clearly indicated that 1μM Torin1 treatment for 2 hours in growth medium is potent enough to inhibit mTOR and subsequently inducing autophagy and therefore this dose was used in all further experiments.
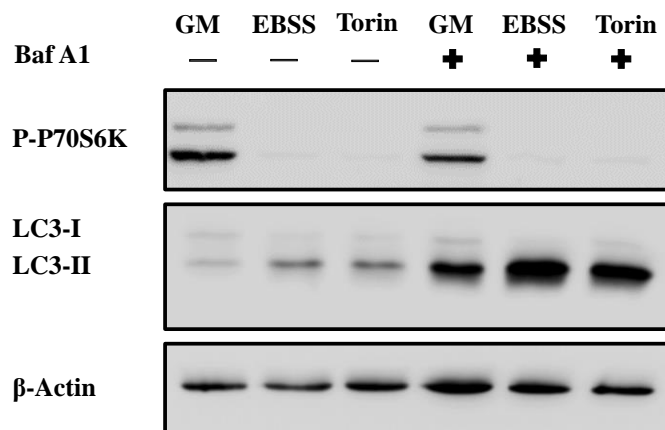


**Figure 7: Torin is an autophagy inducer.** HeLa cells were pre-treated with 100 nM BafA1followed by incubating with EBSS (starvation medium) or treated with 1μM Torin1 in growth medium to induce autophagy and LC3-II conversion was checked. As shown in the western blot, an over and above increase in LC3-II level (Lane 5 and 6) was observed upon induction of autophagy. In the figure, phosho-P70S6K is represented as p-P70S6K, and LC3- Microtubule-associated proteins 1A/1B light chain 3B, EBSS- Earle's Balanced Salt Solution, GM- growth medium.

**Upregulation of autophagy leads to increased recognition and capture of mHTT aggregates by autophagy machinery**

To study the effect of autophagy induction on recognition and capture of mHTT aggregates, HeLa cells were co-transfected with EGFP-HTT-Q74 and RFP-LC3 constructs followed by autophagy induction either by starvation or treatment with Torin1. Cells were then processed for immunostaining with p62 antibody.

The recruitment of p62 on mHTT aggregates was quantitated by measuring the mean intensity of co-localization between p62 and mHTT aggregates (**Fig. 8**). As shown in the microscopic images recruitment of p62 on aggregates was found to be lesser in basal autophagy as compared to autophagy induced conditions. Quantitation of mean intensity of co-localization shows significant increase in association of p62 with aggregates during upregulation of autophagy.

Next, it was expected that this increased association of adaptor protein p62 with aggregates would augment LC3 localization onto these aggregates suggesting their capture by autophagosomes.

Therefore, we quantitated the mean intensity of co-localization between LC3 and mHTT aggregates (**Fig. 9**). In growth medium there were fewer co-localization events of LC3 with aggregates but these events were increased significantly upon autophagy induction either by starvation or treatment with Torin1.

Finally, triple co-localization of LC3, p62 and mHTT aggregates was analyzed. (**Fig. 10**). In congruence with our previous dual localization analysis we noted a likewise significant increase in triple co-localization upon autophagy induction.

These data suggest that upon autophagy induction, either by starvation or Torin1 treatment there was enhanced recognition of mHTT aggregates by p62 and subsequent capture by LC3 as compared to basal autophagy conditions (growth medium).
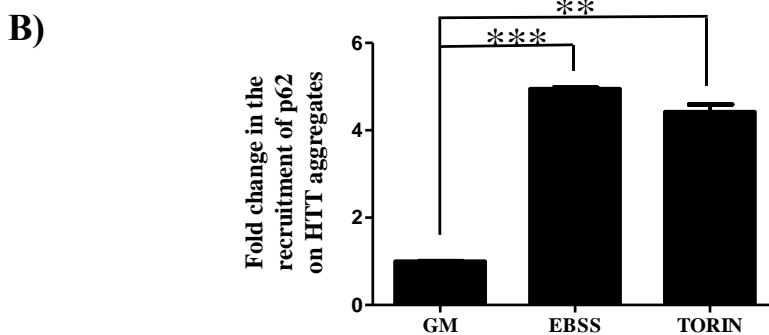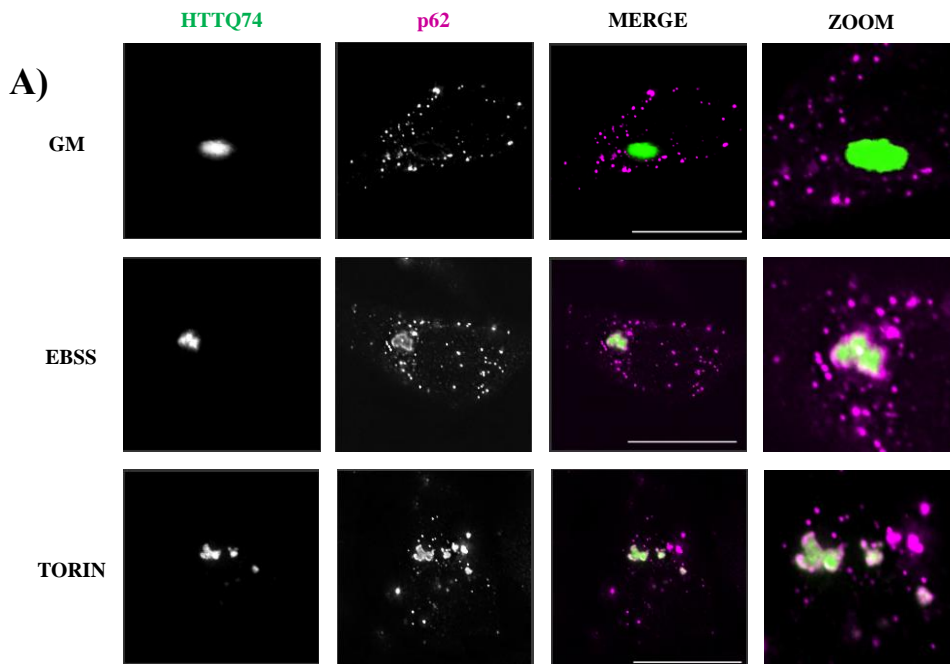
**Figure 8: Upregulation of autophagy helps in increased recruitment of p62 on aggregates. A)** HeLa cells transfected with EGFP-HTT-Q74 were incubated for 2 hours under starvation condition (EBSS) or 1μM Torin in GM to induce autophagy followed by immunostaining with p62 antibody. Microscopic images show lesser recruitment of p62 on huntingtin aggregates in GM whereas an enhanced co-localization was observed in autophagy induced condition (EBSS and Torin treatment) (zoom panel). Scale bar-15μm. **B)** Quantitation of fold change in the recruitment of p62 on huntingtin aggregates which increases significantly in case of autophagy induced condition. GM- growth medium, EBSS- Earle's Balanced Salt Solution, HTT- huntingtin. Unpaired t-test, *** $p \leq 0.001$, N=3, n=20 cells.
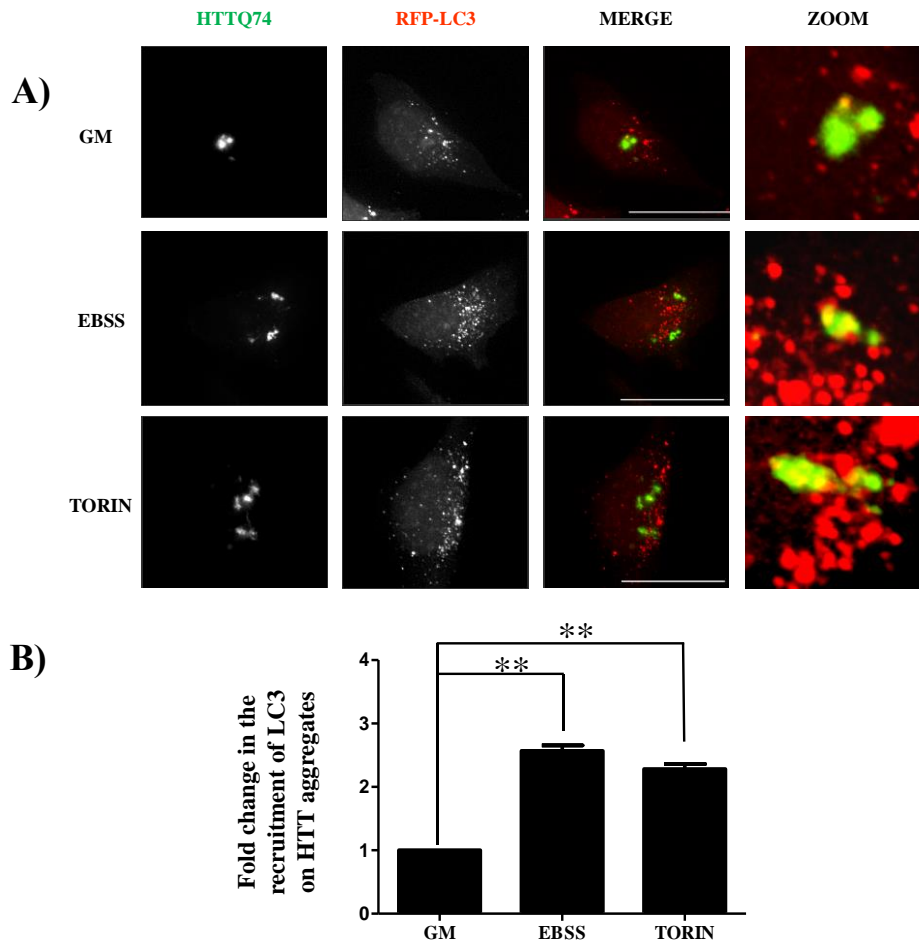
**Figure 9: Upregulation of autophagy promotes recruitment of LC3 on aggregates.**

**A)** HeLa cells were transfected with EGFP-HTT-Q74 and RFP-LC3 and incubated for 2 hours under starvation condition (EBSS) or 1μM Torin in GM to induce autophagy. Microscopic images show lesser co-localization events of LC3 with huntingtin aggregates in GM whereas these events were more in case of autophagy induced condition (EBSS and Torin treatment) as shown in the zoom panel. **B)** Graph showing quantitation of fold change in the recruitment of LC3 on huntingtin aggregates. LC3 recruitment on aggregates increase significantly when autophagy is upregulated. GM- growth medium, EBSS- Earle's Balanced Salt Solution, HTT- huntingtin. Unpaired t-test, **p≤0.01, N=3, n=20 cells.
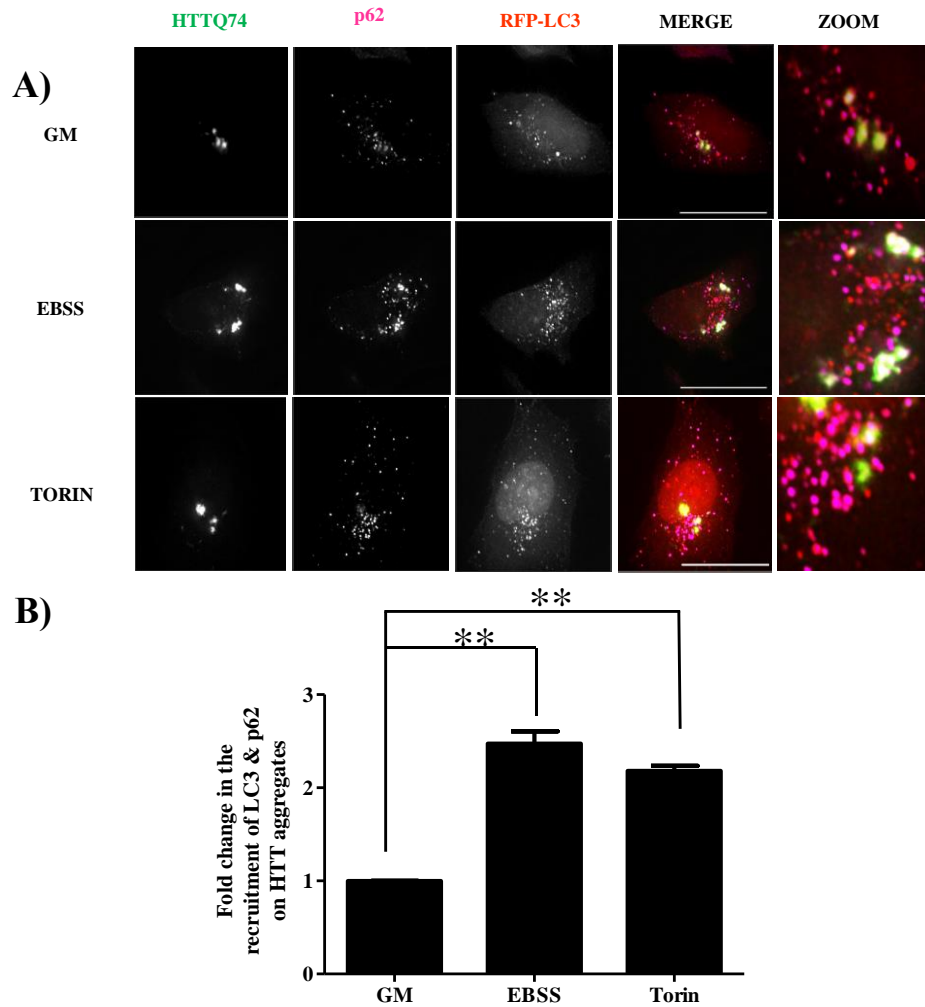
**Figure 10: Upregulation of autophagy augments recruitment of p62 & LC3 on aggregates.** **A)** HeLa cells transfected with EGFP-HTT-Q74 and RFP-LC3 were incubated for 2 hours under starvation condition (EBSS) or 1μM Torin in GM to induce autophagy followed by immunostaining with p62 antibody. Microscopic images show lesser recruitment of p62 and LC3 on huntingtin aggregates in case of GM as compared to autophagy induced conditions (EBSS and Torin treatment). Scale bar-15μm. **B)** Quantitation of fold change in the recruitment of p62 and LC3 on huntingtin aggregates which increases significantly in case of autophagy induced condition. GM- growth medium, EBSS- Earle's Balanced Salt Solution, HTT- huntingtin. Unpaired t-test, ** $p \leq$ 0.01, N=3, n=20 cells.

**Upregulation of autophagy helps in clearance of mHTT**

In the previous sections it has become evident that upregulation of autophagy is conducive to recruitment of autophagy recognition and capture machinery on mHTT aggregates. Now in order to check whether enhanced recognition and capture via autophagic machinery culminates in improved clearance of the mHTT aggregates, we followed degradative products of EGFP-HTT-Q74 as a readout for its degradation.

While analyzing expression of mHTT by western blot in our experiments, one of the serendipitous observation was the presence of an extra band which coincided with the size of only GFP i.e. near 26kDa.

To ascertain lysosomal degradation of a substrate in the field of autophagy, GFP-tagged substrates are followed for their degradation to monitor autophagy flux. Since GFP is relatively more resistant to lysosomal hydrolases, therefore temporal increase or accumulation of free-GFP gives a readout of autophagy flux upon autophagy inhibited or induced conditions.

In accordance with that, HeLa cells were transfected with EGFP-HTT-Q74 and were treated with 1μM Torin1 across various time points (2-8 hours) to induce autophagy (**Fig. 11**). A temporal and significant accumulation of free-GFP was observed notably in 6h and 8h time point as compared to the basal autophagy (growth medium).

This suggest that the full-length EGFP-HTT-Q74 fusion protein is getting degraded which leads to accumulation of free-GFP since GFP is more resistant to the action of proteolytic enzymes of lysosome. The appearance of free-GFP band cannot be an anomaly as lysate preparation was done across various time points, which suggested a gradual turnover of EGFP-HTT-Q74 (compare lane 2 vs lane 6, Western blot picture). This preliminary

observation has to be supplemented with more control experiments where autophagy is inhibited either by chemical modulation such as treatment with 3-MA or genetic modulation for example usage of Atg5 knockout cell line and same experiment has to be carried out in autophagy null background to comment upon autophagy mediated increased cellular clearance of mHTT.

Hence autophagy induction results in better recognition, capture and clearance of mHTT protein.
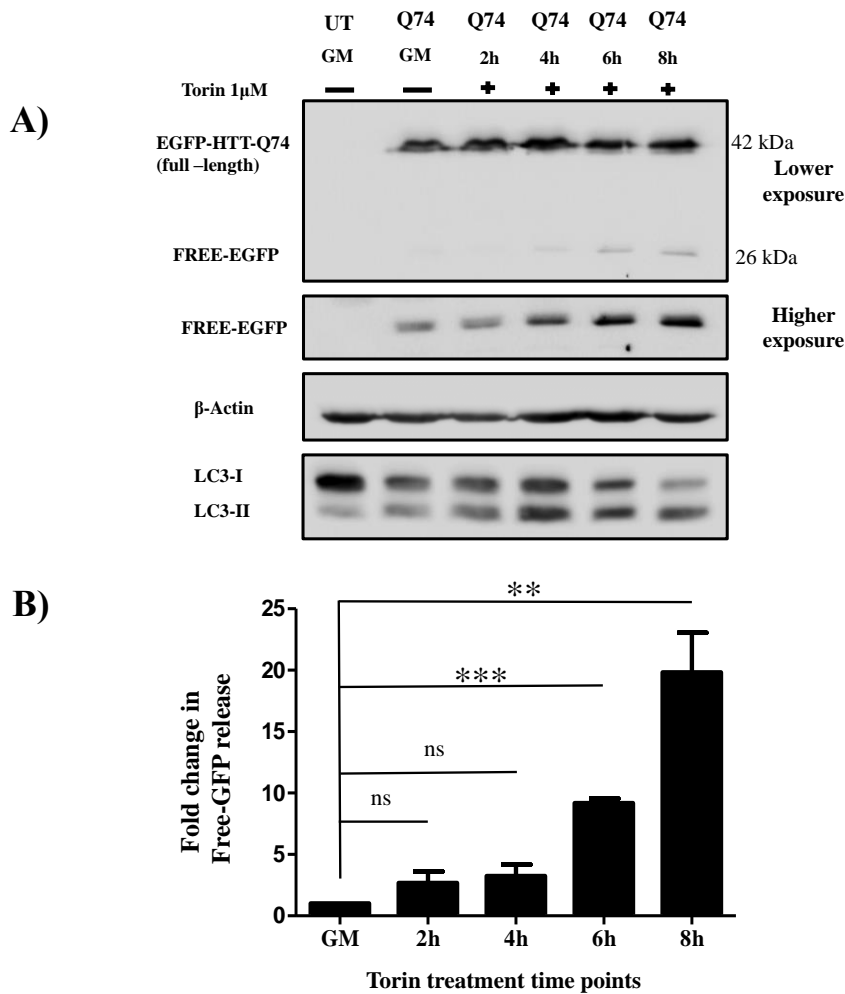
**Figure 11: Upregulation of autophagy helps in clearance of EGFP-HTT-Q74. A)** HeLa cells were transfected with HTT-Q74 followed by treatment with Torin from 2-8 hours to induce autophagy and analyzed by immunoblotting. Western blot shows the full length form of EGFP-HTT-Q74 (42 kDa) and free-EGFP (26 kDa) detected by GFP antibody. The level of free-GFP increases from 2-8 hours of Torin treatment as shown by higher exposure. In addition, LC3-II levels were found to be higher upon Torin treatment thereby confirming that autophagy was induced during the course of treatment. **B)** Graph representing the quantitation in fold change of free-GFP release for different time points upon Torin treatment. This was increased significantly for 6h and 8h time points. GM- growth medium, ns- nonsignificant, HTT- huntingtin. Unpaired t-test, *** p ≤ 0.01, ** p ≤ 0.01, N=3.

**Chapter-4 Generation of tetracycline inducible expression system in HeLa cells for driving toxic aggregate protein expression**

## 4.1 <u>Background information</u>

To study a toxic gene of interest, it becomes mandatory to have a controlled expression to avoid consequences of toxicity like death of the cells upon overexpression. Huntingtin with its expanded polyglutamine tract is toxic to cells since it tends to form aggregates therefore it is indispensable to establish a system where expression of this gene can be regulated. Aggregation of a protein is a dynamic process which involves the formation of various intermediate oligomeric structures. Depending upon the physiological conditions like pH and ionic concentration, misfolded proteins can organize into different species such as molten globule amyloidogenic, small amyloidogenic oligomers, filaments, protofibrils and mature fibrils. The intermediate species are known to be more toxic than the lesser dynamic terminally accumulated species i.e. inclusion bodies. These species tend to sequester diverse cellular proteins during the course aggregation thus affecting vital processes of the cell. To study the susceptibility of these species for degradation by cellular proteolytic machineries it is essential to have a system where expression of such proteins can be controlled. Therefore, an inducible expression system is a good approach to achieve homogeneous expression of a protein of interest and study sensitivity of distinct oligomeric species for one of the components of proteostatic machinery i.e. autophagy.

Such a temporal cell-based model could be useful to study aggregation dynamics. For this particular requirement Tet-on system was used and a sequential approach was taken wherein the primary aim was to make stable cell line expressing reverse tetracycline transactivator followed by putting gene of interest into the same cell line.

## 4.2 Generation of stable cell line expressing reverse tetracycline transactivator

In order to generate stable HeLa cell line expressing reverse tetracycline transactivator (rtTA), antibiotic based selection was used to select stable cells having genomic integration of this plasmid since it has puromycin as antibiotic selection marker. Firstly, a kill curve was performed on HeLa cells to standardize the concentration of puromycin to be used for selection of stable transfectants since cells in which transfection does not lead to a stable genomic integration of plasmid would die due to antibiotic selection pressure. Transfected cells were then put under antibiotic selection pressure to select stable transfectants.


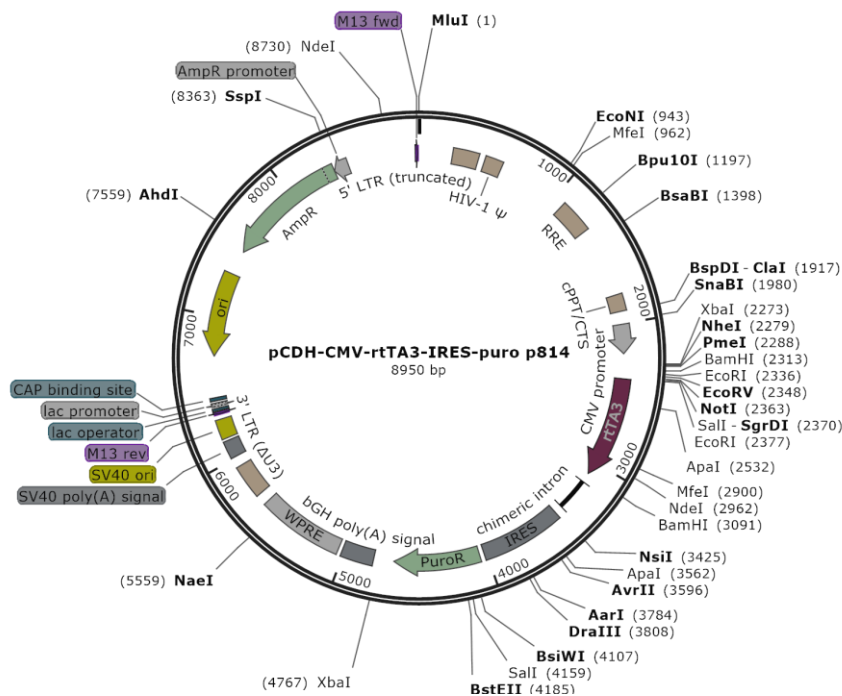
**Figure 1: Map of rtTA plasmid.** The plasmid map for reverse tetracycline transactivator showing characteristics of plasmid in detail. MluI restriction site was used to linearize the plasmid before transfection into HeLa cells. This plasmid has puromycin as antibiotic selection marker which was used for selection of HeLa cell lines stably expressing rtTA plasmid. (Source- Dr. Malini, JNCASR)

### 4.2.1 Methodology

HeLa cells were plated and grown on a 60 mm dish until 60-70% confluency was reached. 2.5 µg of rtTA linearized plasmid DNA was transfected into the cells and after 48 hours, growth medium was replaced by medium containing puromycin antibiotic at a concentration of 500 ng/ml (as standardized). Replacement of media containing selection antibiotic was done every 2-3 days until single colonies of cells appeared. Cells were then picked up from single isolated colonies and were transferred to one well of a 6-well plate, multiple single colonies in this way were transferred to individual wells of a 6-well plate and were maintained in antibiotic containing medium. Cells were allowed to grow followed by trypsinization and expansion of each colony. Four of these colonies were used to make cell stocks and were frozen. Validation of clones will be discussed in the following sections.

### 4.3 Generation of plasmids having huntingtin constructs under tetracycline inducible promoter

### 4.3.1 Background information

In order to have a controlled expression of a gene of interest, it is essential that it should be cloned under an inducible promoter e.g. tetracycline inducible promoter in this case. This bipartite system requires constitutive expression of a transactivator (rtTA) from one plasmid and an inducible expression of gene of interest cloned under tetracycline inducible promoter form another plasmid. Since a stable HeLa cell line expressing rtTA was generated, constructs driving inducible expression of huntingtin in response to tetracycline were further generated with an aim to have a double stable HeLa cell line.

## 4.3.2 Methodology

pTRE-tight mammalian expression vector (Clontech) is a response plasmid that can be used to express a gene of interest in a Tet-On or Tet-off system. This vector was used as a backbone to clone EGFP-HTT-Q23 and EGFP-HTT-Q74 (plasmid map in chapter-3) under tetracycline inducible promoter. Compatible restriction sites i.e. 5'- NotI and 3'- HindIII were generated on overhangs of inserts through PCR and subsequently cloning was done as explained in materials and methods.
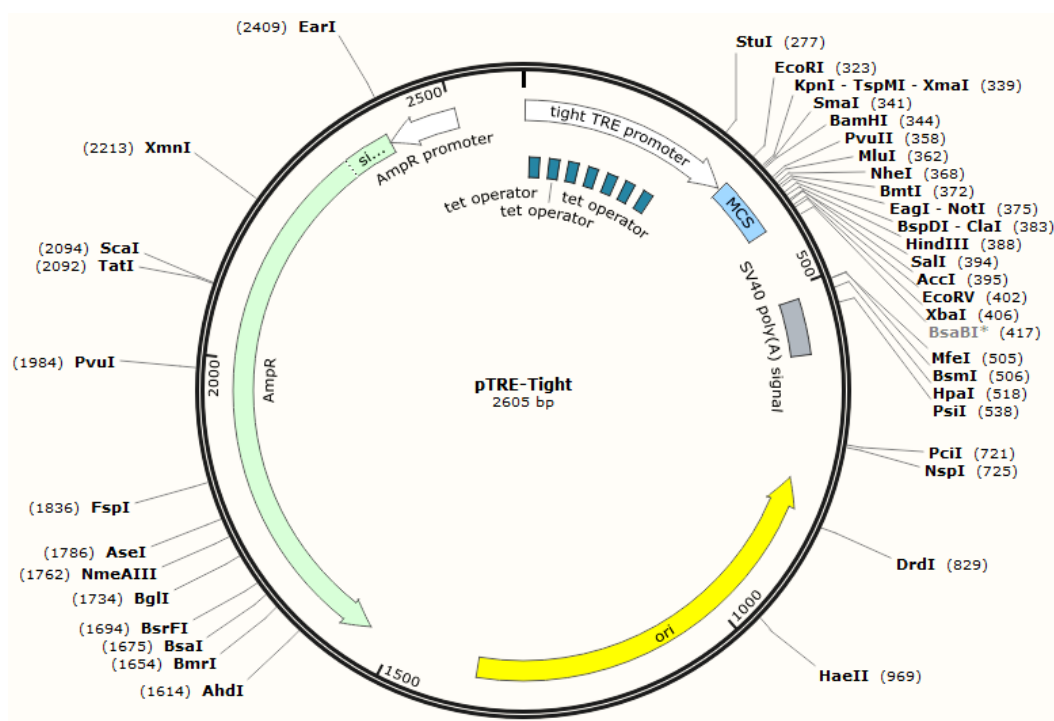


**Figure 2:  Map of pTRE- tight plasmid**. The plasmid map for pTRE-tight vector showing characteristics of plasmid in detail. Huntingtin constructs were cloned under the tetracycline promoter of this plasmid between NotI and HindIII sites.

### 4.3.3 Results

Cloning was confirmed by PCR amplification and restriction digestion. For confirmation of positive clones containing PTT-EGFP-HTT-Q23 insert, clone 3.1 and 3.2 were tested by PCR which resulted in a band of 933 base pairs (bp) corresponding to that of the positive control thereby confirming the clones.

Further confirmation was done by double digestion using HindIII and NotI enzymes. Insert release was observed as can be seen in the agarose gel picture (Figure-3). Similarly, clone confirmation was done for PTT-EGFP-HTT-Q74 where a band of 1086 bp was observed upon PCR and restriction digestion for two of the clones tested.



**Figure 3: Clone confirmation of PTT-EGFP-HTT-Q23.** EGFP-HTT-Q23 was cloned under tetracycline inducible promoter of pTRE-tight vector. **A)** Agarose gel electrophoresis picture showing a 933 bp band corresponding to that of the positive control thus confirming the clones tested to be positive. **B)** Gel picture showing insert release from clone 3.1 and 3.2 upon double digestion with HindIII and NotI enzyme. M-marker, +ve-positive, UD-undigested and DD-double digested.
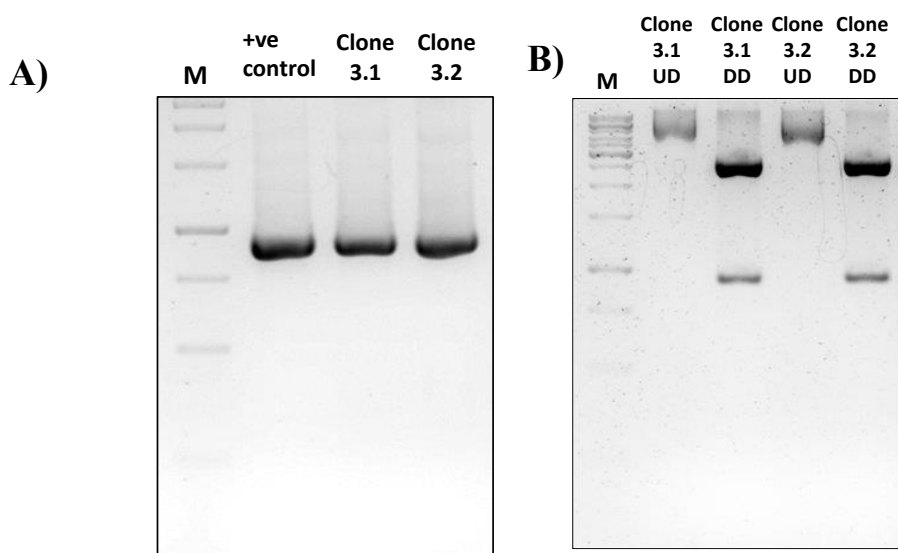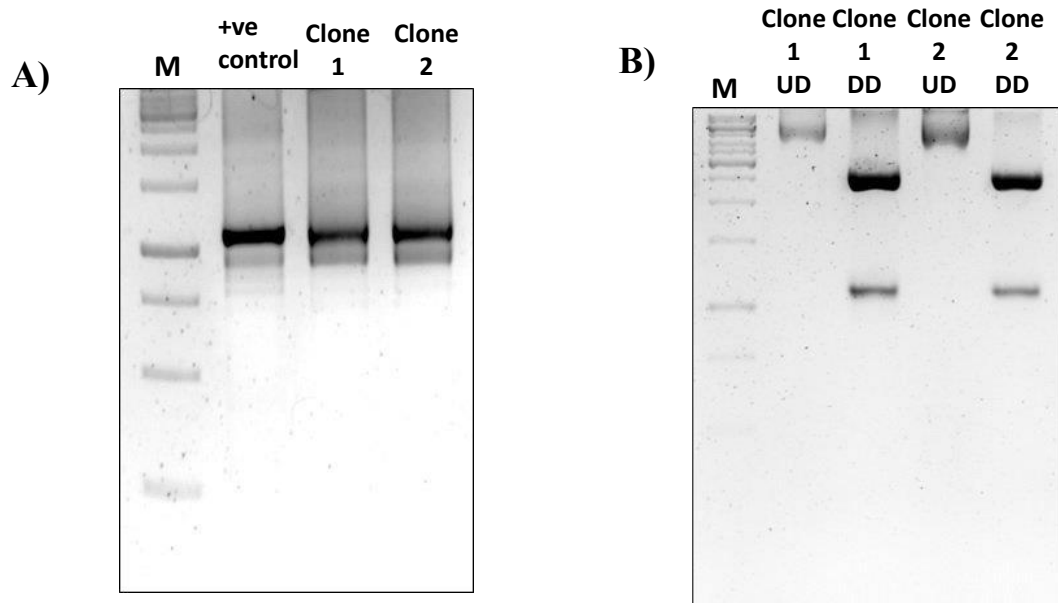
**Figure 4: Clone confirmation of PTT-EGFP-HTT-Q74.** EGFP-HTT-Q74 was cloned under tetracycline inducible promoter of pTRE-tight vector. **A)** Agarose gel electrophoresis picture showing a 1086 bp band corresponding to that of the positive control thus confirming the clones tested to be positive. **B)** Gel picture showing insert release from clone 1 and 2 upon double digestion with HindIII and NotI enzyme. M-marker, +ve- positive, UD-undigested and DD-double digested.

**Clone confirmation by microscopy**

Microscopy was done to further confirm the clones, wherein HeLa cells were co-transfected with rtTA plasmid and the plasmid DNA of the clones to be tested. This was followed by Doxycycline induction (1µg/ml) for 24 hours. In clones (3.1 and 3.2) of PTT-EGFP-HTT-Q23 a diffused expression of EGFP was observed in the doxycycline induced condition whereas no expression was observed in the uninduced condition. Hence

confirming successful expression through Doxycycline inducible promoter therefore the clones were verified by microscopy and were found to be positive.



**Figure 5: Clone confirmation of PTT-EGFP-HTT-Q23 by microscopy.** Microscopic images cells co-transfected with rtTA and clone 3.1 or 3.2 showing a diffused expression of EGFP upon induction with Dox for 24 hours. Whereas no expression could be observed in case of uninduced condition. Scale bar - 15μm. UI-uninduced.

In case of PTT-EGFP-HTT-Q74, both the clones showed aggregate formation in co-transfected cells upon induction which corroborated with the kind of phenotype associated with huntingtin having more number of glutamines but no expression was observed in uninduced condition. Hence the clones tested were found to be positive.



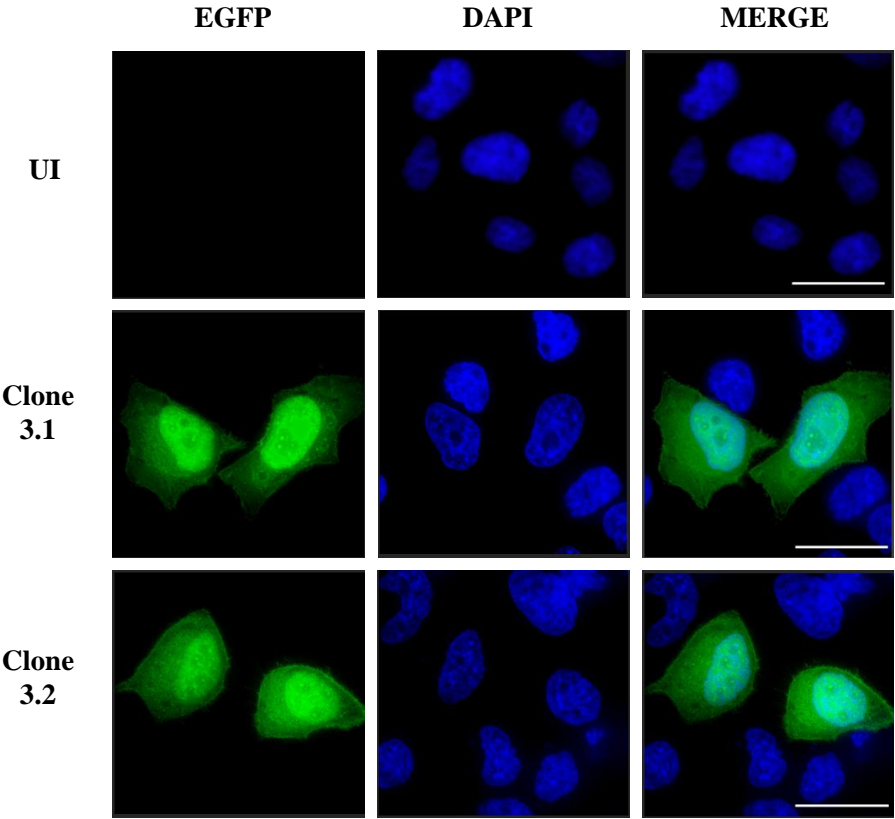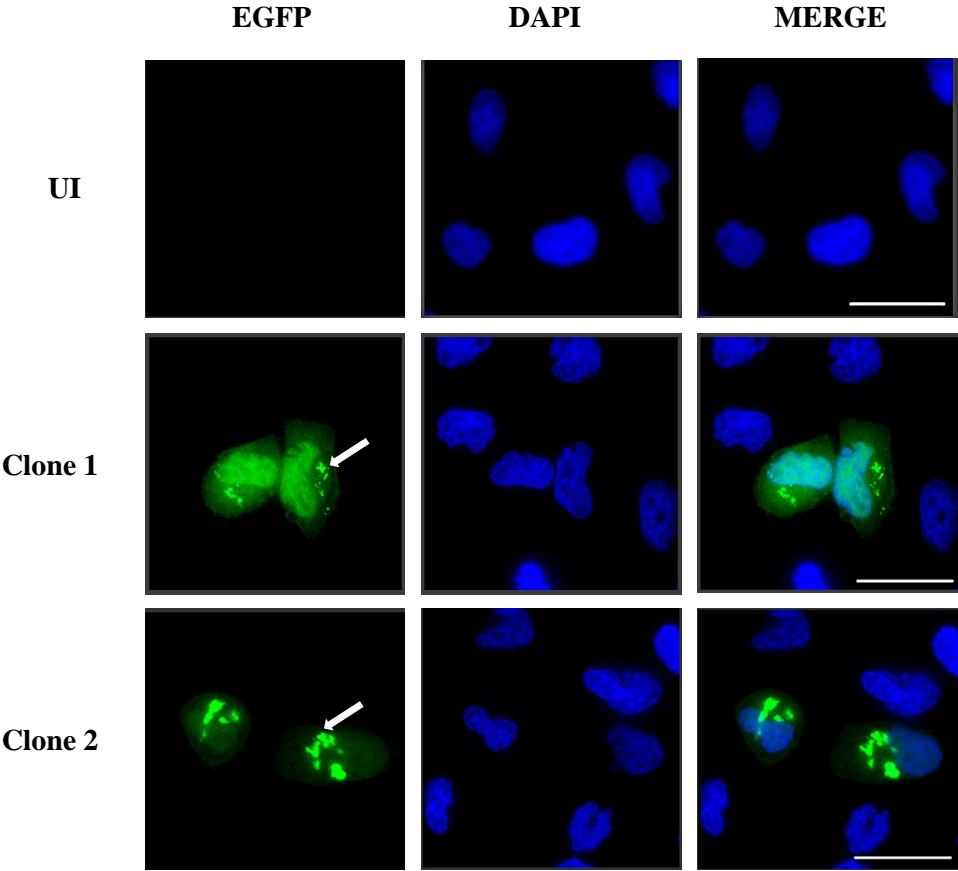**Figure 6: Clone confirmation of PTT-EGFP-HTT-Q74 by microscopy.** Microscopic images cells co-transfected with rtTA and clone 1 or 2 showing aggregate (arrows) formation upon induction with Dox for 24 hours. Whereas no expression could be observed in case of uninduced condition. Scale bar - 15μm. UI-uninduced.

## 4.4 <u>Validation of reverse trans-activator expressing cell line</u>

## 4.4.1 Validation by microscopy

**Results**

To validate the stable HeLa cell line expressing rtTA a temporal study was done to monitor expression of gene driven by tetracycline inducible promoter. For this experiment, rtTA expressing HeLa cell line was transfected with PTT-EGFP-HTT-Q23 construct as mentioned in materials and methods. Doxycycline induction was done across time points ranging from 6-24 hours. A time dependent increase in the expression of PTT-EGFP-HTT-Q23 was observed which saturated at around 18 hours as depicted in the graph below. Almost no expression was observed in the case of uninduced condition.
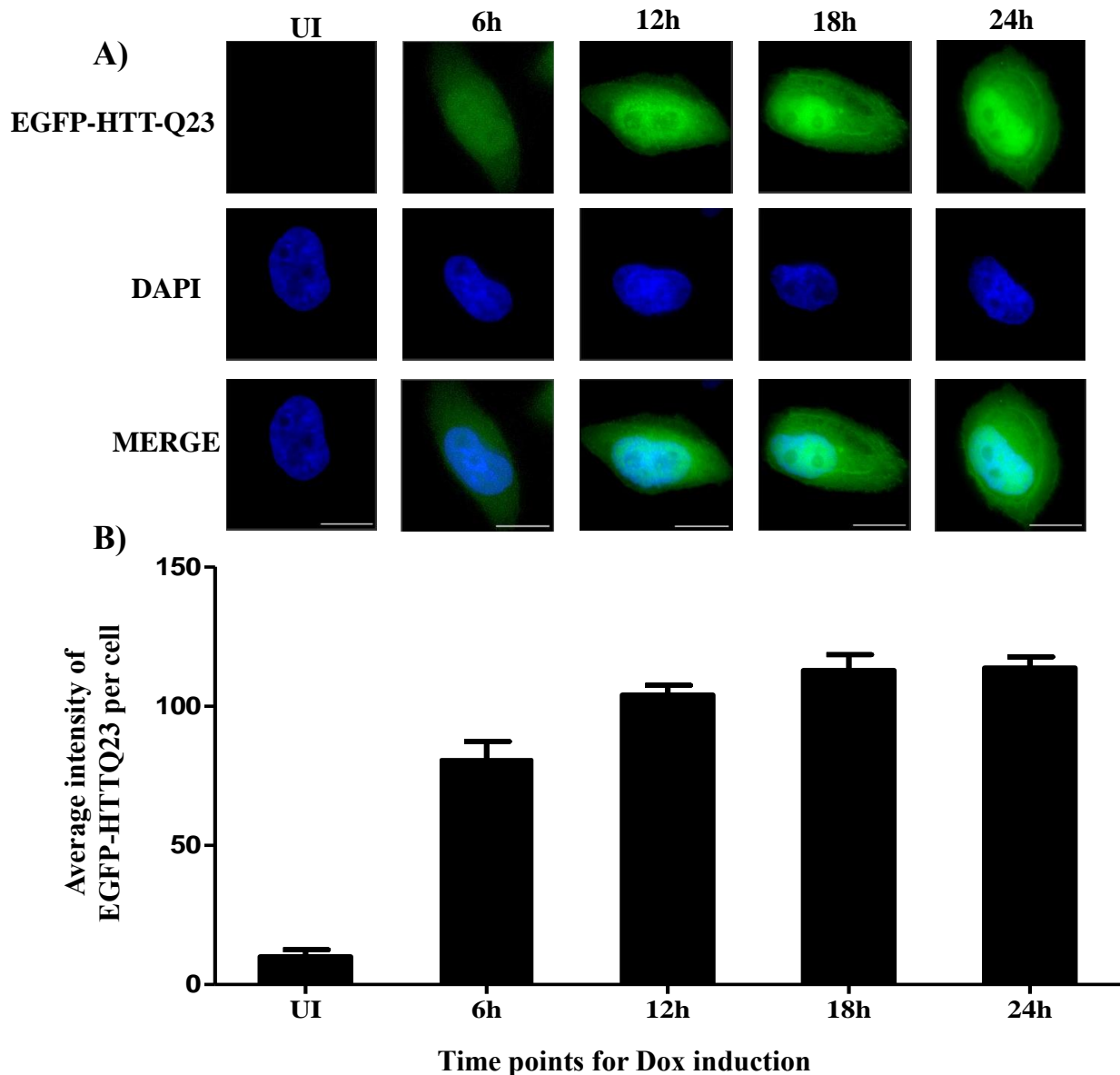
**Figure 7: Validation of reverse trans-activator expressing cell line by microscopy. A)** Microscopic images of stable rtTA HeLa cell line transfected with PTT-EGFP-HTT-Q23 construct showing expression upon Dox (1μg/ml) induction across various time points. Scale bar-15μm. UI-uninduced. **B)** Graph representing quantitation of average intensity of expression of PTT-EGFP-HTT-Q23 per cell across various time points. n=20 cells. Quantitation was done using ImageJ software.

## 4.4.2 Validation by Western blotting

**Results**

In order to validate rtTA HeLa cell line by Western, cells were plated and transfected in a 6-well plate followed by induction with doxycycline at a concentration of 1μg per ml for different time points.

A time dependent increase in expression of PTT-EGFP-HTT-Q23 was observed. No expression was seen in case of uninduced condition.

The same experiments have to be done with PTT-EGFP-HTT-Q74 to check its time-dependent aggregation and also to study susceptibility of various higher order structures such as monomer, oligomer and aggregates for their autophagy mediated degradation.

A)

| | UT | Q23 UI | Q23 6h | Q23 12h | Q23 18h | Q23 24h |

EGFP-HTT-Q23

β-Actin

B)
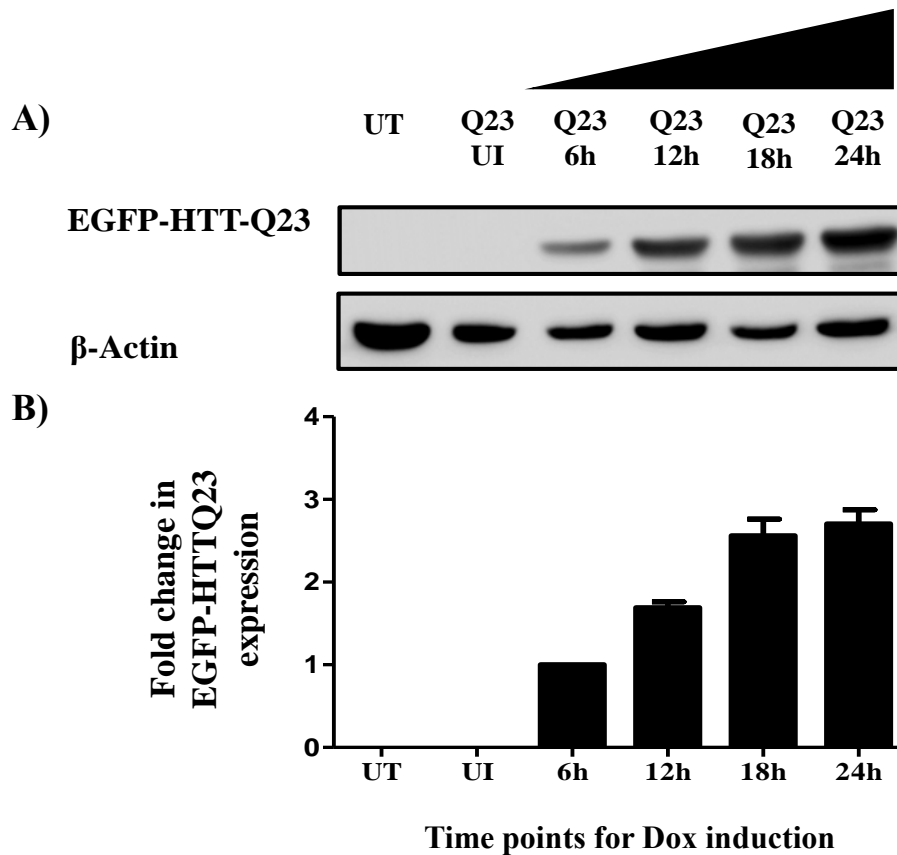
**Figure 8: Validation of reverse trans-activator expressing cell line by Western blotting. A)** HeLa cell line stably expressing rtTA was transfected with PTT-EGFP-HTT-Q23 construct. A time-dependent increase in expression of HTT-Q23 upon Doxycycline (1μg/ml) induction detected by using antibody for EGFP, across various time points as shown in the western blot with β-actin as loading control. **B)** Graph representing quantitation of the Western blot showing a temporal increase in the fold change in expression of HTT-Q23 with no expression observed in uninduced condition. UT-untransfected, UI-uninduced. N=2.

**Chapter-5**

**Conclusion and future direction**

Cellular integrity is substantially dependent on maintenance of protein homeostasis or proteostasis. At a given time in a cell, there is a fine balance between protein synthesis and degradation which is achieved by the synergistic action of several proteostatic machineries. Proteostatic network has various branches like UPS, ERAD and autophagy-related pathways which work in synchrony to maintain a pool of healthy and functional proteins in the cell. A breakdown in this network can happen if a cell is exposed to conditions like stress or any other pathophysiological conditions (neurodegenerative disorders) which can lead to accumulation of damaged cell organelle and misfolded proteins. In most of the NDs, accumulation of aggregate-prone proteins is observed which give rise to higher order structures like oligomers and aggregates due to decline in the capacity of proteostatic network. The aggregates so formed are cytotoxic in nature and clearance of same is one of the ameliorative approaches in the field of neurodegeneration. The large size of the aggregates and other higher structures limit their clearance by UPS but they are known to be a substrate for autophagy.

Autophagy is cellular degradative process which involves capture of cargo in a double membrane bound vesicle called as autophagosome. These autophagosomes fuse with lysosome resulting in degradation of the captured cargoes. Subsequently degradative products or basic subunits are recycled back to the cytoplasm.

In the present study we have used polyglutamine protein, mHTT to study aggrephagy in detail. mHTT is a cause of HD which is one of the nine polyglutamine disorders where a trinucleotide expansion in huntingtin gene translates to poly-Q tract in the protein which

makes highly hydrophobic and cause aggregation. Upregulation of autophagy by using small molecule or non-small molecule approaches have been shown to be beneficial in terms of clearing of aggregates and improving motor phenotype in cell line and mouse model of HD.

In our studies, we established a cell based model of HD in HeLa cell line to investigate incompetency of autophagy machinery at various steps in the context of cargo recognition, capture and clearance. We have shown that upon autophagy induction by using a known autophagy inducer Torin1 there was increased recruitment of autophagy adaptor p62 on mutant huntingtin (mHTT) aggregates. An increase in number of co-localization events of LC3 with mHTT aggregates was also observed, suggesting enhanced recognition and capture of mHTT upon autophagy induction as compared to basal autophagy condition. Additionally, a temporal induction of autophagy showed an increased accumulation of degradative product (free-GFP) of mHTT hinting towards its enhanced clearance from the cell however further experiments have to be done to claim that degradation was exclusively autophagy-dependent.

These preliminary results augment the idea of autophagy induction on effective clearance of proteinaceous aggregates (mHTT in this case) from the cell. Although induction of autophagy has been shown to be promising but non-specific induction of autophagy can cause toxicity in the cells especially neurons. Also overproduction of autophagosomes in the disease conditions where cargo capture and lysosomal degradation is impaired can lead to inefficient clearance of the cargo.
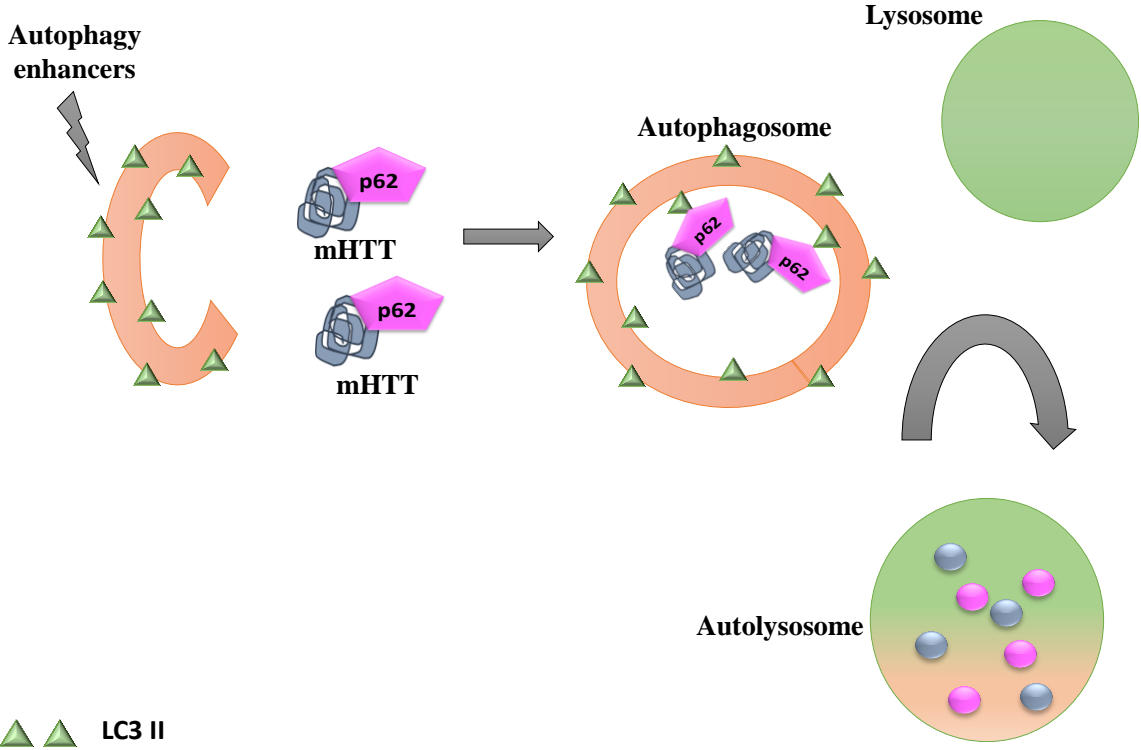


**Figure 1: Model depicting the role of autophagy induction in clearance of mHTT aggregates.**

Since in case of HD, reports suggest a defect at cargo recognition step during autophagy therefore targeting selective autophagy by modulating the activity of autophagy adaptors provides a new avenue in the field of autophagy and neurodegeneration.

This study put forward questions like, what makes p62 and LC3 more competent to capture aggregates effectively upon autophagy induction. Are there any specific post-translational modification involved behind this observation?

The implication of p62 remains a conundrum in HD, since p62 knockout had been shown to have a protective role in a mouse model of HD (Martinez-Vicente, Talloczy et al. 2010)

In the future, it becomes imperative to thoroughly dissect the function and regulation of autophagy adaptors in NDs so that autophagy can be selectively upregulated instead of overall induction of autophagy.

Furthermore, development of inducible expression system for driving polyglutamine containing mutant huntingtin expression will provide us with a good model system to study its aggregation as a function of time and also to ascertain selectivity of various intermediate species for autophagy mediated degradation.

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