Proteostasis regulation in ALS linked protein mediated toxicity

A thesis submitted for partial fulfilment of degree of

Master of Science

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(Biological Sciences)

by

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Declaration

I hereby declare that the work described in this thesis entitled **'Proteostasis regulation in ALS linked protein mediated toxicity'** is the result of investigations carried out by myself under the guidance of Prof. Ravi Manjithaya at Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice 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.

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CERTIFICATE

This is to certify that the work described in this thesis entitled **'Proteostasis regulation in ALS linked protein mediated toxicity'** is the result of investigations carried out by Ms Anindita Pal at Autophagy laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, under my supervision.

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May 2018

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Abbreviations

A_{600}- Absorbance at 600 nm

LC3- Microtubule-associated proteins 1A/1B-light chain 3

RT- Room temperature

^{0}C- Degree centigrade

rpm- rotation per minute

SDS- Sodium Dodecyl Sulphate

PAGE- Poly-acrylamide gel electrophoresis

atg- Autophagy related gene

kDa- Kilo Dalton

µL- micro litre

µM- micro molar

ROS- Reactive oxygen species

Vps- Vacuolar protein sorting

SNARE- SNAP (Soluble *NSF(N-ethylmaleimide-sensitive factor)* attachment protein) receptor

NBR1- Neighbour of BRCA1 gene 1

NDP52- Nuclear dot protein 52 kilo dalton

ALFY- Autophagy linked FYVE protein

Lamp- Lysosomal associated membrane protein

NaOH- Sodium Hydroxide

GFP- Green fluorescent protein

RFP- Red fluorescent protein

FITC- Fluorescein Isothiocyanate

TRITC- Tetramethyl Rhodamine

DAPI- 4’,6- diamidino-2-phenyl indole

Tris- Tris(hydroxymethyl) aminomethane

DTT- Dithiothreitol

h- hours

FACS- Fluorescence Activated Cell Sorting

HTS- High throughput screen

TOR- target of rapamycin

Synopsis

The thesis entitled “Proteostasis regulation in ALS linked protein mediated toxicity” encompasses the proteostasis network in maintaining the cellular quality control system and its implication in the aspect of neurodegeneration. Neurodegenerative disease is often characterised as the accumulation of misfolded proteins which are highly aggregate prone and render toxicity to the neuronal cells. Autophagy, an important proteostasis mechanism, governs a key role in maintaining the balance between the protein aggregate formation and clearance; failing so leads to neurodegenerative diseases like ALS. The following chapters summarize the role of an ALS linked protein in neurodegeneration and its connection to autophagy thereafter.

Chapter 1 is the literature summary about proteostasis machinery and its imbalance in context of neurodegeneration. This chapter narrates about the compromised proteostasis balance in neurodegenerative diseases like ALS and the implication of autophagy thereof.

Chapter 2 contains the details of materials and reagents required in the experiments. The chapter content also includes the methodologies of the experiment performed.

Here we have employed yeast model for Amyotrophic lateral sclerosis (ALS), a neurodegenerative disease, to study the toxic effect rendered by the protein. For this, construct harbouring TDP43 (responsible factor for ALS) was transformed into wild type and Δatg1 yeast strains and subsequent experiments were performed.

In chapter 3 we carried out various experiments like microscopy, spot dilution assay, growth assay to understand the toxic phenotype of the protein in yeast and its aggregate prone nature. Our result showed that there is defect in autophagy for the experimental strain. We sorted the cells by flow cytometry analysis to get the highest population of cells expressing the protein.

Growth analysis revealed significant growth lag as compared to that of Wild type strain. We employed this evident growth lag as a tool to screen a small molecule library (ChemDiv library) for rescue of the growth defect.

Chapter 4 summarizes the results we have found so far and concludes that indeed TDP43GFP shows toxic phenotype in yeast and this can be an appropriate model system to screen for the drugs and perform the experiments.

Chapter 1:

INTRODUCTION

Chapter 1: INTRODUCTION

Preface

The proteostasis network plays pivotal role in maintaining cellular homeostasis. The proteins are translated from the ribosome and folded with the help of chaperone to the nascent polypeptides. But, if any stress response like heat or oxidative stress, or mutation may lead to misfolded nature of protein which is prone to form aggregates (*Mark et al, 2014*). These aggregates may be protective or lethal depending on the conformation- oligomeric, fibrillar or inclusion bodies. The misfolded proteins are taken care of by the intracellular degradation pathway like autophagy, proteasome, multivescicular bodies.

The ubiquitin-proteasome system (UPS) is the primary protein quality control system. It degrades only the short-lived and soluble proteins because of its limited capacity. In contrast, autophagy is a catabolic process responsible for degrading intracellular long-lived proteins, soluble or insoluble misfolded protein aggregates and organelles (*Mizushima et al, 2011*). It is conserved throughout eukaryota, from yeast to human beings. Autophagy can be divided into different classes like- Macroautophagy, Microautophagy, Chaperone-mediated autophagy. Autophagy can be selective for specific organelles. Nucleophagy is the degradation of the nucleus; Ribophagy is the term for degradation of the ribosomes, and ERphagy is the degradation of the endoplasmic reticulum.

Overview:

1.1 Protein misfolding and aggregation

A protein must fold properly to attain correct minimal-energy configuration to arrive at its native, functional form. This process occurs in micro- to nano- seconds. *Levinthal* stated that protein rapidly folds through nearby amino acids interaction, thus limiting the available conformational space. Protein folding follows a funnel-shaped energy landscape to attain the minimal energy and most stable conformation. Thus, proteostasis involves the global regulation of transcription, translation, folding, trafficking, processing, assembly/disassembly, localization, and degradation (*Soto et al, 2012*), thereby encompassing the protein quality control in the cell (Fig 1.1).

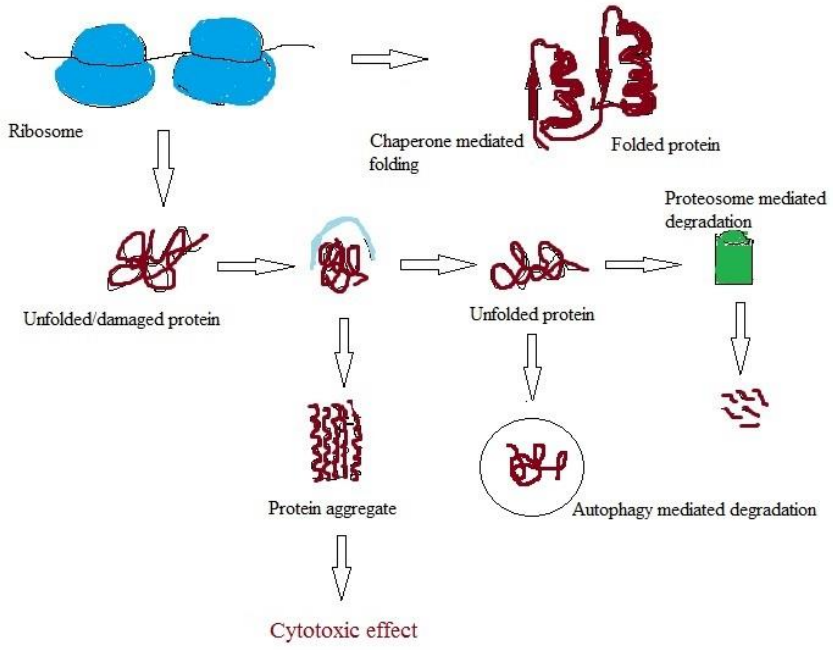
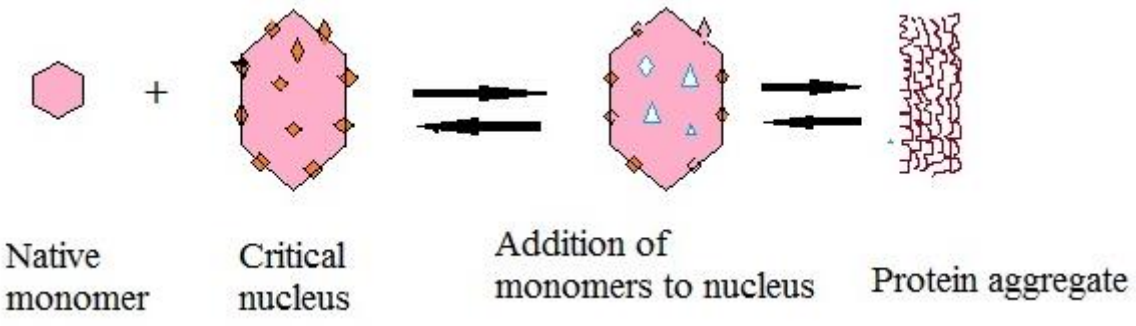
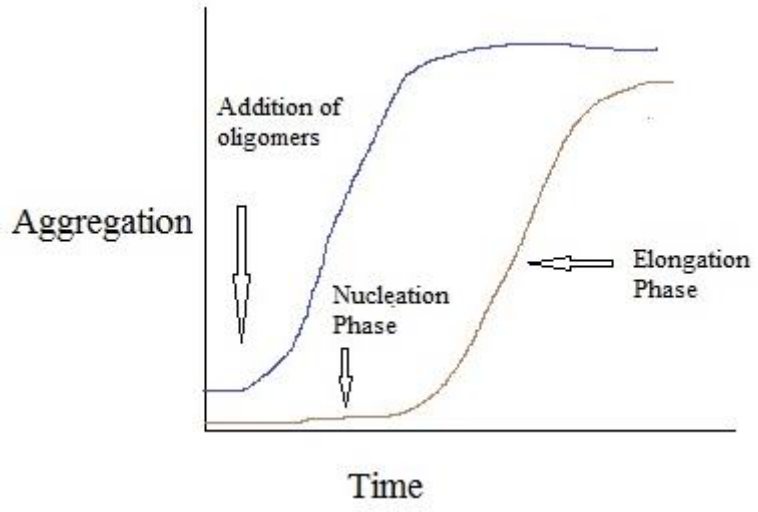


Fig 1.1: The proteostasis network. Schematic representation of protein misfolding and aggregation.

Protein misfolding occurs when a protein cannot fold into its native form primarily due to aggregation propensity of amino acid sequences. Disturbance in the balance between the generation and degradation of the misfolded protein starts building up and burdens the cellular quality control system. This process involves many steps, like nucleation step, exponential step (Fig 1.1.1). The nucleation step is the rate-limiting step for formation of aggregates *(Chaturvedi et al, 2016).*



(A)



(B)

Fig 1.1.1: Mechanism showing oligomerisation of monomeric protein into matured fibrillar form. A. Nucleation dependent fibril formation. B. Graphical representation of fibril formation via seeding. The mechanism includes two phases- nucleation phase and elongation phase.

1.1.1 Factors promoting protein misfold

Proteostasis declines as a function of time which provides the explanation of ageing is responsible for neurodegeneration (*Vilchez et al, 2014*). Molecular chaperones and degradation machinery are the first check-points to solubilize, refold or degrade the misfolded proteins. Thus, aged cells show inability to transform metastable proteins into properly folded structure leading to protein aggregation.

Genetic and environmental factors are also responsible for misfolded protein aggregation. Mutation in the certain region of the gene causes loss of function of that protein and leads to proteotoxicity (*Mark et al, 2014*). Also, the post-translational modification in the protein

governs alteration of different cellular signalling cascades, which in turn, causes pathogenicity. The other reasons of intracellular and extracellular protein aggregates accompany oxidative stress response and inflammatory response.

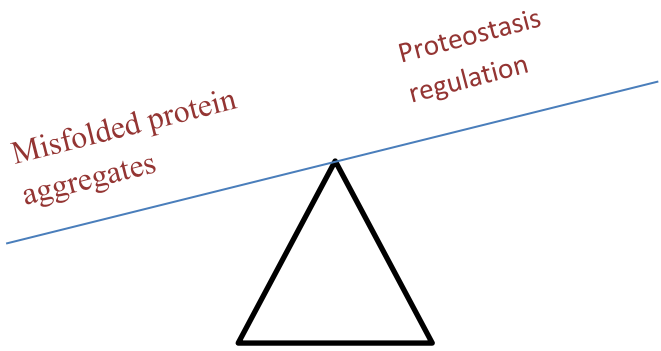


Fig 1.1.2: Schematic representation of stress and response with respect to proteostasis impairment. Constant production or accumulation of protein aggregates due to disease or ageing renders stress response refractory, enhancing proteome imbalance.

1.1.2 Cellualr quality control to maintain homeostasis

Protein homeostasis is regulated by maintaining the balance between the refolding and degradation of the misfolded protein (Fig 1.1.2). The misfolded proteins, which are resistant to degradation, are sequestered in a specific region of the cell with the aid of chaperone and autophagy. Being a permanent post-mitotic cell, neurons employ different quality control process to take care of the aggregates.

Aggresomes are the most common insoluble perinuclear inclusions which encompass ubiquitinated proteins that are actively transported to MTOC (microtubule organising centre) by dynein through retrograde transport. Increased incidence of misfolded proteins leads to their accumulation in two subcellular compartments, named JUNQ and IPOD (Fig 1.1.3). JUNQ (Juxtanuclear Quality Control) is the compartmentalized structure composed of soluble misfolded proteins, chaperones and proteasomal subunits (*Kaganovich D et al., 2008*). JUNQ apparently serves as the temporary storage site for misfolded ubiquitylated proteins that cannot be folded or degraded because of the limited capacity of the ubiquitin–proteasome system under certain stress conditions. In contrast, IPOD (Insoluble Protein Deposits) is a perivacuolar peripheral compartment contains non-diffusible insoluble aggregated substrates, giving the name Insoluble protein deposit. IPOD is also found in non-stressed cells and results from the accumulation of aggregation-prone, mostly non-ubiquitylated substrates that are finally

sequestered from the cytoplasm to protect the cell from the consequences of their potential toxicity. It is interesting to speculate that IPOD is localized next to vacuole and autophagic vesicles to facilitate the degradation of the insoluble protein aggregates by autophagosome

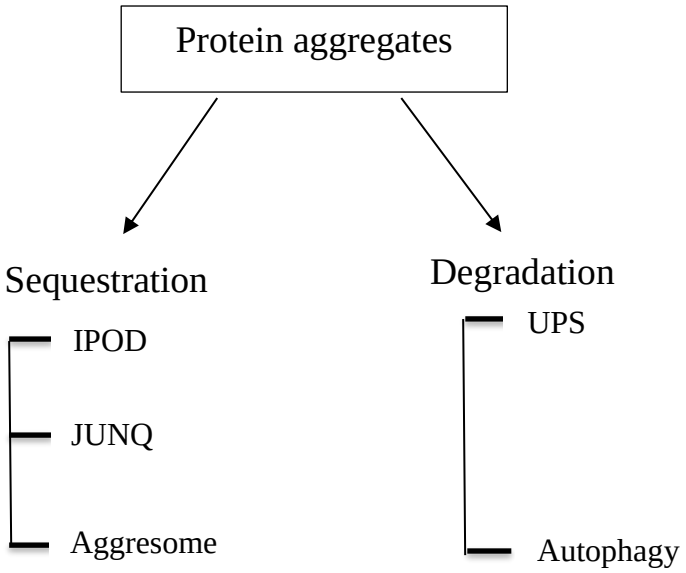


Fig 1.1.3: Strategies to maintain protein homeostasis in a cell. Protein aggregates are sequestered inside aggresomes, JUNQ or IPOD to protect the cells from the proteotoxicity. Aggregates are cleared or degraded through two major pathways- autophagy and UPS.

1.2 Autophagy: a brief outlook

The term 'autophagy' is derived from the Greek words 'auto' meaning "self", and 'phagein' meaning "to eat". This 'self-eating' mechanism is responsible for bulk turn-over of cytoplasm and involved in degradation of superfluous organelles, misfolded protein aggregates, long-lived proteins and invading pathogens. It is an adaptive response to replenish nutrients and energy during stress. *Christian De Duve* (1963) coined the term autophagy and explained its morphological process. In 1992, the scientific breakthrough happened with the demonstration of autophagy in yeast by *Yoshinori Oshumi*. He described that nutrient-deficient conditions induce extensive autophagic degradation of cytosolic components in the vacuoles of yeast cells. The degradation products like, amino acids and other basic building blocks, are recycled back to the cytoplasm and used up the cell (*Klionsky, 2007*). Oshumi's group carried out a yeast genetic screen and identified 15 different complementation groups that were defective in

carrying out the process of autophagy (*Tsukada & Ohsumi, 1993*). This pioneering work was followed by other group and several different core autophagy gene were identified (*Barth and Thumm, 2001*) and led to the discovery of selective autophagy pathways like Cvt pathway, pexophagy, mitophagy and aggrephagy (*Farre et al., 2008; Hutchins &* Klionsky, 2001; Kanki et al., 2009a; Kondo-Okamoto et al., 2012).

Aggrephagy is a selective autophagic process where protein misfolded aggregates are degraded. In pathophysiological context, aggrephagy has a wide-range of implications in case of neurodegeneration, where it imparts quality control mechanism to clear up the aggregates.

1.2.1 Types of autophagy

Autophagy can be classified as macroautophagy (herein,autophagy), microautophagy and chaperone mediated autophagy (CMA). In microautophagy, the vacuolar membrane engulfs the components near its vicinity and degrades them (*Cesen et al., 2012*). In chaperone mediated autophagy, a motif KFERQ on protein substrates is recognised by the chaperone Hsc70 and is translocated into the lysosomal lumen for degradation by lysosome associated membrane protein 2A (Lamp-2). Macroautophagy is the major form responsible for bulk degradation of cargo (*Bandyopadhyay et al., 2008*).

Autophagy can be non-selective or selective for its cargo (*Reggiori et al., 2012*). Genetic screens conducted in yeast *S. cerevisiae* have identified a large number of autophagy genes usually referred to as ATG (ATG1 to ATG37). The core autophagy genes involved in autophagosome formation are associated with all the autophagy related pathways (general and selective). In selective autophagy, the autophagosomes capture and degrade specific cargo such as mitochondria (this selective process is known as mitophagy), peroxisomes (pexophagy), ribosomes (ribophagy), protein aggregates (aggrephagy), pathogens (xenophagy), part of the nucleus (nucleophagy), endoplasmic reticulum (reticulophagy), and others. Apart from the core machinery, selective autophagy requires some specific factors which tag the cargo on one end and interact with the autophagosome on the other and thus serve as a bridge (*Johansen & Lamark, 2011; Noda et al., 2010*).

1.2.2 Steps of autophagy

The molecular components of autophagy are mostly described in yeast e.g.- *Saccharomyces cerevisiae, Pichia pastoris* for its amenability towards genetic manipulation. Broadly the process (Fig 1.2.1) can be divided into 6 major steps like-

Induction

Vesicle nucleation

Autophagosome expansion

Cargo entrapment

Autophagosome fusion

Degradation of cargo.

1.2.2.1 Induction

The common trigger for inducing autophagy is 1) nutrient starvation during lack of essential nutrients in yeast, 2) Using autophagy modulator- inducer or inhibitor in the upstream or downstream of the signaling cascade. In yeast, nitrogen starvation is the potential trigger for induction (*Takeshige K et al., 1992*). In plants, nitrogen or carbon starvation elicits autophagy (*Yoshimoto K et al., 2004*). The response to the depletion of amino acids in mammals varies extensively. The main reason of this difference is the variation in the amino acid metabolism profile in different cell types. Therefore, regulation of autophagy in mammals is highly complicated. For example, in *ex vivo* perfused liver, Ala, Tyr, Pro, Trp, Met, Phe, His, Gln, and Leu suppresses autophagy (*Mortimore GE et al., 1987*).

The known autophagy inducers are- rapamycin, torin, trehalose etc. Rapamycin and torin both regulate the mTOR. The mTOR (mammalian Target of Rapamycin), a master regulator of nutrient signaling induces autophagy upon its inhibition by rapamycin or CCI-779 in yeast (*Noda T et al., 1998*) and in animals (Ravikumar B *et al*., 2004). mTOR inhibits autophagy by hyperphosphorylating Atg13, lowering its affinity towards Atg1 and hence their interaction is greatly abrogated (*Kamada Y et al., 2000*). In nutrient starvation condition, or upon treatment with rapamycin, Atg 13 is dephosphorylated, interacts with Atg 1, stimulates its kinase activity and recruits other proteins like Atg 17, Atg 31, Atg 29 to form a complex (*Kamada et al, 2010*). However, other signalling pathways like Akt, PKA/Sch9 kinase and AMPK also play a role in regulating autophagy (*Arico et al., 2001; Budovskaya et al., 2004; Liang et al., 2007*).

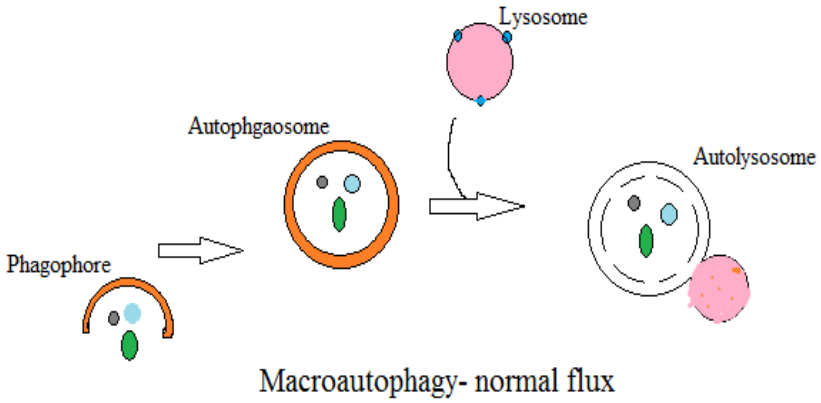


Fig 1.2.1: An overview of macroautophagy

1.2.2.2 Autophagosome nucleation

On autophagy induction, nucleation of the proteins involved in the process takes place at a site called the Pre-Autophagosomal Structure/Phagophore Assembly Site (PAS). Atg17 is the first protein that gets recruited to the PAS (*Ragusa et al., 2012*). It acts as a scaffold for the autophagy machinery to assemble during general autophagy. Atg11 performs similar function in selective autophagy (*Suzuki et al., 2007*). Atg17 interacts with Atg31 and then with Atg29 to from a ternary complex. Atg17 also interacts with Atg13 which in turn recruits Atg1 to PAS (*Reggiori & Ungermann, 2012; Yamamoto et al., 2016*). Atg1 is needed for the transport of Atg9 vesicles from PAS (*Rao et al., 2016*). Atg9 is a transmembrane protein which shuttles between the peripheral sources like ER, mitochondria and plasma membrane, and is responsible for bringing the membrane source to PAS for the developing autophagosome (*Imai et al., 2016; Mari et al., 2010; Reggiori et al., 2005b*). Atg23 and Atg27 are responsible for the anterograde transport of Atg9 vesicles to PAS. Retrograde transport of Atg9 vesicles is mediated by Atg1, Atg2 and Atg18 (*Reggiori et al., 2004*). Atg9 vesicles are about 30-40 nm in size. Three such Atg9 vesicles with about 27 Atg9 molecules are required for autophagosome formation (*Yamamoto et al., 2012*).

There are two Phosphatidylinositol (PtdIns) kinase complexes which are downstream of Atg1 complex. Complex I functions in autophagy while complex II functions in endosomal pathway. Both the complexes have Atg6, Vps15 and Vps34. Beclin 1 is the mammalian homologue of Atg6. Atg14 is integrated specifically into complex I whereas Vps38 is specific for complex II (*Obara & Ohsumi, 2011*). Atg14 directs the localisation of complex I to PAS, a perivacuolar structure. This is brought about by interacting with Atg17/Atg11, a scaffold protein on which other autophagy proteins assemble. Vps34 forms Phosphatidylinositol 3-phosphate (PtdIns-3P)

which becomes the platform for binding of FYVE domain containing proteins like Atg18 resulting in recruitment of Atg18-Atg2 complex at PAS (*Obara & Ohsumi, 2008; Obara et al., 2008*).

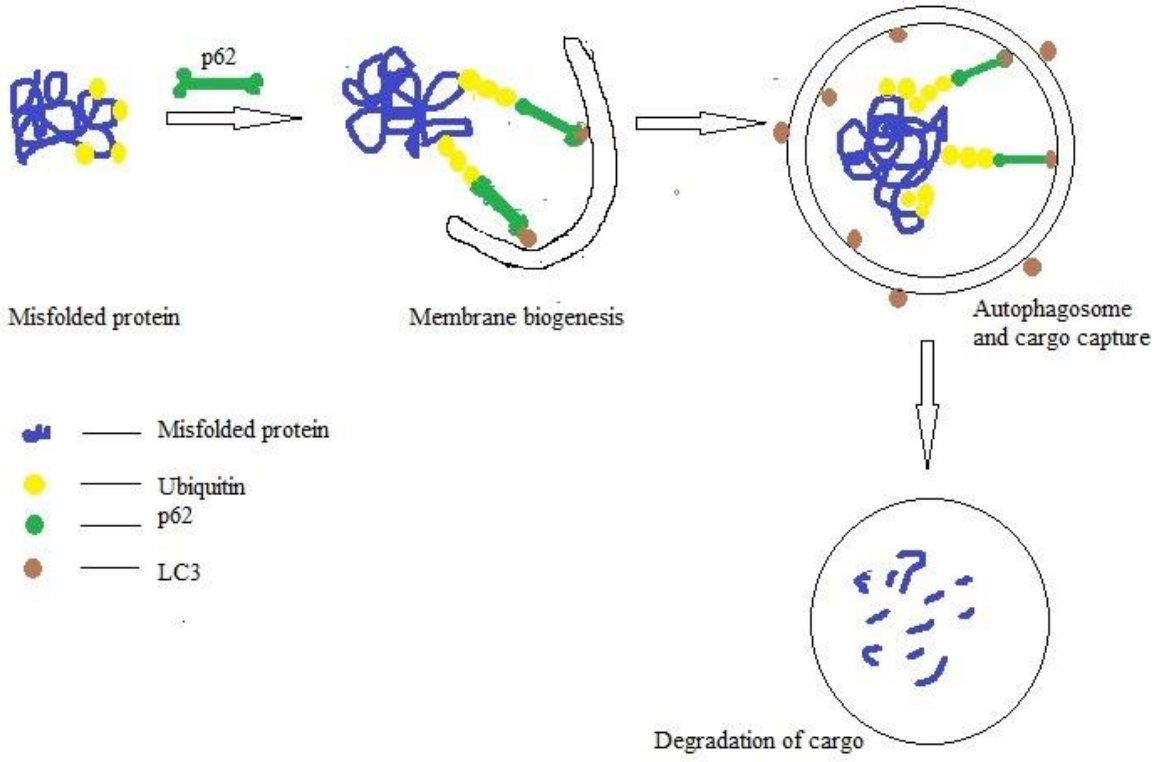


Fig 1.2.2: Substrate recognition and selective autophagy. Misfolded protein aggregates are selectively targeted for degradation. The adaptor proteins like p62 are recruited on the ubiquitinated cargo and it interacts with LC3 on the autophagosome followed by the fusion with a lysosome to mediate the degradation

1.2.2.3 Autophagosome elongation

The initiation of the autophagosome formation happens with the appearance of isolation membrane at PAS. Atg8 is one of the primary proteins associated with the autophagosome throughout its lifespan, from biogenesis to degradation (*Meiling-Wesse et al., 2004*). Atg8 is inserted in the autophagosomal membranes by conjugation to PE (Phosphatidylethanolamine) with the help of two ubiquitin like conjugation systems: the Atg7–Atg3–Atg10 conjugation system and the Atg5–Atg12– Atg16 (*Mizushima et al., 1998a; Nakatogawa, 2013*). The first ubiquitin like system results in the processing of Atg8 (*Kirisako et al., 2000*). Atg4 is a cysteine

protease that helps in conjugation of Atg8 with PE by cleaving the C-terminal Arg residue and exposes the Gly for conjugation. This processed form of Atg8 is then activated by Atg7 and then conjugated to PE with the help of Atg5–Atg12– Atg16 complex (*Fujita et al., 2008; Ichimura et al., 2000*). PtdIns-3P helps in recruitment of Atg5-Atg12-Atg16 complex to the phagophore (*Suzuki et al., 2007*).

1.2.2.4 Capture of protein aggregates as cargo

Apart from general bulk cytosolic degradation (macroautophagy), selective autophagy involves specific sequestration of the cargo for elimination (Fig 1.2.2). Protein aggregates inside a cell are heavily ubiquitinated and eventually become a burden to the proteasome, since hydrophobic nature of aggregates clogs its tunnel. In mammalian system, protein aggregates are initially recognised by chaperone complex (Hsp70, HspB8 and Bag3) and ubiquitinated by E3 ligases that recruits adapter proteins like p62, NBR1, NDP52 and ALFY. p62, the first known adapter molecule that binds to both polyubiquitin tagged protein aggregates and autophagic component LC3 (Light chain binding protein 3), traps cargo into autophagosomes. NBR1 recognizes only protein aggregates but NDP52 identifies ubiquitin-coated intra cellular pathogens as well (*Thurston TLM et al., 2009*). These adapter proteins contain LIR (LC3 Interaction Region) motif. Adapter proteins interact with LC3 (mammalian homolog of Atg8) while forming autophagosomes and hence trap the protein aggregates into it. CMA (Chaperone-mediated autophagy) also targets protein to lysosomes, involving the Hsp70 chaperone which recognise KFERQ motif on proteins and delivers them through LAMP2 protein receptor for lysosomal degradation (*Cook C et al., 2012*).

1.2.2.5 Fusion of autophagosome with vacuole/lysosome

The next step involves vesicle targeting, docking and its fusion with vacuole, which can be referred to the spatiotemporal regulation of autophagosome. The known molecular players include Vam7, Vam3, Ykt6, and Vti1, SNAP, NSF, Sec17-19, Ypt7 and HOPS complex (*Ishihara et al., 2001; Nair et al., 2011*). Double membranous autophagosomes fuse with vacuole with its outer membrane generating single membrane ‘autophagic bodies’ in the vacuolar lumen (*Kihara A et al., 2001*) for the degradation of its contents. In mammalian cells, the autophagosome fuses with lysosome to generate ‘autolysosome’. Apart from Rab and SNARE’s, cytoskeletal machinery is required for the mobility of autophagosomes.

1.2.2.6 Degradation and recycling

Autophagosomes are delivered into the vacuolar lumen as single membrane structures known as autophagic bodies. These autophagic bodies are degraded by the action of vacuolar proteases like Prb1 and Pep4 (*Takeshige et al., 1992*). Aut5/Cvt17 (Atg15) was identified to be an important component of the degradation machinery owing to its lipase activity (*Epple et al., 2001*). In yeast, Atg22 (Aut4) was identified as an amino acid effluxer which pumps amino acids out of the vacuole (*Suriapranata et al., 2000*).

1.2.3 Perturbed autophagy in neurodegenerative diseases

Proteostasis imbalance is the most common phenomenon in case of neurodegeneration. Autophagy is not only neuroprotective but also maintains neuronal plasticity. During development, autophagy plays a major role in shaping form and function of neurons as observed during selective degradation of proteins during synapse formation (*Nixon, 2013*). This is evident from the fact that brain is the primarily affected organ in most of the lysosomal disorders and mutations in genes related to autophagy is very commonly found in neurodegenerative diseases (*Settembre et al., 2008*). Dysregulation of autophagy drives protein aggregates formation. For example, neuron specific knockdown of autophagy proteins like Atg5 or Atg7 in transgenic mice leads to formation of ubiquitinated inclusion bodies which render neurotoxicity and neuronal death. This is implicated in the short life span of the transgenic mice compared to the wild type (*Komatsu et al, Hara et al, 2006*). Protein aggregation is the hallmark of neurodegenerative diseases like Alzheimer's, Parkinson's, Huntington's and ALS disease *(Webb et al, 2003, Nixon et al, 2005, Ravikumar et al, 2002, Barmada et al, 2014*).

1.3 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (herein, ALS) is a rare neurodegenerative disease. It is characterised as a motor neuron disease with the symptoms of defect in walking, eating, swallowing and breathing. With the advent of whole exome sequencing, there is tremendous increase in new discoveries of disease causing genes like *SOD1, TARDBP* (TDP43), *FUS, OPTN* (Optineurin), *UBQLN2* (Ubiquilin 2), *DCTN1* (Dynactin)and *VCP* (Valosin-containing protein) (*Ramesh and Pandey, 2017*).

Like other neurodegenerative diseases, a key pathological hallmark of ALS is the mislocalization of proteins and presence of cytoplasmic aggregates in motor neuron and surrounding cells, causing defects in the machinery maintaining protein homeostasis. The first evidence of protein aggregates was described in familial ALS (fALS) patients with SOD1 mutation (*Rosen et al, 1993*). The SOD1 positive aggregates are ubiquitinated and fibrillized and found in the post-mortem spinal cord samples of sporadic ALS (sALS) patients (Watanabe et al, 2001).

FUS was also found to be major protein aggregates in both fALS and sALS. In post-mortem tissues of FUS mutation carriers, FUS was shown to be enriched in cytoplasmic inclusions within the motor neurons and glial cells (Kwiatkowsky et al, 2009). In the juvenile cases, FUS aggregates appear to have a filamentous structure that are associated with small granules (*Baumer et al, 2010*).

The most common genetic cause of ALS is mutation in *C9orf72* gene (chromosome 9 open reading frame 72), characterised by hexanucleotide repeat expansion (HRE) of GGGGCC in the first intron of the gene (*DeJesus-Hernandez et al., 2011*). The inclusions were first isolated from post-mortem neuron in the pyramidal, frontal and temporal cortices as well as in the hippocampus.

The other discovery of ALS-associated mutations in genes encoding for proteins involved in protein degradation pathways are *OPTN*, *UBQLN2*, *DCTN1* and *VCP*. OPTN inclusions were first detected in spinal cords of non-SOD1 fALS and sALS patients (*Deng et al, 2011*) and UBQLN2 inclusion was first detected in spinal cord of both fALS and sALS patients with the mutation in *SOD1, TDP43, FUS* or *C9orf72* (*Deng et al, 2011; Williams et al, 2012*). (Ref table 1.3.1).

Characteristics of protein aggregates in ALS

Types	Classification	Tissue specification	Characteristics pathologies	References
SOD1	fALS	Spinal cord	Lewy body like, fibrillized, ubiquitinated	*Shibata et al, 1996*
	sALS	Do	Do	*Watanabe et al, 2001*
TDP43	fALS	Spinal cord, frontal cortex	Skein like, ubiquitinated inclusions	*Giordana et al, 2010*
	sALS	Spinal cord, neocortex, hippocampus, glia	Skein like, ubiquitinated, hyperphosphorylated, C terminal fragments	*Arai et al, 2006* *Neumann et al, 2006*
FUS	fALS	Oligodendrocytes, spinal cord, motor cortex, hippocampus	Basophilic, tangle like, ubiquitinated inclusions	*Meckenzie et al, 2011*
	sALS	Spinal cord, glia	Skein like, basophilic, ubiquitinated inclusions	*Baumer et al, 2010*
OPTN (Optineurin)	fALS	Spinal cord	Ubiquitinated inclusion	*Maruyama et al, 2010*
	sALS	Spinal cord, frontal cortex	Skein like, ubiquitinated inclusion	*Maruyama et al, 2010*
UBQLN2 (Ubiquilin 2)	fALS	Spinal cord	Skein like, ubiquitinated inclusion	*Williams et al, 2012*

	sALS	Spinal cord	Skein like, ubiquitinated inclusion	*Deng et al, 2011*
C9orf72	fALS	Hippocampus, cerebellum, neocortex	Ubiquitinated inclusion	*DeJesus-Hernandez et al, 2011*

Table 1.3.1: Overview of characteristics of protein aggregates by SOD1, TDP43, FUS, OPTN, UBQLN2, C9orf72 in familial and sporadic cases of ALS

The predominant proteins involved in the pathology is TDP43. TDP43 inclusions are found in both familial and sporadic ALS cases. TDP43 (TAR DNA binding protein of 43 kDa) consists of 414 amino acid. The N-terminal of the protein has NLS (nuclear localization signal) and NES (nuclear export signal), followed by two RNA recognition motifs, RRM1 and RRM2. The NLS and NES region allows TDP43 protein to shuttle between nucleus and cytoplasm (*Ayala et al, 2008*). The RRM1 is responsible for binding to different RNA targets (*Buratti and Baralle, 2001*) whereas, RRM2 is required to bind to DNA and to maintain the protein structure (*Kuo et al, 2009*). The C-terminal region is composed by the Glycine-rich domain (GRD) which is involved in most of the protein-protein interaction (*Buratti et al, 2005*). The GRD domain includes glutamine/asparagine (Q/N) motif which is responsible for both protein-protein interaction (*Budini et al, 2012*) and TDP43 aggregation (*Fuentealba et al, 2010*)

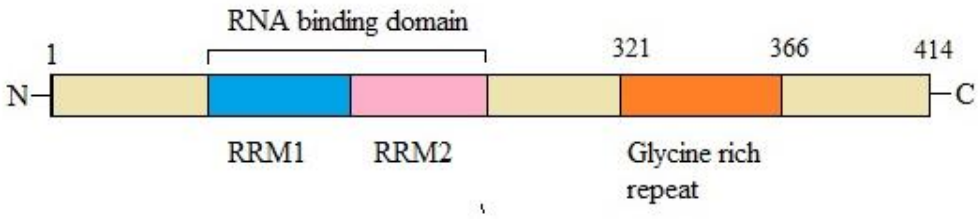


Fig 1.3.1: TDP43 protein. The blue and the pink colour represent the two RNA recognition motif, RRM1, and RRM2 respectively. The yellow region is the Glycine-rich region responsible for the protein-protein interaction.

The C-terminal region is predominantly involved in forming aggregate and showing cytotoxic effect in consequence (*Buratti and Baralle, 2009*). In physiological conditions, TDP43 shuttles between the nucleus and the cytoplasm (*Ayala et al, 2008*) and regulates several processes that

impact on the cellular fate of different RNAs (*Aulas et al, 2012; Buratti and Baralle, 2012; Ratti and Buratti, 2016*). These processes are:

(i) RNA transcription: it includes the regulation of RNA transcription through the direct binding of TDP-43 to promoter regions.

(ii) mRNA alternative splicing: the most studied and known function of TDP-43 is the regulation of alternativemRNA splicing. TDP-43 binds to exon or intron regions and recruits other RNA binding proteins (RBPs) such as hnRNPs or SRs proteins (heterogeneous nuclear ribonucleoproteins and serine-arginine proteins, respectively).

(iii) RNA stabilization: RNA stabilization has been proposed as a function of TDP-43. TDP-43, binding todifferent mRNAs or miRNAs inhibits their premature degradation and possibly favours their translocation from the nucleus to the cytoplasm.

(iv) miRNAs synthesis and processing: TDP-43 has been implicated in the synthesis and processing of different miRNAs by interacting with Drosha and Dicer complexes. It has been reported that TDP-43 down-regulation affects the cellular levels of several miRNAs.

(v) Stress granules localization/regulation

The mislocalization of the protein from nucleus to cytoplasm is referred to toxic in nature. In the pathological condition, TDP43 forms cellular aggregates mainly in cytoplasm which attributes toxicity to the cell (*Arai et al, 2006*). The two-proposed mechanism of toxicity is-

(a) the "gain-of-function" hypothesis, which suggests that cytoplasmic TDP-43 aggregates, or the presence of missense mutations in TDP-43, provokes cell death by cytotoxic effects (*Johnson et al., 2008; Zhang et al., 2009*).

(b) the "loss-of-function" hypothesis, which suggests thatTDP-43 aggregates act as a "sink" by additionally sequestering functional TDP-43 (nuclear and cytoplasmic). This causes a generalized impairment in TDP-43 activity that affects the whole cell physiology.

1.3.1 Regulation of TDP43 by autophagy

Several studies have shown that autophagy vacuole protein p62 and LC3 co-localize with the TDP43 protein aggregates in ALS patient spinal cord sample (*Mizuno et al, 2006*). Apart from p62 and LC3, the autophagy regulator proteins such as- Vasolin containing protein (VCP) and Optineurin (OPTN) are reported to be co-localized with TDP43, p62 and ubiquitin in spinal motor neurons of sporadic ALS patients (*Ayaki et al, 2014*). Caccamao and group in 2009,

reported that inhibition of autophagy by treating 3-Methyl adenine (3-MA) promotes accumulation of full length as well as 35 kDa and 25 kDa truncated protein in N2a and SH-SY5Y cells. Conversely, upon treatment with rapamycin, which is an inducer of autophagy, degradation of different forms of TDP43 was enhanced. TDP43 is implicated in regulation of autophagy. It has been reported by *Bose et al* in 2011 that TDP43 binds to Atg7 mRNA via its RRM1 domain and down-regulation of TDP43 decreased atg7 mRNA level. Consequently, cells lacking TDP43 shows impairment in basal and stimulated autophagy as indicated by the reduction in LC3-II level and accumulation of p62, ubiquitinated inclusion bodies.

Several model systems are available to study the proteotoxic mechanism of TDP43 and the reason behind it. Amongst, yeast proteotoxic model is the simplest one to carry out the preliminary experiments.

Therefore, in the experimental strategy, the cytoplasmic localization of the protein and its toxic function in the yeast are focused.

Johnson et al (2008) reported that TDP43 if overexpressed in *S. cerevisiae* forms aggregates and shows proteotoxic effects, the same strategy was followed here.

1.4 Yeast as a proteotoxic model

Yeast is a unicellular eukaryotic organism which has membrane-bound organelle like mammals. It shares plethora of conserved cellular processes with that of the higher eukaryotes like unfolded protein response, organelle biogenesis and turn over, transcription and translational machinery. Though yeast does not have homologue of the proteins responsible for aggregate and neurodegeneration, but yeast shows similar toxicity phenotype and cellular damage if that protein is overexpressed (*Khurana and Lindquist, 2003*). Also, yeast amyloid is like neurodegenerative amyloids in mammalian cells, which can be stained by thioflavin T or Congo red. Advantage of yeast to use as a tool for proteotoxicity is because of its short generation time, simplicity and availability of genetic resources.

Therefore, in the following experiments, yeast was used as a TDP43 proteotoxic model. The ectopic or overexpression of implicated protein recapitulates the pathophysiology in yeast like a mammalian cell.

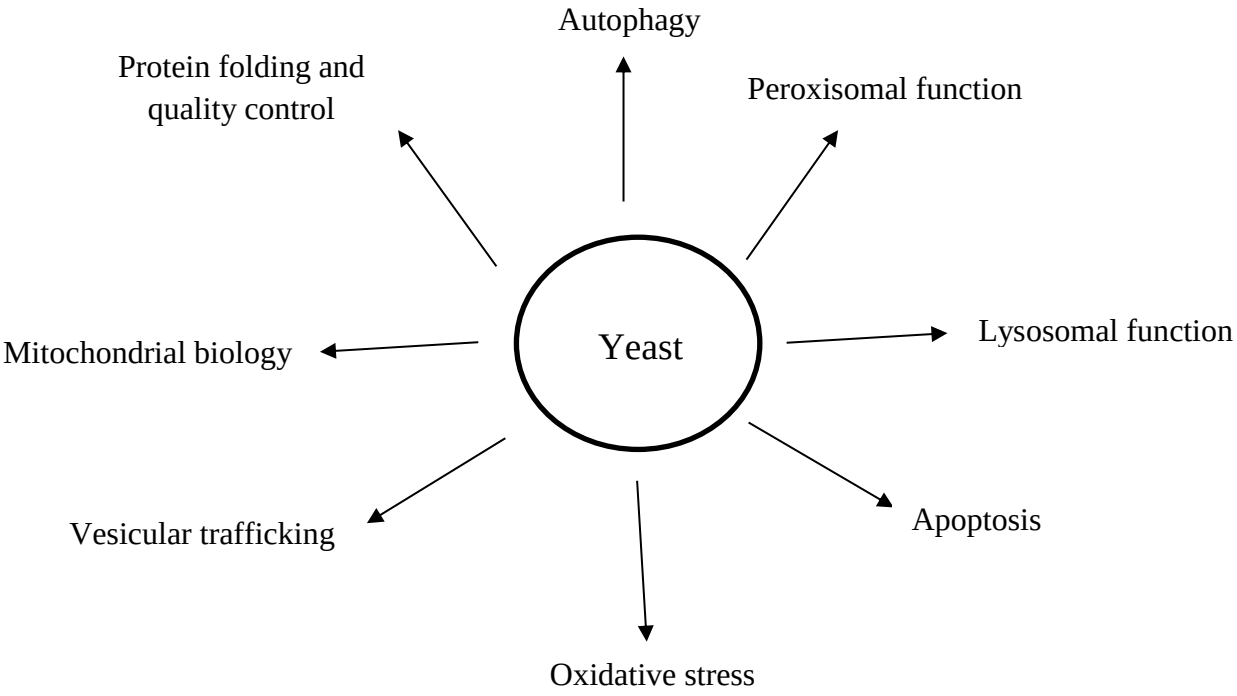


Fig 1.4.1: The various conserved processes of yeast. Yeast has a variety of cellular phenomena like proteostasis machinery which is homologous to that of mammalian cells.

1.4.1 List of yeast proteotoxic model

Disease name	Protein implicated	Pathophysiology	Reference
Amyotrophic lateral sclerosis	TDP43, SOD1, FUS	Protein aggregation impairing growth of the proteotoxic yeast cell	*Johnson et al, PNAS, 2008*
Parkinson's disease	α-Synuclein	Do	*Outerio et al, Science, 2003*
Alzheimer's disease	β- Amyloid	Do	*Caine et al, FEMS Yeast research, 2007*
Huntington's disease	HTT	Do	*Korbitsch et al, PNAS, 2000*

1.5 Research focus and objective

In neurodegeneration, the challenge lies in both understanding disease progression and therapeutic interventions. The field is progressing into enabling innovative aspects towards modeling disease and diagnostics by different targets.

Amytropic lateral sclerosis is a rare neurodegenerative disease. The worldwide annual incidence of ALS is 1.9 per 100000 while in India, the prevalence rate is 6 per 100000 of total population. Only 10% of the cases are familial (inherited from parents), the rest 90% cases are sporadic, no family history available. The male to female ratio is 2:1. It is a fatal motor neuron disease, the symptoms are progressive paralysis followed by loss of ability to talk, swallow, walk move and breathe. In general, the life expectancy is 2-5 years after the diagnosis. The available medicine for ALS treatment is Riluzole and recently FDA approved Radicava. Early diagnostics of the disease might aid in preventing the disease and hence effective management is needed. Rather than targeting a specific mechanism or pathway, employing a more global approach towards the disease phenotype itself may stand a better chance for identifying novel effective small molecules that alleviate the disease phenotype. Furthermore, these small molecules will reveal unique insights into new molecular players involved in aggrephagy.

Keeping this rationale in mind, the following objectives were laid down for the project:

1) Employing proteotoxic yeast model of TDP43 to study the toxic consequences.
2) To screen a library of small molecules against the TDP43 proteotoxic model.

Chapter 2: Materials and methods

Chapter 2: Materials and methods

Overview

2.1 Transformation protocol

2.2 Aggregate induction and characterization

2.3 FACS sorting

2.4 Nuclear staining

2.5 Vacuolar staining

2.6 Cell viability assays

2.7 Assay miniaturization for HTS

Yeasts strains, Plasmids and culture conditions

Strain and Plasmid name	Details	Reference
WTBY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	*www.yeastgenome.org*
pRS426	TDP43, C-terminally tagged with GFP, Gal inducible promoter, Ura selection marker	Johnson et al, PNAS, 2008

Table 2.1: Yeast strain and plasmid information

2.1 Transformation protocol

pRS406 plasmid (procured from Addgene # 27467) was transformed into WTBY4741 and *Δatg1*BY4741 following the protocol below.

A single colony of *Saccharomyces cerevisiae* WTBY4741 and *Δatg1*BY4741 (strains were obtained from Gaurav) was inoculated in 5 mL of YPD [Yeast extract + Peptone + Dextrose] media overnight in 30^0C incubator, shaking condition. Next day, the secondary culture was done from the primary inoculum at A_{600} = 0.2 OD/mL and kept in the incubator till it reached A_{600} = 0.8 OD/mL. Then the culture was harvested in a sterile centrifuge tube at 3000 X g at room temperature (RT) for 5 min. The medium was then poured off and resuspended in sterile 25 mL distilled water (ddH_2O) and centrifuged again as above. The supernatant was discarded, and the pellet was centrifuged again. After that, the transformation mix [Transformation mix: 240 µL PEG (50% w/v), 36 µL1M Lithium Acetate, 50 µL ssDNA (2 mg/mL), X µL plasmid DNA, TDP43-GFP (0.1-10 µg) + X µL ddH_2O = 34 µL] was added to the pellet in same order and mixed thoroughly until the cell pellet was completely resuspended. Heat shock was given at 42^0C for 40 minutes. Then the cells were pelleted, and the transformation mix was removed with the pipette. After that, 600 µL synthetically defined media lacking uracil, SD-Ura media [2% Dextrose + 0.17% Yeast-nitrogen base or YNB + 0.5% Ammonium sulphate + Leucine, Lysine, Histidine, Methionine definite concentrations - Uracil] was added to it and 4 dilutions (50 µL, 100 µL, 150 µL, 200 µL) were plated with the selection marker.

2.2 Aggregate induction and characterization

2.2.1 Expression of aggregate protein

The plasmid (Table 1) used in this experiment is under Galactose inducible promoter. The strains were inoculated in synthetically SD-Ura (synthetic media- dextrose, yeast nitrogen base, ammonium sulfate, leucine, lysine, methionine, histidine) as primary culture with appropriate condition. The secondary culture was done at A_{600} = 0.2 OD/mL from the primary inoculum and kept inside incubator till it reached A_{600} = 0.8 OD/mL. Then the cells were washed twice with sterile water and resuspended in SG-Ura [2% Galactose + 0.17% Yeast-nitrogen base or YNB + 0.5% Ammonium sulphate + Leucine, Lysine, Histidine, Methionine definite

concentrations lacking uracil] for induction of aggregated protein for 24 hours. Subsequently, the expression of fusion protein tagged with GFP was observed under fluorescence wide-field microscope (Elite delta vision) using the filters FITC, excitation and emission range is 488 nm and 510 nm respectively.

2.2.2 Fluorescence microscopy

The cells were picked from the plate and grown overnight in SD-Ura media. Next day, it was inoculated in SD-Ura at A_{600} = 0.2 OD/mL and kept inside the incubator until it reached A_{600} = 0.8 OD/mL. Then the cells were washed and suspended in SG-Ura (2% galactose) for 14-16 hour. After that, 1mL culture was taken and pelleted down. It was resuspended in 100 µL of the same culture and mounted on 2% agarose pad for observing under microscope (DeltaVision, GE bioscience). The filters used were FITC, TRITC, DAPI (excitation and emission spectra- 475 nm, 523 nm; 542nm, 594 nm; 390 nm, 435 nm respectively) using the 100X PlanApoN lens. The images were processed using the SoftWorx software (GE healthcare).

2.2.3 TCA precipitation

The cells were induced in SG-Ura for 24 hours and the samples were collected as A_{600} = 3 OD/mL at certain interval. The culture was pelleted and resuspended in 400 µL 12.5% TCA (tri-chloro acetic acid). The samples were stored overnight in -80^{0}C refrigerator. Next day, the samples were centrifuged at 16000g for 10 min at RT. The pellet was resuspended in 80% ice-cold acetone and centrifuged at 16000g for 5 min and the step was repeated once again. Then the pellet was dried until the acetone fully is evaporated. After that, the pellet was resuspended in 40 µL 0.1 N NaOH + 1% SDS (lysis buffer) and mixed thoroughly. 10 µL 5X SDS-PAGE gel loading dye [Tris- 2.5 mL, pH 6.8, SDS 1 g, Bromophenol blue- 50 mg, Glycerol- 5 mL, DTT- 0.7712 g, dissolved in double distilled water of volume 10 mL] was added to it and kept it for boiling at 99^{0}C for 10 min with mild shaking condition. The samples were kept in ice. A 10% SDS-PAGE gel was run loading 10 µL sample in it.

2.2.4 Immunoblotting

Total cell lysates were electrophoresed on 12% SDS-PAGE for TDP43-GFP and transferred onto PVDF membrane at constant voltage of 100 V for 60 minutes (BioRad Transblot). Blots were incubated overnight at 4^{0}C with mouse polyclonal anti-GFP (Roche) antibody (dil-

1:3000), mouse monoclonal anti-GAPDH (GeneX) antibody (1:3000), rabbit polyclonal anti-Ape 1 (a kind gift from Prof. Oshumi's laboratory) antibody (1:3000) for different experiments. Secondary antibody used at 1:10,000 was goat anti-mouse (Abcam) or goat anti- rabbit antibody (Biorad) conjugated to HRP. Blots were developed by using ECL substrate (Thermo Scientific # 34087) and images captured using auto capture program in Syngene G-Box, UK. Image J software (NIH) was used for quantitation of band intensities.

2.3 FACS sorting

The cells were sorted using Flow cytometry to get highest population of GFP positive cells. A_{600} = 0.6 OD/ mL cells were taken and dissolved in 1X PBS (phosphate buffer saline) to do the sorting. The cells were taken in low concentration to avoid clogging in the FACS machine nozzle. The sorted cells were collected in SD-Ura media, and poured on SD-Ura plate.

2.4 Nuclear staining

NucBlue from Invitrogen, equivalent to DAPI, was used to stain the nucleus as 2 drops/mL and incubated for 40 min in 30^0C incubator. Then 1 mL culture was taken and pelleted down. The pellet was resuspended in 100 µL of the remaining culture and mounted on 2% agarose pad for observing under microscope. The images were processed using the SoftWorx software.

2.5 Vacuole stain

FM4-64 (Thermofisher Scientific), an endosomal dye was used to stain the vacuole. The concentration added was 20 µM to the culture and incubated in the same condition as above. After that, 1 mL culture was taken and pelleted down. The pellet was resuspended in 100 µL of the remaining culture and mounted on 2% agarose pad for observing under microscope. The images were processed using the SoftWorx software.

2.5.1 Statistical analysis and image preparation

Statistical analysis was carried out using GraphPad Prism (GraphPad Sofware). Statistical analyses were performed by comparing the means using the unpaired Student t test. Yeast images were prepared using Softworx software (GE healthcare).

2.6 Cell viability assays

2.6.1 Dead cell quantification

Phloxine B (sigma), a vital dye was used to stain the dead cells. The dye was added (5 mg/mL) in the induction media (SG-U) and kept for 14-16 hours in the same condition as above. After that, 1 mL culture was taken and pelleted down. The pellet was resuspended in 100 μL of the remaining culture and mounted on 2% agarose pad for observing under microscope. The images were processed using the SoftWorx software.

2.6.2 Growth assay

TDP43-GFP strain was inoculated in SD-Ura and allowed to grow overnight at 30^0C, 300 rpm). From this culture, cells (A_{600}= 0.2) were again inoculated and then allowed to incubate further till A600 reached 0.8. Then the culture was washed twice with sterile water and the induction media SG-Ura was added to it as A_{600}= 0.2 OD/ mL. After that 80 μL was aliquoted and added to each well of the 384 microtitre plates and mixed thoroughly. The prepared working plates were used for growth assay in a Varioskan Flash automated plate reader (Thermo Scientific).

2.7 Assay miniaturization for HTS (high throughput screen)

2.7.1 Small molecule screen

A small molecule library was chosen for screening against TDP43-GFP proteotoxic model. The compound library consists of 400 compounds which has molecular weight < 500 dalton. The compounds are both water and DMSO soluble. The compounds are novel and its connection within autophagy is not yet established.

The primary growth assay was based on the observed apparent growth lag due to overexpression of toxic protein TDP43. Thus, the main rationale was to screen for small molecules which would rescue growth lag and those will be considered as hits. These hits will be further validated by performing secondary assays.

2.7.2 Strains, Plasmids and Media:

Strain harbouring GFP tagged TDP43 protein was employed. Synthetic media like SD-Ura media were used for primary and secondary culture. For induction of proteins, SG-Ura [galactose (2%)] was used.

2.7.3 Preparation of Intermediate and Working plates:

Compound library stock plates (10 mM, 100% DMSO) were provided in 96 well plates. From this, the intermediate plates (800 μM, 12.5% DMSO) were prepared in 96 well plates. Eventually working plates were made in 384 well plates with final drug concentration of 50 μM in 1.4% DMSO.

2.7.4 Culturing of yeast:

TDP43-GFP strain was inoculated in SD-Ura and allowed to grow overnight at 30^0C, 300 rpm). From this culture, cells (A_{600}= 0.2) were again inoculated and then allowed to incubate further till A600 reached 0.8. Then the culture was washed twice with sterile water and the induction media SG-Ura was added to it as A_{600}= 0.2 OD/ mL. After that75 μL was aliquoted and added to each well containing 50 μM or 5 μL drug (small molecule) in the 384 microtitre plates and mixed thoroughly. The prepared working plates were used for growth assay in a Varioskan Flash automated plate reader (Thermo Scientific).

2.7.5 Small Molecule Screening

The final working plates (50 μM, 1.4% DMSO) were prepared in a 384 well format. In each 384 well plate, the edges were left empty. Two wells containing media were considered blank followed by cultures of untreated WT, uninduced TDP43-GFP, induced and untreated TDP43-GFP and then compounds added to TDP43-GFP in duplicates. Three working plates were used for screening.

The early exponential growth phase culture was used for screening. The culture was grown under standardized conditions (30^0C, 420 rpm) for 48 h in a 384 well format. After 48 h, growth curves of the control strains were plotted. Mid to late exponential phase of WT time point was chosen for analyzing the data. Its corresponding time point of untreated and treated TDP43-

GFP was plotted separately. Small molecules that would rescue or salvage the growth lag by 3 SD units of untreated TDP43-GFP strain were considered as hits.

Chapter 3: Results and discussions

Chapter 3: Results and discussions

Overview:

3.1 TDP43-GFP overexpression drives aggregate formation

3.2 TDP43 is localized to both nucleus and cytoplasm

3.3 TDP43-GFP is overexpression was validated by immunoblot

3.4 TDP43-GFP cells sorted by flow cytometry analysis

3.5 TDP43-GFP overexpression rendered toxicity and attenuated cellular growth

3.6 Vacuolar transport of TDP43-GFP

3.7 TDP43-GFP toxicity was implicated in dysregulation of autophagy

3.8 The small molecule screening

3.1 TDP43-GFP overexpression drives aggregate formation

pRS426 plasmid encoding TDP43-GFP under the gal inducible promoter was transformed into WTBY4741 yeast strain. Positive transformants were screened through microscopy.

By microscopy we observed that the cells overexpressing TDP43-GFP appear as big foci at multiple foci of the cell. The control strain which has only GFP plasmid, showed diffused GFP signal, inferring that overexpression of TDP43-GFP forms aggregate.

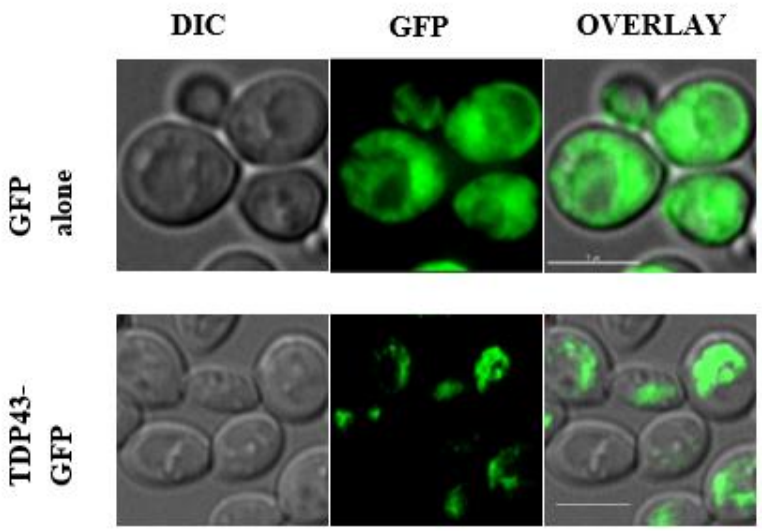


Fig3.1: TDP43-GFP overexpression forms aggregate. Scale bar 5 µm.

3.2 TDP43 was localized to both nucleus and cytoplasm

TDP43 was naturally localized in nucleus but whenever overexpressed it tended to form the aggregate in the cytoplasm, known as mislocalization of TDP43. Below is the microscopic image where TDP43-GFP if overexpressed localize both in cytoplasm and nucleus.

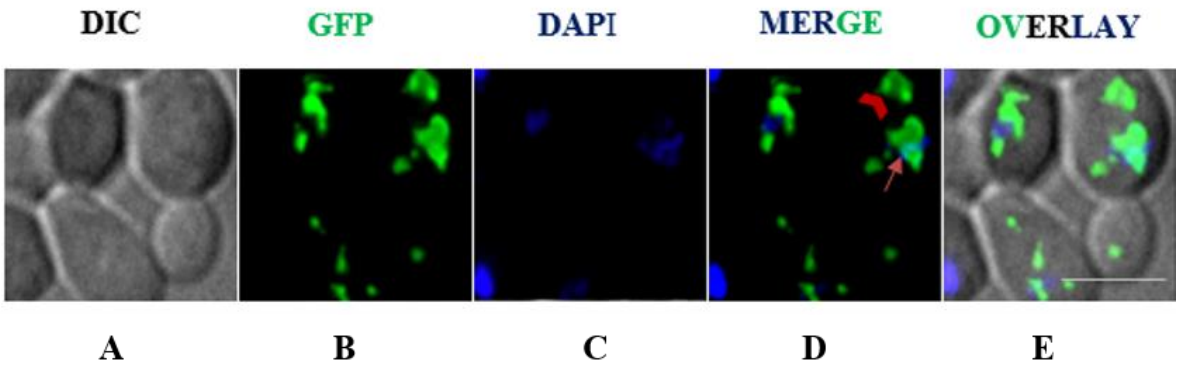


Fig 3.2: - Localization of TDP43-GFP aggregates. A. Deconvoluted image of yeastcells. **B**. TDP43-GFP aggregates visualized using FITC filter. **C.** Nucleus stained with DAPI. **D**. The merged image of GFP and DAPI showing the localization of aggregates is both nucleus and cytoplasmic. Arrow indicates nuclear aggregate while arrow head indicates cytoplasmic aggregate. **E**. Overlay of DIC, DAPI and GFP channels. Filter used- FITC, TRITC, DAPI, POL, Scale bar 5 µm.

3.3 TDP43-GFP overexpression was validated by Immunoblot

TDP43-GFP was overexpressed in wild type yeast cells using Gal inducible promoter. Surprisingly protein was expressed only at the 12th hour. There was no expression of protein till 6 hours. Since we did not take any intermediate time between 6th and 12th hour, it could not be inferred that protein was expressed exactly at 12th hour.

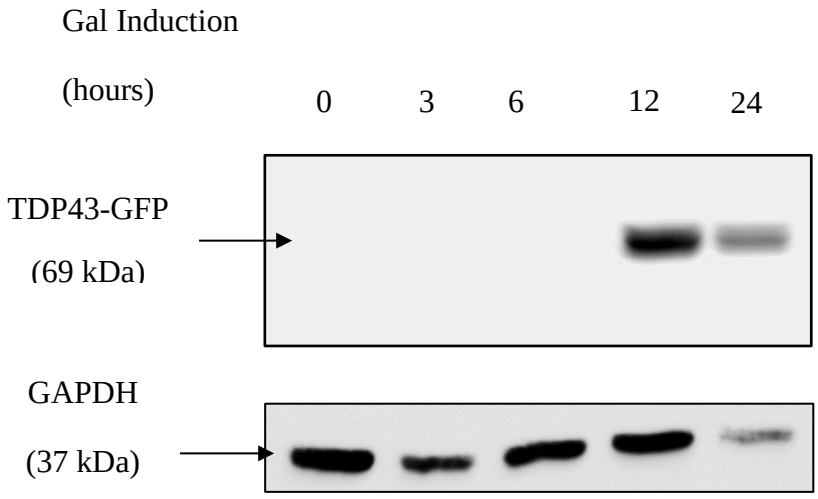


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Fig3.3: - TDP43-GFP expression by immunoblot. Anti-mouse GFP antibody (1:3000) was used for the blot. The protein is expressed after 6 hours. The loading control is anti-mouse GAPDH antibody (1:3000).

3.4 TDP43-GFP cells were sorted by flow cytometry analysis to get highest population of GFP positive cells

The proteotoxic yeast strain harbouring TDP43-GFP formed aggregate when it was overexpressed under gal promoter. So, the cells were induced with galactose for 12-14 hours and then sorted by flow cytometry to get the pure population of cells expressing GFP of maximum intensity. After that, sorted cells were kept in the media for 30 minutes or recovery purpose. Next it was plated on selection plate (SD-Ura). The colony appeared after 2 days. The big colonies were picked up and again plated on SD-Ura plate. After the cells had grown on plate, those were validated by microscopy, western blot and growth assay.

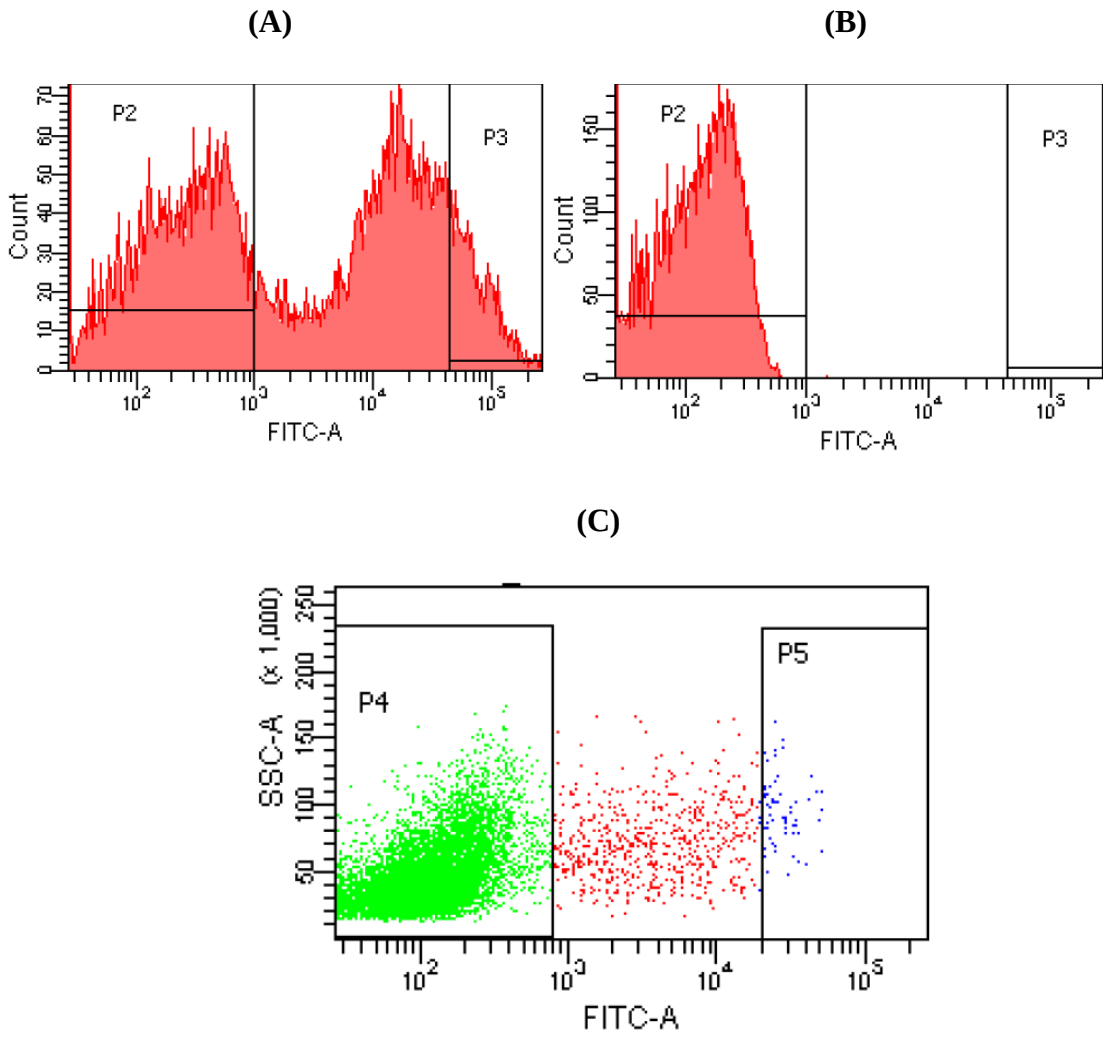


Fig3.4: - FACS sorting of the TDP43-GFP cells. A. Positive control, WtGFP cells which are positive for the GFP expression has been used as positive control. B. Negative control, TDP43-GFP, since expressed under gal inducible promoter, so the protein won't be expressed in dextrose containing media. So, the TDP43-GFP yeast cells in dextrose media were taken as negative control. C. Experimental strain, The P5 region is the highest populated GFP positive cells, while the P4 is the auto fluorescence region.

3.5 TDP43-GFP overexpression rendered toxicity and attenuated cellular growth

TDP43-GFP overexpression shows toxic phenotype to cells which was validated by – microscopy, spot dilution assay, growth assay.

3.5.1 Microscopy- Phloxine B stain

Phloxine B is a lipophilic vital dye which stains both live and dead cells, but the dead cell lacks the efflux pump and therefore unable to throw the dye out of the cell like the live one. Hence, dead cells can be identified and quantified using this dye. The images were taken by the fluorescence microscopy using FITC and TRITC filter.

The dead cell count in overexpressed TDP43-GFP is more than the control one.

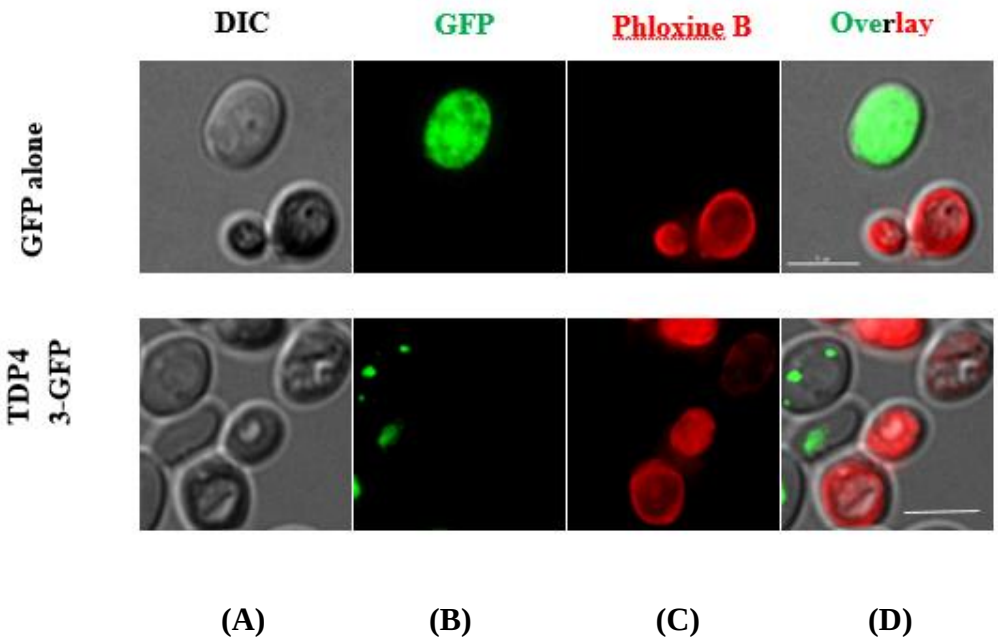


Fig 3.5.1: - Phloxine B stain of vector control and TDP43-GFP phototoxic yeast strain. A. DIC images of both vector control and test strain, i.e. proteotoxic TDP43-GFP yeast cells. **B**. GFP is diffused in vector control while the test strain showed punctate structure of TDP43-GFP aggregate. **C**. Phloxine B stained dead cells. **D**. Merged image of all the channels. Filter used- FITC, TRITC, POL, scale bar 5 μm**.**

The same result is quantitated and plotted below.

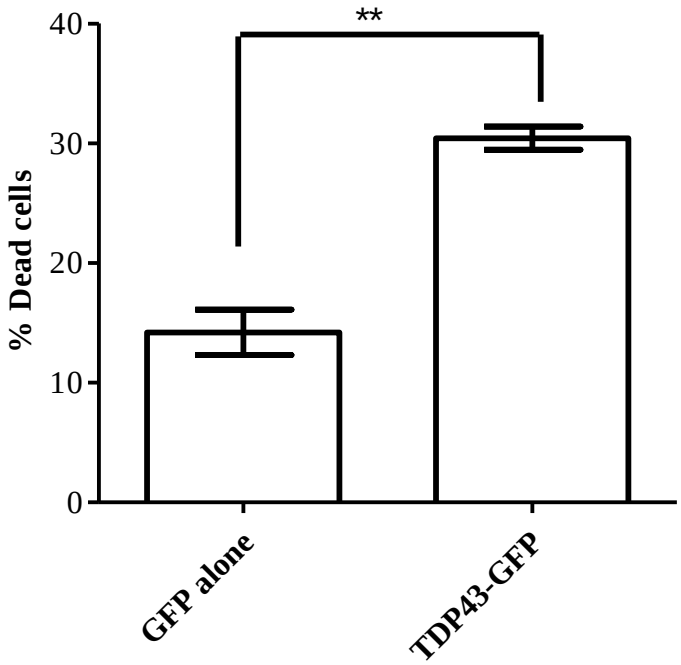


Fig 3.5.1.1: - Percentage dead cells count by PhloxineB stain. The dead cell percentage is higher in TDP43-GFP proteotoxic strain than that of the vector control. P value ≤ 0.01, number of cells= 20, n=3.

3.5.2 Spot dilution assay

TDP43-GFP was spotted in appropriate condition with and without galactose (induction media) and incubated for 48 hours. Then 1 OD cells were taken, and serial dilution was made. 2.5 μL from each of the dilution was spotted on the uninduced as well as induced plate. The uninduced plate containing dextrose in the media had appreciable number of colonies whereas, the galactose induced plate had reduced number colonies than that of the uninduced one implying that overexpression of TDP43-GFP is imparting toxicity to the cell.

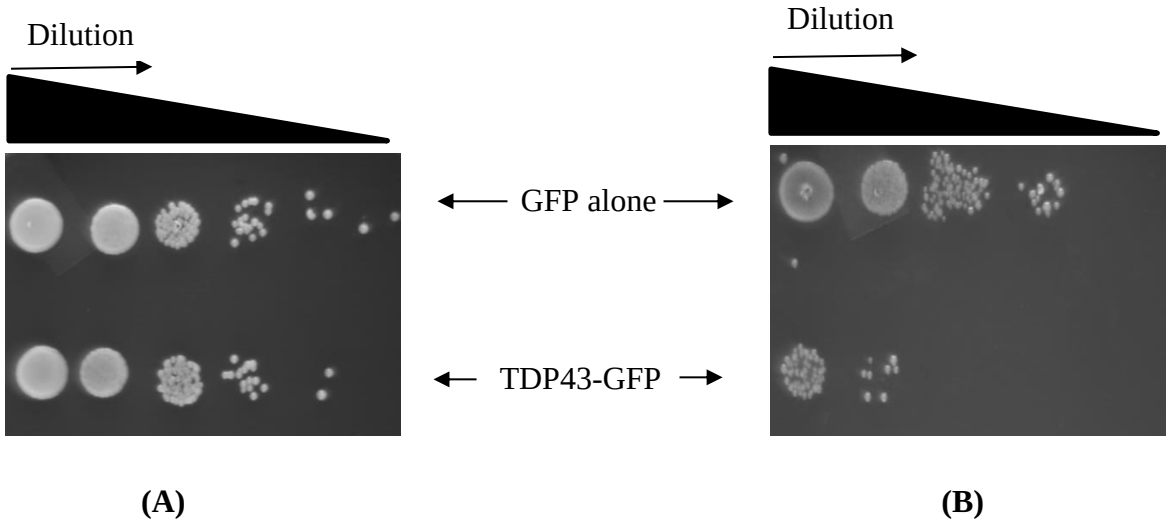


Fig 3.5.2: - Spotting of TDP43-GFP and only GFP plasmid in 6 serial dilution.A. Spotting without galactose induction media. **B**. Spotting with the galactose induction media.

3.5.3 Growth assay

Growth curve of proteotoxic strain is an ideal measurement of its ill effect. Therefore, growth assay for the proteotoxic strain was carried out. In uninduced condition, TDP43-GFP grew with proper lag and exponential phase. But upon induction with galactose, TDP43-GFP was overexpressed and showed severe growth lag than that of the uninduced condition. TDP43 expressing cells barely reached exponential phase. The extended lag phase observed due to TDP43-GFP was about 16 hours inferring that toxicity is rendered by overexpression of TDP43 which attenuates growth.

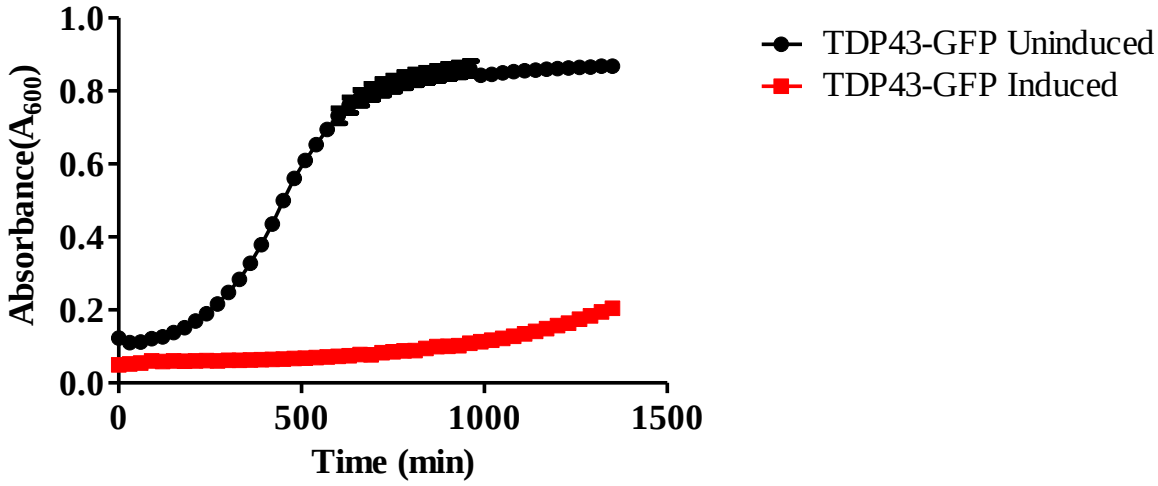


Fig 3.5.3: - TDP43-GFP imparts proteotoxicity and impairs the growth

3.6 Vacuolar transport of TDP43-GFP

Wild type yeast cells harbouring TDP43-GFP was stained with an endosomal stain FM4-64. FM4-64 is transported through endosome, hence it can stain plasma membrane as well as vacuole, if the cells stained for longer time.

In the experiment, TDP43-GFP aggregates were induced for 14-16 hour and stained with FM4-64. The aggregates were not present inside the vacuole over time. This result implies that the aggregates did not undergo vacuolar degradation, probable reason would be the endosomal pathway might have been perturbed due to overexpression of this toxic protein.

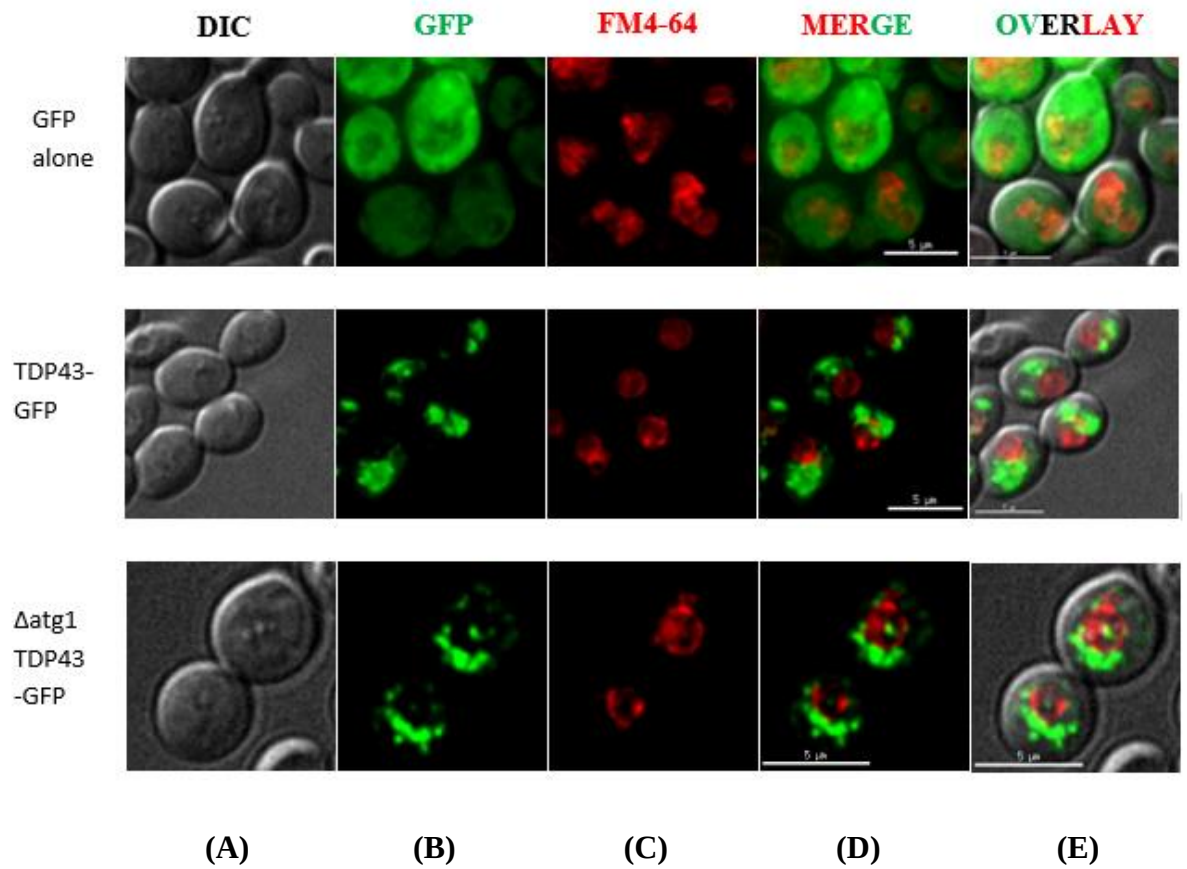


Fig 3.6.1: -TDP43-GFP proteotoxic cells were stained with FM4-64 dye. **A**. DIC images of the strain harbouring the GFP plasmid and proteotoxic yeast cells overexpressing TDP43-GFP and Δatg1TDP43-GFP. B. TDP43-GFP proteotoxic cells, both wild type and knock-out strains form aggregate. C. The vacuole was stained with FM4-64. D. Merged images of all the channel. Aggregate were not present inside the vacuole over time, implies that it does not undergo vacuolar degradation. Filter used- FITC, TRITC, POL, scale bar- 5 µm.

Since starvation triggers autophagy, TDP43-GFP cells were further put into starvation media (SG-N, amino acids depleted media) for 8 hours. Then the images were captured to check the localization of aggregates. Even in starvation induced condition, the aggregates were seen outside the vacuole. The inference could be the autophagy is perturbed in the proteotoxic TDP43-GFP cells.

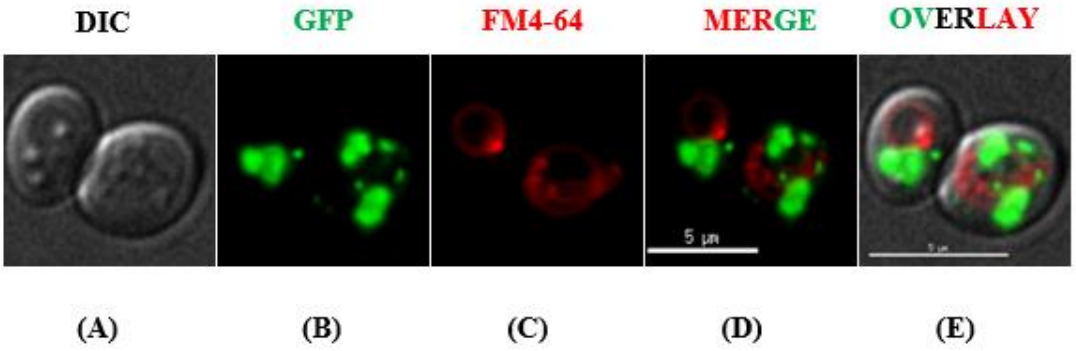


Fig 3.6.2: TDP43-GFP proteotoxic cells did not undergo vacuolar degradation under in starvation condition. A. DIC image of wild type TDP43-GFP cells. **B**. The cells have formed aggregate. **C**. The vacuoles were stained by FM4-64 dye. **D.** Merged image of all the channels where aggregates did not undergo vacuolar degradation. Scale bar- 5 µm.

3.7 TDP43-GFP toxicity was implicated in dysregulation of autophagy

One of the key experiments as a proof of basal autophagy is Aminopeptidase 1 (Ape 1) maturation assay. Aminopeptidase 1 is a specific cargo protein which can be delivered to vacuole from cytoplasm via Cvt or macroautophagy pathway. Ape 1 (Aminopeptidase 1), synthesized as a precursor dodecameric form, pApe 1 (61 kDa) in cytosol, contains a propeptide, which is important for its vacuolar delivery. Inside the vacuole, the propeptide is broken down by resident hydrolase of the vacuole generating mature sized Ape 1 (mApe 1, 55 kDa). Cvt pathway and autophagy are interrelated to each other. Hence conversion of pApe 1 to mApe 1 is a proof of uninterrupted basal autophagy, while the absence of mApe 1 interprets that the basal autophagy is disrupted to some extent.

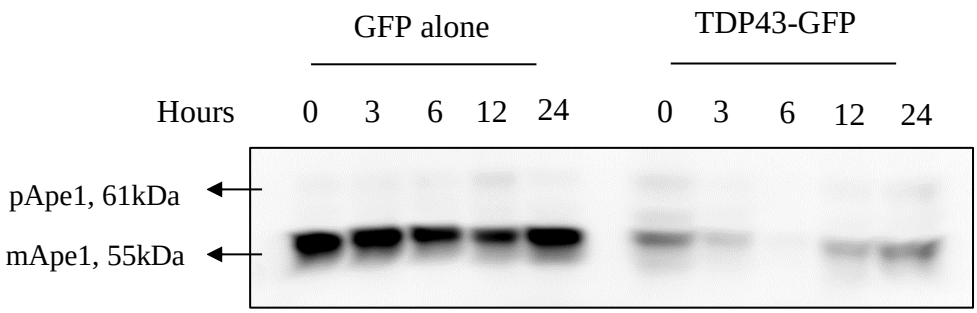
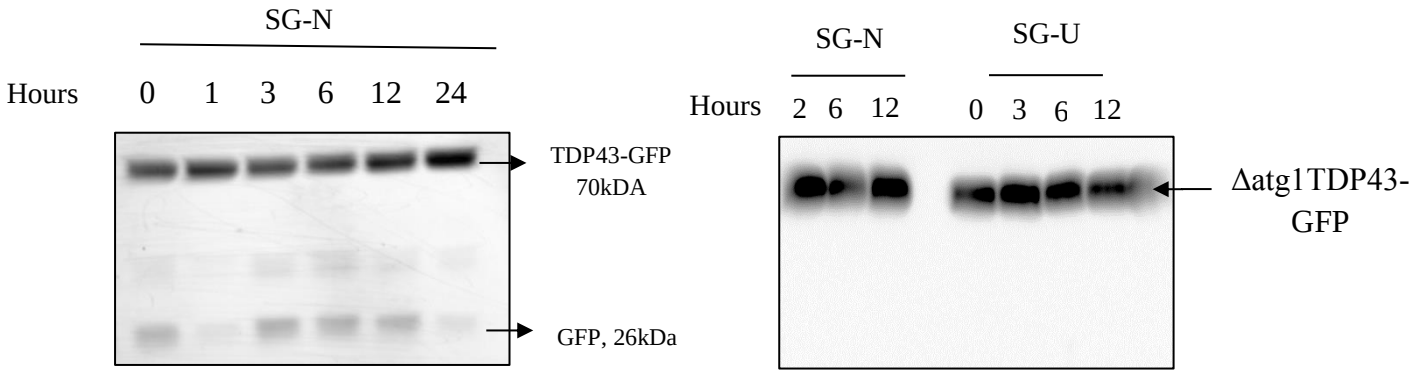


Fig 3.7: Ape 1 maturation assay. The proteotoxic yeast cells expressing TDP43-GFP showed reduced level of precursor Ape 1 which implies that the basal autophagy is compromised. The blot was probed with rabbit polyclonal anti Ape 1 antibody used in 1:3000 dilution.

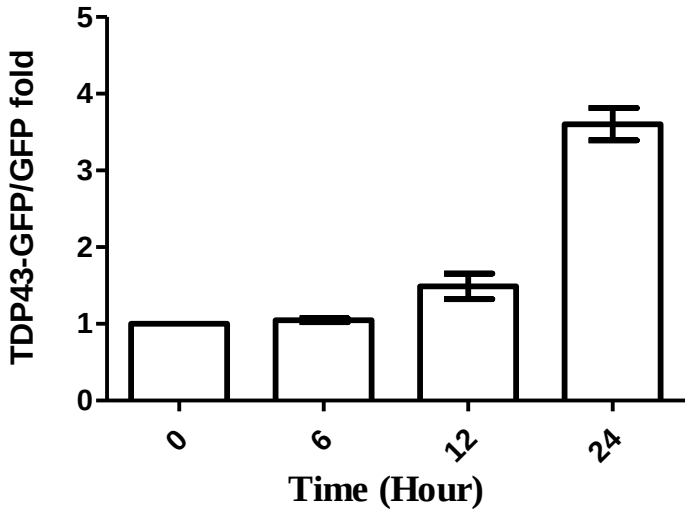
The above immunoblot showed that compared to GFP alone plasmid, the proteotoxic yeast cells which expressed TDP43-GFP has reduced level of mature Ape 1 which inferring that the basal autophagy is impaired in the proteotoxic yeast cells.

3.7.1 Induction of autophagy enhances degradation of protein

Previous experiment showed that the basal autophagy in the proteotoxic yeast cells is disrupted. Therefore, starvation induced autophagy might enhance the degradation and clearance of the toxic TDP43-GFP protein. So, immunoblot was performed to check the expression of protein in nitrogen starvation condition that is SG-N media, where the carbon source is galactose to induce the protein, but the media is nitrogen deprived.



(A) **(B)**



(C)

Fig 3.7.1: Effect of starvation induced autophagy in TDP43-GFP proteotoxic yeast cells. **A.** Wild type TDP43-GFP cells, upon induction, showed degradation of the protein over time. **B.** Δatg1-TDP43-GFP cells, though autophagy was induced did not show any degradation of the protein. Both blots were probed with mouse monoclonal anti GFP antibody in 1:3000 dilution. **C**. The WtTDP43-GFP blot was numerically calculated to get the fold change of TDP43-GFP in respect of free GFP. The free GFP band was absent in Δatg1TDP43-GFP strain, n =3.

Since Atg1 is a core autophagy protein, Atg1 knockout strain expressing TDP43-GFP failed to show enhanced degradation of autophagy upon starvation mediated induction. It can be inferred from the above experiment that autophagy is indeed involved in clearance of toxic accumulation of TDP43-GFP in the proteotoxic yeast cell.

3.8 The Small molecule screening

Small molecules are defined as organic compounds with low molecular weight (<900 Daltons) but not polymers, which might have biological functions. These are involved as tools to probe biological function like enhancers or inhibitors of protein activity or a pathway (Veber DF *et al*., 2002) and in the development of new therapeutic agents as well.

The main advantages of Small Molecules Drugs (SMDs) over large biologicals are that they can be administered orally, cross cell membrane barrier easily and be rapidly absorbed. Some molecules are given as 'Prodrug'. Prodrugs are defined as a pharmacologically inactive but readily transformed to active compounds when administered into body (Stella VJ *et al*., 1985). Secondary metabolites are one of the common examples of small molecules. These are biologically active natural compounds produced by plants, fungi and bacteria, for example- alkaloids, steroids etc.

3.8.1 Drug Discovery:

The drug discovery process encompasses

1) high throughput screening of compounds against biological target or disease phenotype,
2) hit identification,
3) optimization of attained hits to reduce side-effects or increase affinity/selectivity,
4) efficacy or potency test for attained leads on animal disease model.

The identification and validation of target or disease phenotype is crucial initial step of drug discovery. The pre-requisites for a reliable assay are as follows (Hughes JP *et al*., 2011),

1) The assay should be pharmacologically relevant with desired potency.
2) Reproducibility.
3) Cost effective.
4) Quality of assay as depicted by Z' factor.
5) Effects of solvents or compounds in the assay.

3.8.2 Small molecule screen against TDP43 protein

The small molecule screen was done in an unbiased approach to explore new molecular pathway imparting TDP43 toxicity and to unravel the molecule efficacy and potential as therapeutics.

Yeast provides an excellent platform because overexpression of the toxic TDP43-GFP abrogates the growth of the wild type strain. This phenomenon has been employed as a tool to design the small molecule screen. Rationale of this experiment is that the compounds which can rescue the growth lag is considered as Hit and can be investigated further to understand the nature of proteotoxicity.

3.8.3 Standardization of Growth assay in 384 well Format:

The growth assay was standardized in a 384 well format for various parameters like initial A_{600} = 0.08, culture volume = 80 µl and 420 spm (spin per minute) using Varioskan Flash. All parameters were standardized to avoid the A600 drop due to settling of cells. For instance, low or high culture volume leads to settling of cells eventually and leads to drop in A600.

3.8.4 DMSO- Final Working Concentration:

Small molecules were dissolved in DMSO. As DMSO would be toxic to cells, it was important to titrate the growth assay with different DMSO concentrations. Strain WT-GFP was assayed with various final DMSO concentrations such as 10%, 5%, 2%, 1%, and 0.5 %. Higher concentrations of DMSO (10% and 5%) severely hampered the growth whereas, yeast cells could tolerate till the presence of 2% DMSO concentration. Therefore, final DMSO concentration 1.4% was used for the assay in the working plates.

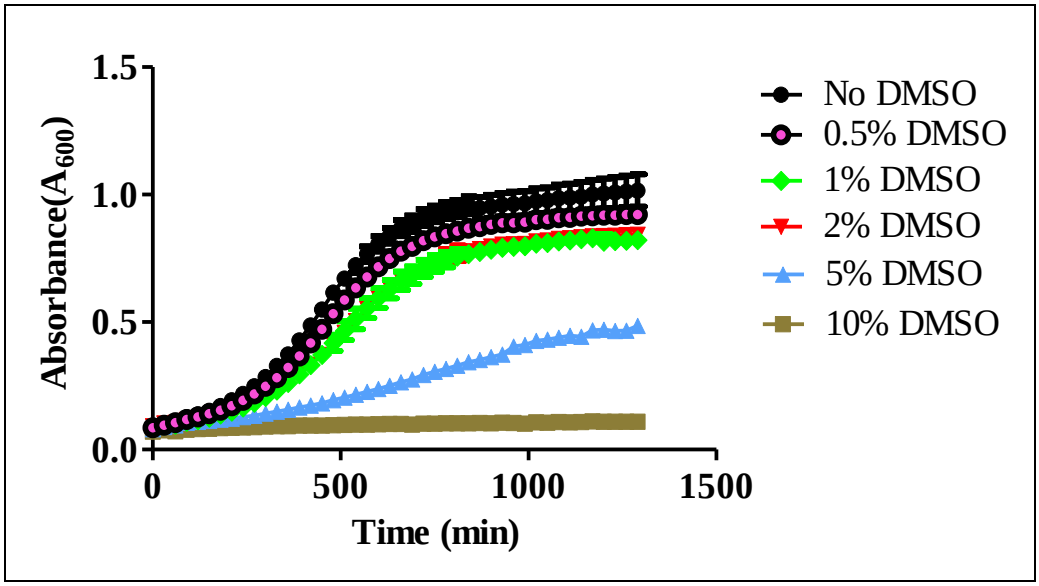


Fig 3.8.4: Assay with DMSO vehicle. The strain WT GFP was assayed with different DMSO concentrations (10%, 5%, 2%, 1%, and 0.5%). As expected, WT-GFP strain shows poor growth at about higher (10% and 5%) DMSO concentrations. But cells could tolerate up to 2% DMSO concentration.

3.8.5 Small molecule screen result

The small molecule screening assay did not yield very significant result. A time-point in the mid-exponential phase was chosen and the absorbance values were plotted for all the controls and compounds. A statistical 3 SD (standard deviation) box was plotted.

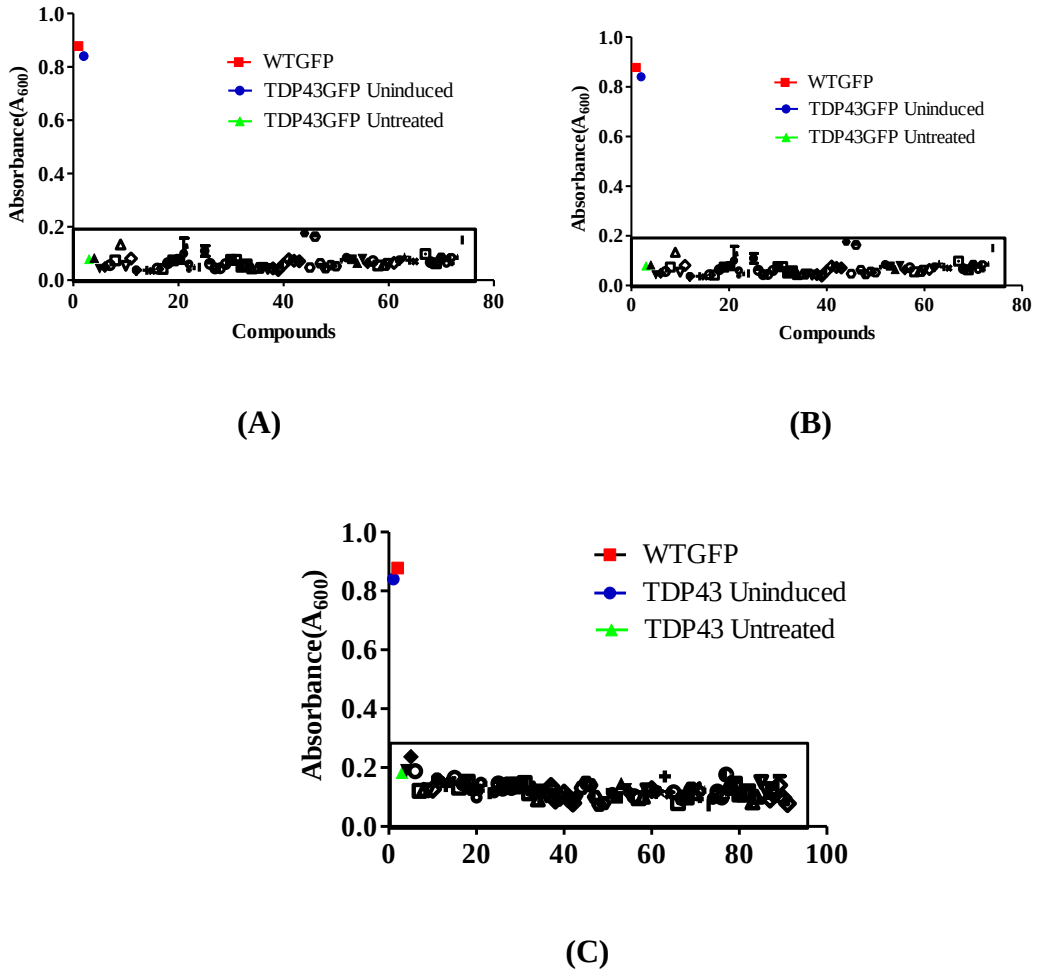
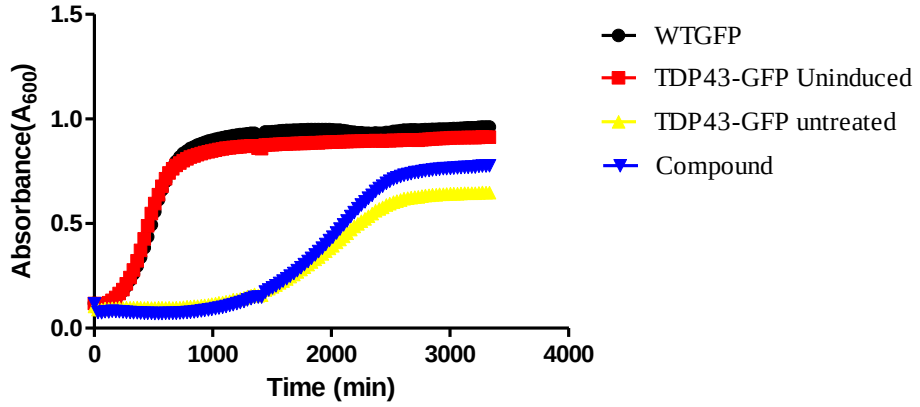
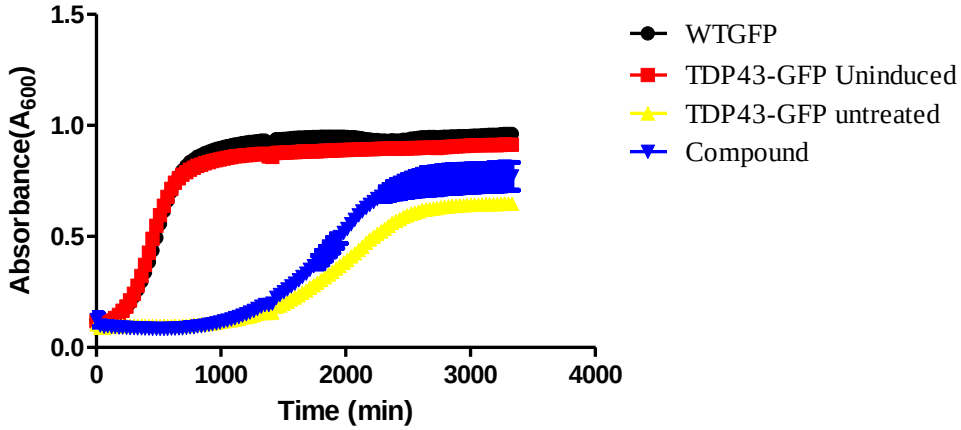


Fig 3.8.5.1: Representative box plot of 3 working plate. The box represents statistical 3 SD unit. The experimental strain is TDP43-GFP in galactose media (induced) in untreated condition. The two control strains are TDP43-GFP in uninduced condition (in dextrose media) and WTGFP, where GFP is constitutively expressed.

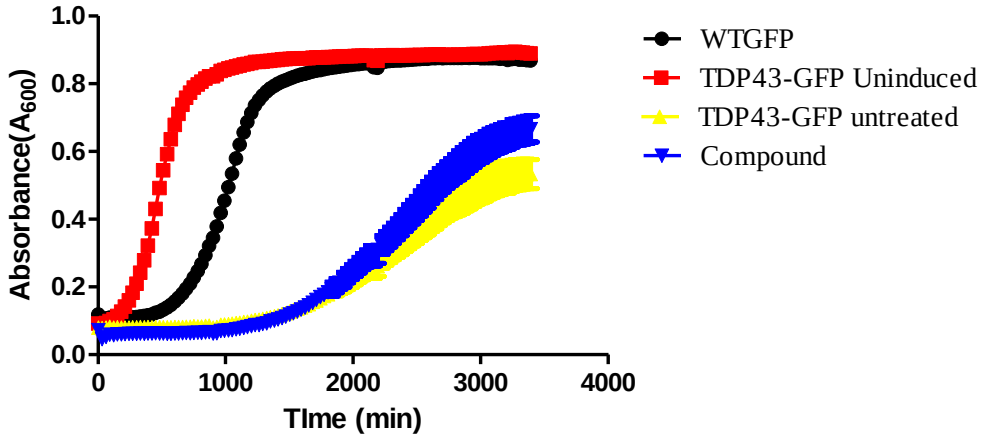
The above plots showed the compound set which were screened against the yeast proteotoxic model of TDP43-GFP. None of the compounds succeeded to cross the statistical 3 SD (standard deviation) unit which implied that the compounds failed to rescue the growth lag observed in the proteotoxic strain. However, three compound treatments showed some promising result compared to TDP43-GFP strain in untreated condition. Those compounds were plotted separately to understand the effect.



(A)



(B)



(C)

Fig 3.8.5.2: Growth assay for the three different compounds against the TDP43-GFP protetotoxic strain

The experiment was carried down of a duration of 58 hours. At the later time-point the experimental strain did not show the growth lag, rather started showing the growth like the control strain. So, the effect of the compounds, whether it is rescuing the growth lag, is not very clear. But the compounds, to some extent, showed growth rescue which could be investigated further by conducting several assays.

Chapter 4: Conclusion and future directions

Chapter 4: Conclusion and future directions

TDP43 is a multifaceted protein governs many cellular processes like mRNA translation and takes part in splicing mechanism. It has distinct role in autophagy. It has been reported that knockdown of TDP43 mRNA can decrease the atg7 activity and vice versa. Hence it maintains a feedback loop and regulates autophagy. The overexpression of TDP43-GFP in yeast forms intracellular aggregates. The aggregates are localized mostly in the cytoplasm albeit the aggregates are found in the nucleus also. The mislocalized aggregates render the toxicity in two possible way (*Lee et al, 2012*)-

1) Loss of TDP43 normal function- TDP43 normally is localized inside the nucleus where it plays crucial role in mRNA translation and splicing. Also, it interacts with two important proteins, Raptor and Dynactin-1 to maintain the autophagic regulation. Whenever it is mislocalized in the cytoplasm, the normal function of TDP43 is compromised. Hence, there is dysregulation of autophagy which renders toxicity. In consequence, the yeast cells growth is attenuated.
2) Gain of toxic function of TDP43- TDP43, whenever mislocalized, becomes aggregate prone. These aggregates are toxic in nature and affect many cellular processes in consequence.

Xia et al, in 2016, reported that the TDP43 downregulation in HeLa or SH-SY5Y cell line promotes nuclear translocation of Transcription Factor B or TFEB, a master transcription factor, which plays key role in regulating the expression of gene controlling lysosome biogenesis and autophagy induction (*Napalitano and Ballabio, 2016*). TDP43 controls the level of Raptor, which is required to governing the activity of mTOR by stabilising its mRNA. Thus, silencing TDP43 inactivates mTOR by decreasing Raptor levels (*Xia et al, 2016*). Phosphorylation of TFEB by mTOR is important to maintain the cytoplasmic localisation of TFEB. Due to downregulation of TDP43 decreases mTOR dependent phosphorylation of TFEB and induces the translocation of TFEB in the nucleus. This augmented nuclear localisation of TFEB triggers increase in gene expression of *lamp1, lamp2, atg5, beclin-1* and other autophagy related genes. In consequence, LC3II, autophagosomes, lysosomes are also accumulated. But in contrast, downregulation of TFEB attenuates Dynactin-1 level (*Xia et al, 2016*), which is important in autophagosome-lysosome fusion. In summary, down-regulation

of TDP43 facilitates autophagy through TFEB translocation in nucleus but decrease in activity of Dynactin-1 impairs the progression of autophagy flux in consequence.

In our result, we showed, due to overexpression of TDP43 protein, it is predominantly present in the cytoplasm and forms aggregates. These protein aggregates do not undergo complete vacuolar degradation even in induced autophagy condition for 12 hours. In case of, Δ*atg1*TDP43-GFP strain, the aggregates were localized throughout the cytoplasm. These protein aggregates render toxicity to the cell. The read-out of the toxic effect is abrupt decline in the growth of yeast cells. Thus, the model can be considered a standard model system to study the proteotoxicity in the cell. Therefore, we used the model system to screen a small molecular library with the aim of finding a novel molecular player linked to TDP43 in ALS.

Since the compound library was very small and we did not get any significant hit from the screen analysis, a large library of compound need to be screened in next level. The compounds which will be considered HIT, undergo GFP-Atg8 processing assay, LC3 maturation assay, RFP-GFP tandem assay etc to understand the effect of autophagy modulation. Also, the mammalian cell-line model harbouring the TDP43 plasmid along with its endogenous expression will help to shed light on the mechanism of proteotoxicity in higher order organism TDP43 inclusions are found in other neurodegenerative diseases like Fronto temporal lobar dementia (FTLD) and Alzheimer's disease (Tau-pathy). The reason behind the intersection of these protein aggregates is yet to dissect out. So, it will be interesting to dig out the underpinning mechanism of forming the inclusion bodies. Thereafter, drug combating TDP43 pathology is a major challenge. In this case, the compound library screen can usher a new sight to the therapeutic intervention to TDP43 linked protetotoxicity.

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