Studies on the role of Rab-GTPase Ypt1p in the nuclear mRNA surveillance in *Saccharomyces cerevisiae*

A Thesis submitted for the degree of

Doctor of Philosophy

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by

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Under the Guidance of

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Studies on the role of Rab-GTPase Ypt1p in the nuclear mRNA surveillance in *Saccharomyces cerevisiae*"** submitted by **Sri. Sunirmal Paira,** who got his name registered on 21st July, 2017 for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon his own work under my supervision and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Studies on the role of Rab-GTPase Ypt1p in the nuclear mRNA surveillance in *Saccharomyces cerevisiae*"** is entirely original and was carried out by me under the direct supervision of **Prof. Biswadip Das**, Department of Life science and Biotechnology, Jadavpur University, Kolkata, India.

I further declare that the contents of this thesis have not been the basis for the award of any degree, diploma, fellowship, associateship or any other similar title of any university or Institution.

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"Success is a Journey, not a destination. The doing is often more important than the outcome."

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ABSTRACT

Nuclear degradation of pre-*HAC1* mRNA and subsequent targeting of the resulting precursor mRNA plays a vital role in the activation as well as attenuation of Unfolded Protein Response (UPR) in *Saccharomyces cerevisiae*. Unfolded Protein Response (UPR) is an intracellular signalling pathway that responds to stress causing a burden of unfolded proteins (ER stress) in the ER lumen. To mitigate this situation, baker's yeast *Saccharomyces cerevisiae* produce a huge burst of Hac1p, a transcription factor that activates genes encoding ER chaperones. Hac1p is encoded by *HAC1* pre-mRNA harboring an intron and a bipartite element (BE) at its 3′-UTR, which undergoes a reversible and dynamic intra-nuclear 3′→5′ mRNA decay by the nuclear exosome/CTEXT at various phases of UPR. Accelerated decay, in absence of stress leads to a pre-*HAC1* mRNA pool, the majority of which lacking the BE, thereby undergoing inefficient targeting to Ire1p foci consequent to diminished splicing and lower production of mature Hac1p. ER-stress leads to its diminished decay, producing an increased abundance of pre-*HAC1* mRNA population carrying an intact BE leading to its accelerated recruitment to Ire1p foci followed by increased splicing and increased production of mature Hac1p.

Rab-GTPaseYpt1p, which was previously implicated in the ER to Golgi shuttling of proteins was also demonstrated to be involved in the regulation of UPR signalling dynamics by promoting the decay of *HAC1 m*RNA. This finding suggests a potential involvement of Ypt1p in the regulatory mechanism for selection and recognition of the *HAC1* pre-mRNA by the nuclear exosome/CTEXT. In this study we show that physical association (as detected by RNA-immunoprecipitation) of Rab-GTPase Ypt1p with *HAC1*-pre mRNA facilitates the recruitment (as detected by ChIP) of NNS/CTEXT/nuclear exosome on to the pre-*HAC1* mRNA and stimulates its nuclear decay in absence of stress. In presence of stress, reduced amount of Ypt1p becomes associated with the pre-*HAC1* thereby lowering the recruitment of exosome/CTEXT/NNS (as detected by Co-immunoprecipitation) on to it, which leads to the production of huge amounts of pre-*HAC1* mRNA with an intact BE. Regulation by Ypt1p relies on its characteristic nuclear localization in absence of ER-stress causing its strong association with pre-*HAC1* mRNA at its 3′-UTR that promotes a sequential recruitment of Nrd1-Nab3p-Sen1p (NNS) complex → CTEXT → nuclear exosome onto the pre-*HAC1* mRNA that eventually stimulates its selective nuclear decay. During induction of UPR, Ypt1p rapidly relocalizes to the cytoplasm with its consequent dissociation from pre-*HAC1* mRNA thereby causing a decreased recruitment of NNS/CTEXT/Nuclear exosome with its consequent diminished 3′→5′ degradation by the exosome. This reversible mechanism ensures a timely activation of UPR and its prompt attenuation following the accomplishment of homeostasis. These findings will help to gain an insight into the mechanism of intra-cellular signalling pathway UPR that is accomplished by differential degradation at the post-transcriptional level.

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CHAPTER 1

Introduction

1.0. INTRODUCTION

Efficient and productive translation of the genetic information into proteins requires fail-safe biogenesis of mRNA(Fujiwara et al., 2012).To achieve a fail-safe nuclear mRNA biogenesis, genetic information has to undergo various processing events in the nucleus. These events start with transcription through capping of the nascent message at 5′-end, splicing to remove introns, and a maturation at 3′-end of mRNA involving a site specific-cleavage and polyadenylation(Satarupa Das & Das, 2013; Nick J Proudfoot et al., 2002)(Fig. 1.1) followed by their export to the cytoplasm. All protein-encoding genes are transcribed by the 12-subunit DNA dependent RNA polymerase II (RNAPII). Most of the pre-mRNA processing events and nuclear export of mRNA from nucleus to cytoplasm are extensively coupled to transcription(Köhler & Hurt, 2007), which is partly mediated by phosphorylation status of specific serine residues in the C-terminal domain of Rpb1p subunit of RNA polymerase II enzyme. It was proposed that functional coupling between the transcription and a given biogenesis event minimizes the possibility of formation of inappropriately processed transcripts and enhances the efficiency and likelihood of production of export competent productive mRNPs (Aguilera, 2005; Bentley, 2005; Bird et al., 2004; Reed & Cheng, 2005). Despite tight coupling, however, improperly processed transcripts/faulty messages still arise, which are selectively targeted, degraded and rapidly eliminated by various mRNA quality control and surveillance mechanisms (Giordano Caponigro & Parker, 1996; *Differential Effects of Translational Inhibition in Cis and in Trans on the Decay of the Unstable Yeast MFA2 MRNA - PubMed*, n.d.; McCarthy, 1998b). In addition to these nuclear events, heterogeneous ribonucleoproteins (hnRNPs) and various maturing factors take part in mRNA processing, export and degradation. As of now, more than thirty hnRNPs in humans and ten in baker's yeast *Saccharomyces cerevisiae*were identified(Dreyfuss et al., 2002), all of which

bind to growing and transcribing mRNAs in a transcription independent manner. The association between hnRNPs and maturing transcripts determinates the fate of an mRNA during the nuclear events. The export competent mRNAs then undergo nuclear export, arrive in the cytoplasm followed by their translation and degradation. There is also a surveillance mechanism (Fig.1.1) present for the improperly-processed mRNAs in the cytoplasm.

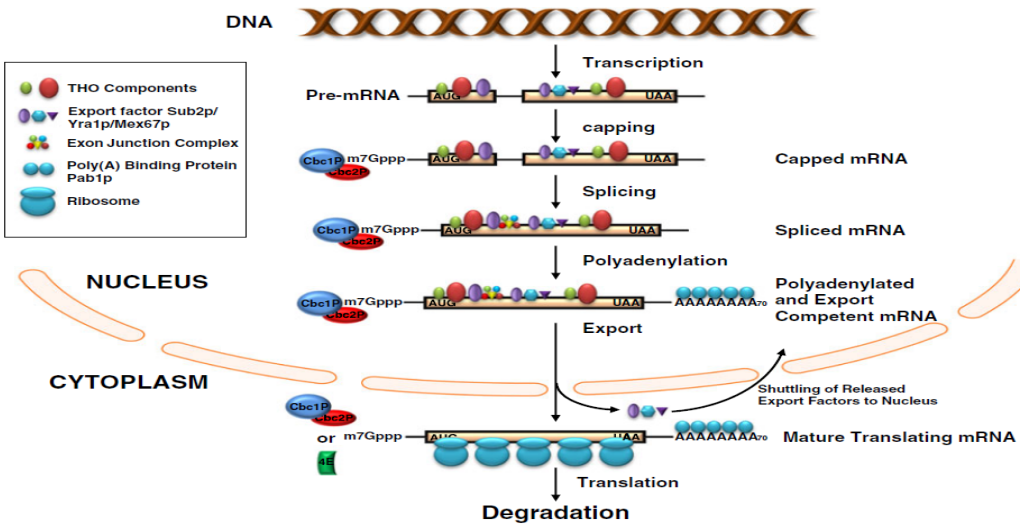

Figure1.1.Nuclear Routes of Processing and Turnover for RNA Pol II Transcripts and mRNA life-cycle in eukaryotic cell. *Nuclear processing of Pol II transcripts includes capping, excision of introns, and addition of a polyA tail. Schematic view of the nuclear and cytoplasmic phases of mRNA life cycle, namely transcription, capping, splicing, polyadenylation, nuclear export, translation and finally degradation in cytoplasm. Various mRNA binding proteins, presented as coloured symbolson the transcript body, are deposited onto/remain associated with maturing transcripts during different stages of nuclear phase of life. Each symbol is either annotated directly in the diagram or denoted in the legend box. Note that THO components/maturing factors/mRNA-binding proteins are released from mRNA once the mRNA matures and become export competent. Similarly, export factors are also released from transcript body once mRNA arrives at the cytoplasm and finally shuttle back nucleus. In the cytoplasm, messenger RNAs may remain associated either with nuclear CBC (while undergoing pioneer round of translation) or with eIF4E (while undergoing subsequent steady state translation) which is indicated in the cytoplasmic phase of the diagram. Also for simplicity, other mRNA binding proteins which remain associated to translating mRNAs in the cytoplasm are not shown except for cap-binding complex (CBC), and translation initiation factor 4E. AUG and UAA indicate the beginning and end of the open reading frame (ORF) carried by the message. All vertical downward arrowheads represent various biogenesis steps as mentioned in the figure.****Adapted fromDas& Das, 2013).***

1.1. The Eukaryotic transcription machinery and RNA polymerase:

The eukaryotic transcription machinery is more complex than prokaryotes and it relies mainly upon three different RNA polymerases, RNA polymerase I, II and III.RNA polymeraseII (RNAPII)is present in the nucleoplasm and it is responsible for transcription of protein coding genes, mRNA processing genes, snRNAs (small nuclear RNAs), rRNA processing

genes, snoRNAs (small nucleolar RNAs) and micro RNAs, and functions in mRNA silencing(Pikaard et al., 2008; WILLIS, 1993; Zhang et al., 2007). Another two polymerases of Eukaryotic transcription machinery are RNA polymeraseI, found in the nucleolus and polymeraseIII, present in the nucleoplasm. RNA polymeraseIII synthesizes 5S rRNA, transfer RNAs (tRNAs) and some snRNAs. RNA polymeraseI transcribes various large ribosomal RNA precursor genes (28S, 18S and 5.8S rRNAs)(Drygin et al., 2010; WILLIS, 1993).The RNAPII consists of 12 subunits, named Rpb1-Rpb12, most of the genes encoding various subunits of RNA polymerase II (RNAPII) are essential for transcription and cell viability. The two largest subunits of RNAPII, Rpb1(~200 kDa) and Rpb2(~150 kDa) are homologues to β′ and β subunits of the bacterial RNA polymerase respectively(Young, 2003). The subunits of RNAPII are categorized into three domains: the core domain(Rpb1, 2, 3 and 11), which has homologous counterpart in prokaryotic RNA polymerase, catalytic domain (Rpb5, 6, 8, 10 and 12), which are common to all three polymerases and subunits (Rpb4,7 and 9) which are specific to RNAPII but are not essential for transcription elongation(Hahn, 2004).The largest subunit (Rpb1) of RNAPII has a C-terminal domain, which extends as a tail. This CTD is unique, highly flexible and it contains series of repeats of the heptapeptide sequence: Tyr-Ser-Pro-Thr-Ser-Pro-Ser(Fig. 1.2)(Gnatt et al., 2001).The number of repeats is species specific, for example in *Saccharomyces cerevisiae*it ranges from 26-27, in *Drosophila melanogaster*it exists as 43 repeats, in *Caenorhabdite elegans* it exists as 34 repeats and in humans it is 52 repeats (Fig. 1.2)suggesting that with the increasing complexity of genomes the length of the repeat increases(Hampsey, 1998).

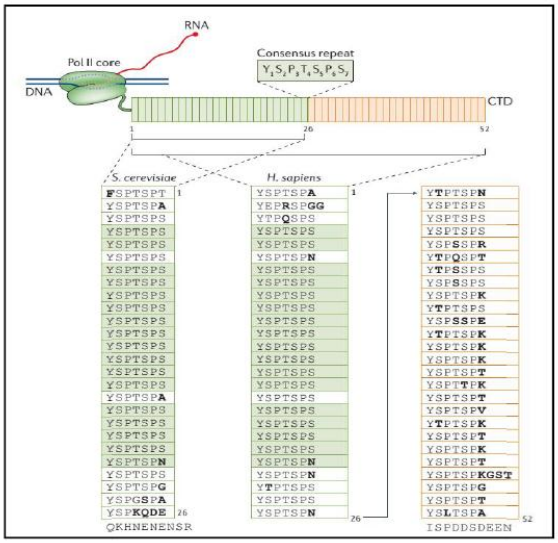

Figure 1.2: The conservation of the CTD of RNAPII: *The peptide sequences in the core and the carboxy-terminal domain (CTD) of saccharomyces cerevisiae and human RNA polymerase II are shown. Each rectangle represents one CTD repeat and the repeat sequence consensus is shown above. The yeast CTD is composed of 26 mostly consensus repeats, whereas the human CTD is composed of 52 repeats, the first 26 being highly similar to the yeast CTD whereas the remaining 26 are more divergent from the consensus sequence. Identical repeat sequences between yeast and human CTDs are highlighted in green; note the high degree of conservation between S. cerevisiaeand the first half of the human CTD.((**Adapted from Harlen & Churchman, 2017)***

Recruitment of various factors including promoters and mediators during various stages of transcription relies on the nature of post-translational modifications of the CTD heptad repeats. Among various post-translational CTD modifications, phosphorylation of specific serine residues is a crucial in pre-mRNA maturation. The CTD of RNAPII acts as a loading platform for several transcription and mRNA processing events. RNAPII mainly exists in two different forms: phosphorylated and non-phosphorylated, dubbed RNAPIIO and RNAPIIA respectively. The elongation of transcription is initiated by the phosphorylation of Ser5 of the CTD by TFIIH. The phosphorylation of Ser5 dissociates the mediator complex from RNAPII (Fig. 1.3). After mediator complex is released, the newly phosphorylated Ser5 recruits several enzymes to the 5′-end of the newly synthesized RNA to facilitate 5′-cap formation and a number of processing factors to the 3′-end of the transcript, specifically to the poly(A) sites followed by promoter clearance(Søgaard & Svejstrup, 2007). The level of Ser5P peaks

around the transcription start site (TSS) near the promoter and a lower level of Ser5P is maintained throughout the transcription elongation process.Ser2 of the heptapeptide is phosphorylated by Cdk9 (CTDK1), subunit of the positive transcription elongation factor b (P-TEFb). Increased level of Ser2P leads to the transcription of the whole gene. At the 3′-end of the gene, protein phosphatase (PPase) removes Ser5P and Ser2P dominates towards the end of the transcription termination (Fig. 1.3). While RNA polymerase II reaches at the end of the transcription termination site Ser2P undergoes dephosphorylation and this dephosphorylation of Ser2P facilitates RNA pol II (Fig. 1.3)to reinitiate transcription(Bowman & Kelly, 2014; Chapman et al., 2008; Egloff & Murphy, 2008a). The eukaryotic transcription process is divided into three phases, initiation, elongation and termination.

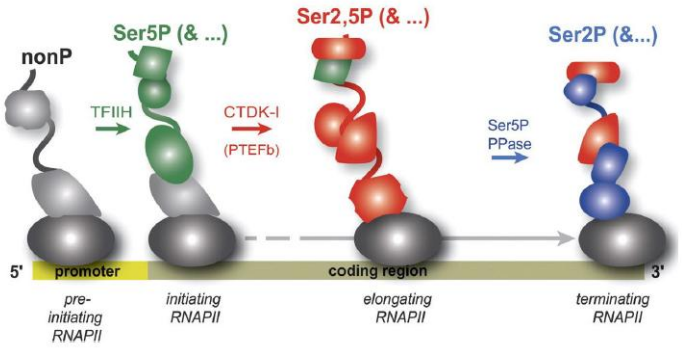

Figure 1.3: Phosphorylation pattern of CTD of RNA pol II during various process of transcription. *CTD phosphorylation patterns dictate which factors associate with RNAPII. RNAPII (gray oval) is depicted at four positions along a gene, and at each position its CTD (wavy line) is presented in a different color to indicate different phosphorylation states: Gray indicates non-phosphorylated repeats; green indicates Ser5P repeats; red indicates Ser2P, 5P repeats (doubly phosphorylated); and blue indicates Ser2P repeats. Proteins bound to a type of repeat are indicated in the same color as the repeat.****(Adapted fromPhatnani & Greenleaf, 2006)***

1.1.1 The Initiation ofEukaryotic Transcription:

The initiation of RNAPII mediated gene transcription starts with recruitment of RNAPII and the recognition of promoter by a series of accessory and transcription factors, known as

general transcription factors (GTF).The transcriptional co-factors like co-activators/co-repressors do not bind to promoter regions constitutively but rather act at promoters of specific subset of genes.There are two types of transcriptional cofactors: onetype of cofactors modify the histones or remodel the chromatin in an ATP-dependent manner(Sterner et al., 1999) and second type of cofactors (mediator complex) function mainly to activate or recruit GTFs and other key protein complex (Conaway & Conaway, 2011).Transcription factors are generally of two types: basal or general transcription factors (TFIID, TFIIA etc.), which attach to gene promoter and cell specific regulatory transcription factors, which bind to regulatory sequences like silencer and enhancers. The transcription start site is determined by general transcription factors. TFIID is a complex made up of the TATA-Binding Protein (TBP) and 12 TBP associated factors. After TFIID has attached to the core promoter, pre-initiation complex (PIC) is formed by the attachment of the remaining general transcription factors. These GTFs bind to the complex in the order TFIIA, TFIIB, TFIIF/RNAPII, TFIIE and TFIIH. After pre-initiation complex formation, TFIIH having a DNA helicase activity melts the DNA to form the open complex (OC). In the presence of ATP, TFIIH phosphorylates CTD of RNAPII to initiate transcription. Once the first phosphodiester bond is formed, RNAPII is released from the promoter to facilitate downstream transcription.

1.1.2 The Eukaryotic Transcription Elongation:

Recent genome wide studies suggest that a number of developmental genes contain stalled RNAPII at their promoter proximal position of the genes(about 20-50 nucleotide downstream of TSS)(Aida et al., 2006; Barboric & Matija Peterlin, 2005; Saunders et al., 2006; Sims et al., 2004). Negative elongation factors initiate this stalling of RNAPII in promoter proximal position while the positive elongation factors help in rescuing RNAPII from the paused state. This unique type of stalling mechanism minimizes the necessity in recruitment of RNAPII in

the promoter region. This type of novel RNAPII stalling is increased at highly regulated genes during development of embryo (Muse et al., 2007; Zeitlinger et al., 2007). Once the mature RNAPII is committed to productive elongation, the stable RNAPII complex transcribes hundreds of kb of DNA without dissociating from the template(J. Singh & Padgett, 2009). As the transcription elongation goes on, the CTD of RNAPII recruits and deposits various types of enzymes and proteins like THO proteins, capping enzymes, splicing factors, mRNP proteins and 3′-end processing factors etc. at various segments of nascent transcript as they undergo transcription elongation (McCracken et al., 1997).

1.1.3 The Eukaryotic Transcription Termination:

Transcription termination is very crucial step for both, the release of nascent transcript as well as release of RNAPII from the transcription site and also the recycling of RNA pol II for re-initiation(Logan et al., 1987). Two types of transcription termination model are established. The first model known as ‘allosteric’ model proposes that during 3′-end polyadenylation RNAPII is stalled and this stalling of RNAPII causes conformational change in itself, which leads RNAPII to lose its processivity and dissociate from the template(Calvo & Manley, 2001; Logan et al., 1987).The second model known as ‘torpedo’ model, which proposes that a 5′→3′ exonuclease Rat1p(Xrn2p) is involved in the release of RNAPII from DNA template(Connelly & Manley, 1988). The functional interaction between the functional poly(A) site and some essential subunits of cleavage factors lead to couple the 3′-end processing and transcription termination(Gross & Moore, 2001; Zaret & Sherman, 1982).

1.2 Post-transcriptional mRNA modification events:

To become a mature export competent mRNP, the nascent pre-mRNA needs to undergo three major extensive modifications like 5′-end capping, splicing and polyadenylation. Most of

these modifications take place co-transcriptionally(Egloff & Murphy, 2008b; Fuda et al., 2009; Kim et al., 2009; Koch et al., 2008). If any of these mRNA modification events fails to complete successfully, the mRNA is not exported to the cytoplasm and is degraded within the nucleus. Proper processing events serve as triggers to recruit appropriate protein factors, which are essential for the mRNA export. Depending on the phosphorylation status of the CTD of RNAPII, all of these modifications are coupled to transcription process and this acts as a bridge to coordinate the protein factors that are involved in maturation of an export competent mRNA(Nick J Proudfoot et al., 2002).

1.2.1. Cappping:

Capping at the 5′-end of the nascent mRNA constitutes one of the earliest mRNA processing event in the life of eukaryotic mRNA which involves methylation at 7^{th} position to form 7-methylguanosine cap through a 5′-5′ triphosphate bridge. A dimeric capping enzyme catalyzes the first step of RNA capping and associates with the CTD of RNAPII (Hirose & Manley, 2000). One subunit of this dimeric capping enzyme removes the γ-phosphate from the 5′-end of the nascent mRNA emerging from the surface of an RNAPII. The other subunit of capping enzyme transfers the GMP moiety from GTP to the 5′-diphosphate of the nascent mRNA, hence forming the guanosine 5′-5′triphosphate structure. Finally, a separate enzyme transfers a methyl group from S-adenosylmethionine(SAM) to the N^7 position of guanine at the 5′-end, then it is called**cap0**. In yeast, mRNAs contain mainly cap0 but it may vary in many higher organisms. The ribose of the adjacent nucleotide can also be methylated to give **cap1** structure. Methylations at the second and third nucleoside along with the methyl group of the RNA molecule produce cap2, cap3 structures and so on (Furuichi, 2014; Furuichi & Shatkin, 2000). There are so many significance and implications of 5′-cap structure, like

protection from degradation, transport of mRNA from nucleus to cytoplasm. As soon as 5′cap structure formed, it is associated with a heterodimeric cap binding complex, consisting of a 80 kDa large subunit CBP80 (Cbc1p in yeast) and a 20 kDa (Cbc2p in yeast) small subunit CBP20. This CBC structure protects mRNAfrom 5′-3′ exoribonuclease activity. In addition, this cap structure plays an important role both in the splicing of cap-proximal intron, 3′-end processing and nuclear export and the mRNA quality control(N J Proudfoot et al., 2002). In the cytoplasm, it is also serving as the binding site for translation initiation factor eIF4E(Lewis & Izaurralde, 1997).

1.2.2 Splicing:

The next co-transcriptional modification is removal of introns right after the capping of mRNA. In lower eukaryotes like *S.cerevisiae*, only 5% of genes contain introns while in higher eukaryotes like metazoans, and mammals multiple introns are variably interspersed between coding sequences. Spliceosome, a complex of small ribonuclear protein (snRNP) catalyses splicing of introns in a series of reaction.The pre-mRNA contains various *cis*-acting elements such as 5′-splice site (donor site), the branch point(near the 3′-end of the intron), the polypyrimidine tract and the 3′-splice site(acceptor site), which are crucial for splicing reaction.The Spliceosome is a large RNA-Protein complex composed of five small ribonuclear proteins: U1, U2, U4, U5 and U6. Interaction between these RNA components and various *cis* acting elements plays a vital role in their precise recognition. For the assembly and execution of the spliceosome, a number of proteins including U2AF and SF1 are required. In addition to this, other auxiliary sequences within the pre-mRNA body like a sequence within an exon, called Exonic Splicing Enhancer(ESE) helps in deposition and assembly of Spliceosome (Cartegni et al., 2002; Cramer et al., 1999; H. X. Liu et al., 1998;

Sun et al., 1993).The major spliceosome splices out two types of introns containing GU at the 5′-splice site and AG at the 3′-splice site. In the first step of two trans-esterification reactions, the U1 snRNA pairs with the GU sequence at the 5′-splice site by RNA-RNA base pairing. At the same time, ASF/SF2, U2AF and SF1 proteins bind to the polypyrimidine tract and the branch point site respectively. These associations of splicing factors and other accessory factors initiate the splicing by constituting commitment complex(E-complex).The association of U2-snRNPs with the branch site converts the E-complex to the pre-spliceosomal complex(A-complex)(Herzel et al., 2017). At this stage, the 5′-splice site comes in close proximity to the branch point through the association between U2-snRNP and U1-snRNP. Next, attachment of U5-snRNP and U4/U6-snRNP with the A-complex converts pre-spliceosomal complex to the B1-complex. After that, U1-snRNP is released from B1-complex, B2-complex is formed by release of U4 and it requires hydrolysis of ATP. Dissociation of U1 and U4 from U6-snRNP of B2-complex facilitates U6-snRNP to freely pair with the U2-snRNP forming the active site(C-complex). This U6/U2 of the C-complex catalyse the trans-esterification reaction by bringing the 5′-splice site, 3′-splice site and the branch point in close proximity and leading to the formation of a lariat structure. At the next step, 3′-splice site and 5′-splice sites are cleaved and exons are ligated by hydrolysis of ATP. It is believed that, lariat formation and debranching of lariat structure is mediated by U6-snRNP.

1.2.3 The 3′-end formation:

The final stage of the pre-mRNA processing event is consisting of a 3′-end cleavage and template-independent polyadenylation, which together prompts a pre-mRNA to undergo export and translation.With the exception of replication-dependent histone genes, virtually all

eukaryotic protein coding mRNAs harbour a series of adenosine residuesat their 3′-end termini(approx. 60-90 residues in yeast and 250-500 residues in mammals) to form a poly (A) tail(Nick J Proudfoot et al., 2002). The addition of poly (A) tail to the nascent mRNA is mediated by template independent RNA polymerase, poly (A) polymerase, Pap1p. This polymerase acts at an internal site, which is created from the cleavage of the nascent pre-mRNA to which the poly (A) tail is added. A single processing complex undertakes both the cleavage and polyadenyation. A *cis*-acting signal sequence, 5′-AAUAAA-3′in the mRNA directs the cleavage and polyadenylation reaction, which is located between 10 and 30 nucleotides upstream of the polyadenylation site. Very often, the dinucleotides 5′-CA-3′is immediately followed by the site of cleavage that in turn is followed 10 to 20 nucleotides later by a GU-rich region. The multi-subunit protein complex CPSF and CstF binds to the poly (A) signal sequence and the GU-rich region respectively. The binding of CPSF to the poly(A) signal sequence arrests RNAPII transcription(Glover-Cutter et al., 2008) followed by binding of CstF to the GU-rich region(Nag et al., 2007; Park et al., 2004). The binding of CstF to the nascent mRNA induces the release of stalled RNAPII and the CPSF mediated cleavage. The endonuclease activity of CPSF (consisting of the cleavage factors CFI and CFII) generates the proper 3′-terminal structure.Cleavage is followed by polyadenylation in a tightly coupled manner.The efficiency of 3′-end polyadenylation is closely influenced by the phosphorylation status of RNAPII CTD. Moreover, CPSF is physically associated with the elongation complex by interaction with the RNAPII body and CstF is functionally linked with CTD.

1.2.4 The Nucleocytoplasmic Export of mRNA:

Once all the covalent modification events take place properly, the correctly processed export competent mRNPs follow their transport through the nuclear pore complex (NPC).The transport receptor recognizes export-competent mRNAs and associated protein molecules to facilitate their export out of the nucleus. The transcribing and maturing mRNAsare loaded with mRNP assembly factors co-transcriptionally, including THO complex, mRNA export factors TREX (TRansciption-Export) complex, like Sub2p, RNA binding protein Yra1, the mRNA export receptors Mex67p-Mtr2p for proper packaging and export of mRNA(Kohler & Hurt, 2007; Luna et al., 2008). Importantly, TREX was reported to export spliced mRNAs more efficiently than unspliced mRNAs or mRNAs derived from cDNAs (Luo & Reed, 1999),which indicated that the transcription, splicing and nuclear export are strongly integrated. Interestingly, in yeast,the serine-arginine rich (SR) proteins Npl3,Gbp2 and Hrb1 play major roles in pre-mRNA splicing by the association of the latter two proteins with the TREX complex(Kohler & Hurt, 2007). Moreover, Gbp2p and Hrb1p, together with the mRNA binding protein, Nab2p constitute the second category of mRNA export adaptors that is typically accomplished by their interactionwith Mex67-Mtr2receptors(Gilbert & Guthrie, 2004; Iglesias et al., 2010; Strasser et al., 2002).The nascent mRNAs, synthesized by RNAPII are folded co-transcriptionally and assembled into pre-mRNP complex, when they are bound by THO/TREX complex co-transcriptionally. In the first step, the THO/TREX complex is bound to the elongating RNAPII. Next,the cap binding protein (CBC) and several RNA binding proteins are loaded and recruited on to the nascent transcript followed by recruitment of Mex67-Mtr2 mRNA export receptor (Fig.1.4) on to the maturing mRNP via interaction with the Yra1(Sträßer & Hurt, 2001).The Sub2 and THO proteins are released from the mRNA body when the export receptor binds to the mRNP. For export of an mRNA from the

nucleus, it has to be licensed by export factors and nuclear processing events. Improperly processed mRNAs are retained in the nucleus and it is recognized by nuclear mRNA surveillance mechanism, hence it's degraded by nuclear exosome(Brodsky & Silver, 2000; B. Das et al., 2000; Libri et al., 2002; Zenklusen et al., 2002). Previous studies revealed that the nuclear transport of mRNA is directional because a fully maturated mRNA is exported and docked first to the nuclear face in the 5′ to 3′ direction (Visa et al., 1996).In *Saccharomyces Cerevisiae*, two conserved mRNA export factors Dbp5(ATP dependent DEAD-box RNA helicases), Gle1(activator of Dbp5) and IP6 are important for maintaining directionality of cytoplasmic release(Alcázar-Román et al., 2006).There are two forms of Dbp5, an ATP-bound closed form and an ADP-bound open form. The ATP-bound closed form makes the sandwich of RNA cargo whereas the open form of Dbp5 releases the RNA from its bound state (Ledoux & Guthrie, 2011).The cytoplasmic nucleoporin components Nup42 and Nup159 maintains the Gle1 distribution in more concentrated form at the cytoplasmic face of Nuclear Pore Complex(NPC) and this skewed distribution of Gle1p is crucial for maintaining the directionality of mRNA transport. Enhanced ATPase activity of Dbp5 removes Mex67-Mtr2 from mRNA as well as remodels the protein profile of the exporting mRNAs. This new protein profile of the mRNP prevents the mRNA re-entry into the nucleus following the same route. Recruitment of Dbp5 onto mRNA takes place at an early stage in the transcription process(Estruch & Cole, 2003)and Mex67-Mtr2 is removed from mRNA by Dbp5 only after the transit through the Nuclear Pore Complex(NPC), but not in the Nucleus.

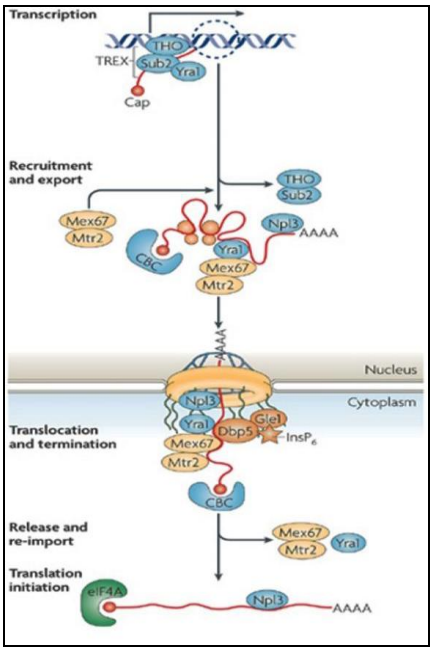

Fig1.4: The mRNA export mechanism in Yeast(Köhler & Hurt, 2007)**.**

1.3 Translation of mRNA:

Once in the cytoplasm, the most obvious destiny of mature exported mRNP is to be engaged into translation, which is the most complicated among all the processes involved in genetic information transfer from genes to proteins. Broadly, translation of an mRNA into protein occurs in three successive steps, initiation, elongation, termination, and ribosome recycling. Translation initiation is the most complex as it requires the largest number of protein factors. During initiation, the small ribosomal subunit (40S) and eukaryotic initiation factors (eIFs) and methionyl tRNA, together form a complex onto the mRNA.This complex scans the5′-UTR of mRNA and subsequently recognizes the initiation codon. During elongation, decoding takes place when the amino-acyl-tRNAs enter the A (acceptor) site of the translating ribosomes. The ribosome catalyses the peptide bond formation, if they are correct cognate tRNA. During the transpeptidation, the peptidyl group on the P site of tRNA is

transferred to the aminoacyl group in the A site through the peptide bond formation. During translocation, A site and P site tRNAs are transferred to the P site and E site respectively accompanied by their bound mRNA. Protein synthesis ends with the termination step, when one of the three stop codons (UAG, UAA and UGA) is encountered by the elongation complex and the finished peptide is released from the ribosome. In the final stage, the 80S ribosome is disassembled and released from the mRNA and dissociates into its subunits, which are recycled thereby setting the stage for another round of polypeptide synthesis (Kapp & Lorsch, 2004).

1.4 mRNA turnover:

Notably, the intracellular concentration of mRNA is regulated not only by the rate of changes in the synthesis of nascent mRNAs but also by the changes in the rate of decay of mRNA (Tavazoie et al., 1999). Once the pre-requisite rounds of translations are over, every mRNA has to undergo degradation by the default decay pathway. Like other cellular processes, the mRNA decay and turnover takes place in a much regulated and finely controlled fashion with the assistance of a variety of RNA degradation systems. Messenger RNAs encoding transcriptional machinery and transcription factors(Y. Wang et al., 2002) are quickly turned over suggesting that mRNA degradation may play a major role in the rapid regulation of these central gene expression processes(G Caponigro & Parker, 1996b, 1996a; McCarthy, 1998a). The decay of mRNA has four distinct functions. First, the turnover maintains a basal level of cellular RNA expression. Second, it eliminates aberrant mRNAs via their selective decay which may lead to the accumulation of harmful polypeptides (Doma & Parker, 2007; Fasken & Corbett, n.d.; J Houseley et al., 2006; Parker, 2012). Third, it removes the byproducts of gene expressions such as excised introns, external or internal spacers, etc. Fourth, degradation of intra and intergenic promoter associated anti-sense RNAs which are

considered as transcriptional noise(Françoise Wyers et al., 2005). Broadly mRNA degradation systems are divided into two forms (a) The general default degradation pathways and (b) the specialized mRNA degradation pathways.

1.4.1 General default degradation pathways:

The default decay pathway starts with the process of deadenylation and this progressive deadenylation rates are specific for each mRNA species. This step provides a timing mechanism that confers that each mRNA species have a defined life-time rather than a stochastic half-life. Eventually all mRNAs are targeted for degradation at a specified cytoplasmic location, known as mRNA processing bodies or P-bodies in *S.cerevisiae* (Sheth & Parker, 2006). The process of deadenylation,(Fig.1.5) involving the shortening of the poly (A) tail is usually carried out by two interrelated protein complexes *S. Cerevisiae,* Ccr4p/Pop2p/Not complex(the major complex) and Pan2p/Pan3p complex(the subsidiary complex)(Tucker et al., 2002). Not1p serves as a scaffold for the docking of additional subunits Ccr4p, Caf1/Pop2, Caf40, Not2 and Not3/5. The two subunits, Ccr4 and Caf1/Pop2 exert catalytic activity, both acting as 3′-5′ poly(A)-specific exoribonucleases (Wahle & Winkler, 2013). Deadenylation primarily leads to the decapping of the mRNA, which involves the removal of the 5′-methyl guanosine (Cap) structure by the Dcp1p-Dcp2p complex(Decker & Parker, 2012; Schwartz & Parker, 2000; She et al., 2004). The deadenylated and decapped transcript body is then degraded either in a 5′→3′ direction via the major pathway by cytoplasmic exoribonuclease Xrn1p or in 3′→5′direction via the minor pathway by cytoplasmic exosome and Ski complex(Doma & Parker, 2007; Poole & Stevens, 1995).

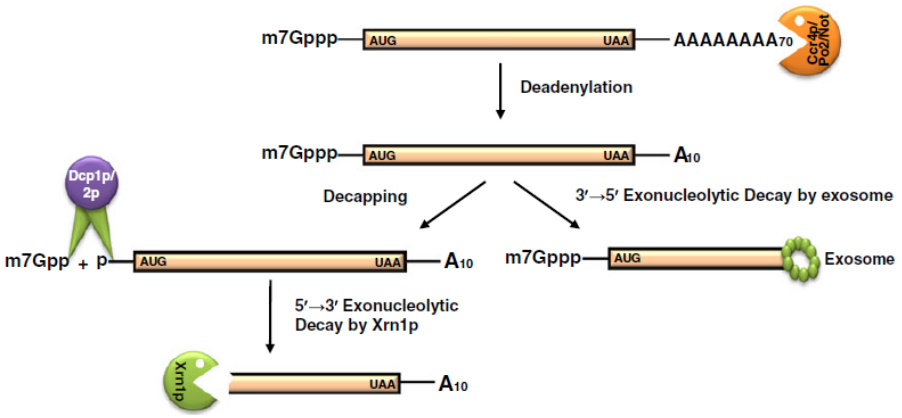

Figure.1.5: Default pathway of mRNA degradation in S. cerevisiae.*Almost all mRNAs undergo decay by the deadenylation dependent pathway. In this mechanism the poly (A) tail is gradually and progressively shortened by a deadenylase activity by Ccr4/Pop2/Not complex. Following deadenylation, two mechanisms can degrade the mRNA. The major mechanism follows through decapping by Dcp1p/2p followed by 5′→3' decay by Xrn1p. The minor mechanism involves 3′→5′ decay by the exosome. AUG and UAA indicate the beginning and end of the open reading frame (ORF) carried by the message. Only relevant decay components are shown by annotated symbols. Proteins which remain associated to translating/degrading mRNAs during different stages of decay are not shown.****(Adapted fromS Das & Das, 2013)***

1.4.2 Specialized mRNA degradation pathways:

Specialized mRNA degradation pathways are very selective mRNA quality control systems, which collectively contribute to the fidelity of gene expression by promptly eliminating the aberrant/defective mRNA species(Doma & Parker, 2007; Jonathan Houseley & Tollervey, 2009; Olaf Isken & Maquat, 2008). Cells are equipped with a wide spectrum of quality control mechanisms, which constantly control/regulatethe accumulation of defective/aberrantmRNAs generated during the transcriptional and post transcriptional processing events. These special type of ubiquitous machineries are called mRNA surveillance or mRNA quality control mechanisms (Bousquet-Antonelli et al., 2000; Burkard & Butler, 2000; B. Das et al., 2000; S Das & Das, 2013; O Isken & Maquat, 2007; Torchet et al., 2002).

1.4.3 The mRNA surveillance mechanism in the cytoplasm:

Currently at least three surveillance mechanisms are known to operate in the cytoplasm, (Jonathan Houseley & Tollervey, 2008; O Isken & Maquat, 2007). These are nonsense-mediated mRNA decay (NMD)(Culbertson & Neeno-Eckwall, 2005; Olaf Isken & Maquat, 2008), nonstop decay (NSD)(Vasudevan et al., 2002) and no-go decay (NGD)(Doma & Parker, 2007).

1.4.3.1. The nonsense-mediated mRNA decay (NMD)

This pathway selectively targets and degrades faulty messages that harbour in frame-premature non-sense codon (Losson & Lacroute, 1979). Interestingly, aberrant splicing that results in the emergence of a pre-mature translation termination codon (PTC) in all three reading frame within the transcript body contributes to generation of the majority of the PTC containing aberrant mRNAs (McGlincy & Smith, 2008). In *Saccharomyces*, NMD was originally discovered as a system which detects and degrades PTC containing mRNAs(Losson & Lacroute, 1979). Later studies revealed that, in addition to PTC containing mRNAs, NMD also targets or degrades a wide range of aberrant mRNAs like,(i) translation initiation defective aberrant mRNAs (Welch & Jacobson, 1999), (ii) splicing defective aberrant pre-mRNAs, which contains stop codon within the retaining intron in all three reading frames(He et al., 1993), (iii) Out of frame aberrant mRNA(Welch & Jacobson, 1999)and (iv) mRNAs with uORF(Gaba et al., 2005).The specificity of target mRNA substrate of NMD in yeast and mammal is entirely different that is NMD in mammalian cells targets only newly synthesized translating messages whereas NMD in yeast targets both the messages undergoing pioneer round of translation and the steady state translation(Gao et al., 2005).

In *Saccharomyces*, every mRNA is believed to be continuously monitored during translation for the presence of PTC by the translation apparatus to verify the termination codon is premature or natural. When the translation apparatus senses that the translation terminates due to presence of premature stop codon, the translation elongation complex disassembles and subsequently recruits Upf protein complexes consisting of Upf1p, Upf2p and Upf3p. The recruitment of Upf complexes onto the mRNA then promotes the decapping of the mRNA, mediated by Dcp1p and Dcp2p proteins in a deadenylation independent manner. This decapped transcripts are then degraded by major cytosolic exoribonuclease Xrn1p in 5′→3′ direction (Bond et al., 2001; Cao & Parker, 2003; O Isken & Maquat, 2007; Muhlrad & Parker, 1994). In addition, mRNAs may also be degraded by exosome-dependent mechanisms in 3′→5′ direction. More recent study suggests that the Upf-mediated targeting and subsequent degradation of the NMD substrate occurs in specific cytoplasmic foci which is called as P bodies (Sheth & Parker, 2006). But, how does translation machinery discriminates a premature stop codon (PTC) from natural stop codon? Recent research shows that the NMD is induced by the physical distance between the site of translation termination and the position of poly (A) in *Saccharomyces*. In case of a PTC containing mRNA, this distance was found to be abnormally long in comparison to a normal mRNA. This abnormally long distance is termed as ‘faux’ 3′-UTR and this ‘faux’ 3′-UTR recruits the NMD factors by a less understood mechanism(Amrani et al., 2004).

1.4.3.2: The Non-stop Decay Pathway

Non-stop decay (NSD) pathway selectively targets and degrades the mRNAs lacking a stop codon (O Isken & Maquat, 2007). Premature polyadenylation within the open reading frameis thought to be an important reason for the production of this type of aberrant mRNAs that lacks the stop codon.The faulty transcripts can be generated by the absence/lack of an in-

frame stop codon, which in turn causes translation to move along the poly (A) tail tillthe extreme 3′-termini. In addition to this, NSD also facilitates the release of the ribosome to protect the cells from the defective proteins. Recent evidences suggest that two distinct pathways of NSD function in concert. The most conserved pathways of NSD in yeast and mammalian cells utilizes the cytoplasmic exosome, the *SKI* complex (Ski2, Ski3 and Ski8), and the adaptor protein Ski7 (Van Hoof et al., 2002).It is believed that the C terminus of Ski7 (adaptor protein) binds to the empty A site of the ribosome releasing the ribosome. The cytosolic exosome and the associated *SKI* complex is recruited by Ski7 on to the defective transcript to trigger deadenylation and rapid degradation of the transcript in the 3′→5′direction (Van Hoof et al., 2002). In the absence of Ski7, a second pathway which mediates NSD in *S. cerevisiae*is operated in 5′→3′ direction(Inada & Aiba, 2005). Here, the translating ribosome removes the PABP, which is known to render those transcripts susceptible to decapping and reducing the translational efficiency.

1.4.3.3The No-go mRNA Decay(NGD)

The third cytoplasmic mRNA-surveillance mechanism is the no-go decay (NGD), which is recently discovered in yeast and is less understood. NGD pathway degrades those mRNAs, which are produced from aberrant alternative splicing with a strong secondary structure that prevents translation by inducing ribosome stalling(Doma & Parker, 2007). The two endonucleases, Dom34 and Hbs1 (paralogs of eRF3 and eRF1 respectively) interact with the stalled ribosome and thereby assist in the disassembly of the translational elongation complex. They also trigger mRNA degradation through endonucleolytic cleavage followed by exonucleolytic decay of the transcript body (Doma & Parker, 2007) by the action of cytoplasmic exosome and Xrn1p (Doma and Parker, 2006).

1.5 The mRNA quality control pathways in Nucleus

Before exported out into the cytoplasm, the *bona fide* mRNA needs to be licensed and to maintain a healthy mRNA traffic, the nuclear mRNA quality control mechanism is also operate within the nucleus. Nuclear RNA exosome is the most well-studied RNA degrading machine in the nucleus (C Allmang et al., 1999; Mitchell et al., 1997). More than 90% cellular RNA is degraded by the Nuclear RNA exosome within nucleus (S Das & Das, 2013). The nuclear RNA Exosome targets a wide range of substrate mRNA extending from small non-coding RNAs (snoRNAs, snRNAs, and rRNAs) to large defective or aberrant mRNAs. The RNA Exosome also degrades the non-productive RNA fragments, which are produced during various transcriptional and post-transcriptional events. In order to build up a robust screening and degradation machinery, the nuclear exosome is assisted by a variety of ancillary co-factors (TRAMP, NNS and CTEXT) in *Saccharomyces cerevisiae*. These cofactors are thought to be similar to the MTREC complex of *Scizosaccharomyces pombe* and the NEXT and PAXT complex of mammals.The research carried in this study involves physiological role of CTEXT/NNS and Nuclear Exosome and is therefore briefly discussed in the following section.

1.5.1 The Nuclear RNA Exosome quality control pathway

The Nuclear RNA exosome is an evolutionarily conserved multi-subunit protein complex that catalyses 3′→5′ degradation of different types of RNA substrates. The exosome is primarily localized within nucleolus, (Dez et al., 2006) the site of RNA biogenesis, where rRNA genes are actively synthesized and processed (Andrulis et al., 2002; Casolari et al., 2004). A small part of exosome is also localized in the nucleoplasm as well (Kilchert et al., 2016).

The core exosome consists of nine subunits that are arranged in a two-layered ring structure with a central channel(Chlebowski et al., 2013; Q. Liu et al., 2006; Lykke-Andersen et al., 2011).The top layer consists of three proteins named Mtr3p, Rrp4p and Csl4p in the shape of 'trimeric cap' whereas the bottomlayer composed of 'hexameric ring' consisting of Rrp41p, Rrp42p, Rrrp43p, Rrp45p, Rrp46p and Rrp40p with a central channel. As if the central channel formed by these two layers, it can only accommodate the single-stranded RNA(Chlebowski et al., 2013).This nine subunit arrangement of core exosome is known as Exo-9. The Exo-9 is catalytically inactive, thought to have no role in RNA degradation and it only provides a scaffold via single stranded target RNA interaction. Dis3p/Rrp44p is the 10^{th} subunit, which imparts the catalytic activity to the core exosome (exo-9). Dis3p/Rrp44p is a 110 kDa protein, has both the endonucleolytic and exonucleolytic activities (Lorentzen et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). Dis3p/Rrp44p and Exo-10 together forms the Exo-10^{Dis3p} complex (Chlebowski et al., 2013; Qu et al., 2009). Recent findings suggest that Dis3p is attached with Exo-9 ring through the bottom hexamer on the opposite side of the trimeric cap and is strongly associated with it both in cytoplasm as well as in nucleus. A separate nucleus specific 3′→5′ exoribonuclease, Rrp6p (M W Briggs et al., 1998), is found to be linked with the Exo-10 complex to form Exo-$11^{Dis3p+Rrp6p}$ complex (Christine Allmang et al., 1999; Michael W Briggs et al., 1998; Q. Liu et al., 2006) in *Saccharomyces cerevisiae*. It is believed that the substrate RNA molecules snake through the central channel formed by the core and cap to reach the catalytically active sites of Dis3p/Rrp44p. Alternatively, it is also postulated that substrate RNA may also enter the Dis3p/Rrp44p catalytic site directly bypassing the central core channel of the exosome(Bonneau et al., 2009; H. W. Wang et al., 2007).

Although, the nuclear exosome is able to degrade a wide spectrum of RNA substrates, it lacks the target RNA specificity. This specificity and the degradation accuracy are modulated in various ways based on the nature of the RNA substrate. This modulation of specificity and activity is accomplished by additional protein complexes, dubbed exosomal co-factors. Nrd1p/Nab3p/Sen1p (NNS) complex and TRAMP are two such complexes found in the nucleus of *S. Cerevisiae.* Among them, NNS complex is believed to be known as Exosome Specificity Factor (ESF), which joins with the exosome from time to time and helps its direction towards specific classes of mRNAs(P. Singh et al., 2021).TRAMP complex is the best known example of exosome co-factors, which is composed ofa non-canonical poly(A) polymerase, Trf4p/Trf5p, DExH box RNA helicase Mtr4p (J Houseley et al., 2006; LaCava et al., 2005; Vaňáčová et al., 2005; F Wyers et al., 2005), a Zn-knuckle RNA binding protein Air1p/2p (Kilchert et al., 2016). Recent research from our laboratory demonstrated that in addition to TRAMP, a CBC dependent machinery, called CTEXT also acts as an exosome targeting complex by recruiting 3′-endprocessing- and export-defective nuclear mRNAs to the nuclear exosome (Maity et al., 2016).

1.5.1.1 CTEXT : CTEXT was initially discovered as an independent degradation Pathway (known as DRN, **D**ecay of m**R**NA in the **N**ucleus) during the investigation of the mechanism of genetic suppression analysis of a polyadenylation defect known as *cyc1-512* mutation in budding yeast (B. Das et al., 2000). Recent findings established that CTEXT acts as a critical co-factor complex assisting the nuclear exosome to carry out an efficient and selective degradation of export defective faulty messages (Maity et al., 2016). Using a combination of genetic and biochemical analysis, various components of this exosome assisting co-factor has been discovered, which are known to be the components of CTEXT. These include the Nuclear cap binding complex proteins, Cbc1/2p involved in a variety of mRNA maturation

and processing events(Bohnsack et al., 2002; B. Das et al., 2000; Izaurralde et al., 1992);the eukaryotic translation initiation factor Tif4631/32p involved in translation initiation and the nuclear mRNA surveillance (S Das et al., 2014); Upf3p, a component of the cytosolic NMD pathway(S Das et al., 2014); and Dbp2p, a DEAD-box RNA helicase. In addition to aberrantly long 3′-extended read through *cyc1-512* mRNAs, various kinds of export defective messages like *lys2-187* mRNA (harbouring a *cis*-acting export defect) and nucleus-arrested global messages in *tans*-acting export defective *nup116*-Δyeast strain are also targeted and degraded by CTEXT.

1.6 Unfolded Protein Response (UPR)

UPR represents a major intracellular signalling mechanism that is induced in response to the massive accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER). The ER lumen is the major depot of protein folding, biosynthesis and maturation in eukaryotic cell. Most of the secreted and membrane embedded proteins, which are destined to be secreted outside the cell or anchored on to the cell membrane are translocated into the ER lumen in a co-translational manner (Wickner & Schekman, 2005). ER chaperones or protein modification enzymes facilitate the folding of the translocated proteins and inhibit the protein aggregation and disulphide bond formation in maturing proteins. Unfolded proteins are degraded by ER-Associated protein Degradation (ERAD pathway) machinery. The ER therefore defines a protein folding factory that imposes an exquisite quality control on its products, proper folding, assembling and functional proteins are delivered to their ultimate destinations (Ellgaard & Helenius, 2003). Interestingly, the protein-folding load of ER alters under a variety of conditions such as extracellular environment and stimuli during developmental process and cell cycle progression. To mitigate such conditions, cells activate UPR to produce a copious amount of ER chaperones and heat-shock proteins, which

immediately refold the unfolded mass of proteins within ER lumen to restore homeostasis. Once the ER homeostasis is established, the UPR is attenuated promptly to its basal level.

Three different transducers of UPR sense the protein folding status in the ER lumen and transmit thatinformation across the ER membrane into the cytoplasm (Reviewed in Bernales et al., 2006; Walter & Ron, 2011). In baker's yeast,*S. cerevisiae*, however, only one kind of ER transducer, Inositol Requiring Protein 1(Ire1p) is primarily utilized, that senses the status of the protein unfolding in the ER lumen (Cox & Walter, 1996; Mori et al., 1996).Ire1p and Hac1p constitute two major components of the Ire1p circuit those encode a transmembrane kinase-endoribonuclease and b*ZIP* class of transcription factor respectively. Ire1p has an ER-luminal domain and a cytoplasmic protein kinase domain. Detection of unfolded proteins in the ER lumen leads to the oligomerization of Ire1p across the plane of the ER membrane (Sidrauski & Walter, 1997) thereby stimulating the trans-autophosphorylation of its cytoplasmic protein kinase domain (Sidrauski & Walter, 1997; Welihinda & Kaufmant, 1996). Stimulation of the trans-autophosphorylation promptly activates its endoribonuclease activity leading to the elimination of the intron from *HAC1* pre-mRNA via non-canonical splicing. Following the splicing of the pre-*HAC1* intron the two exons are subsequently ligated by the nuclear tRNA ligase,Rlg1(Cox & Walter, 1996; Kawahara et al., 1997; Mori et al., 1996; Shamu & Walter, 1996; Sidrauski & Walter, 1997; Welihinda & Kaufmant, 1996).

In the absence of ER stress, the intron containing pre-*HAC1* mRNA(*HAC1*^{u}) harbouring its intron remains transnationally inactive due to the formation of a secondary structure between 5′-UTR and the intron (Rüegsegger et al., 2001). UPR activation is accompanied by the translocation of pre-*HAC1*/*HAC1*^{u}mRNA from the site of transcription to the Ire1p foci (Aragon et al., 2009; Korennykh et al., 2009). A stem-loop structure located in the 3′-end of the pre-*HAC1*/*HAC1*^{u}mRNA dubbed the 3′-bi-partite element (BE) plays a crucial role in the

efficient targeting and transport of this pre-RNA from its site of transcription in the nucleus to active Ire1p foci on the ER surface (Aragon et al., 2009). Thus BE plays an instrumental role that is rate limiting for the formation of mature *HAC1* (*HAC1*^{i})mRNA and subsequent activation of UPR in yeast (van Anken et al., 2014). Besides acting as the recruiter of pre-*HAC1*/*HAC1*^{u}mRNA to Ire1p foci for splicing, 3′-BE also serves as a fail-safe mechanism to reduce accidental splicing of *HAC1* pre-mRNA resulting unwanted UPR activation(Xia, 2019).

1.6.1 Importance of Unfolded Protein Response:

UPR has multiple beneficial as well as many harmful effects on cellular physiology. In response to ER stress, cells adapt themselves to the stress condition via the induction of ER chaperones and activation of ERAD pathway to mitigate/eliminate unfolded proteins as a resultof activatedUPR. These mechanisms synergistically ensure (Feldman et al., 2005) the normal protein production, which in turn regulates cellular viability. Moreover, individual branches of UPR was shown to play specialized roles in developmental and metabolic processes, thus implying this pathway to be involved in cellular differentiation of some professional cells including plasma cells, pancreatic cells, hepatocytes, osteoblasts, and myocytes(Kitamura, 2008). Interestingly, UPR is recently implicated in the aging related neurodegenerative disorders such as in Parkinson's and Alzeimer's diseases(Scheper & Hoozemans, 2015). Functioning of neurons was known to be sensitive to protein aggregates for a long time. The brain cells of patients with Alzheimer's disease were reported to contain a huge amount of protein aggregates, which leads to excess ER stress. This activated level of ER stress caused the activated level of PERK and caspase-4, which are the known markers of UPR. Parkinson's disease is another extensively studied neurodegenerative disorder that was also linked to ER stress as supported by the observation of up regulation of expression of ER

chaperones in the brain of Parkinson's patients. Type 1 diabetes was also linked to ER stress which was suggested by the finding that in Akita diabetic mice, *ATF6* and *XBP1* (*HAC1* ortholog in mice) pathways are constitutively active in pancreatic beta cells (Back et al., 2005). Moreover, it was found that the cerebral ischemia up regulates the ER stress in neurons and activates all the branches (including the *ATF6*, *IRE1*, and *PERK*) of UPR (Kitamura, 2008). This activated UPR leads to CHOP-mediated apoptosis of neurons, which strongly indicates a functional connection between ischemic injury and unfolded protein response. Interestingly, a recent report suggests a role of ER stress in some types of glomerular disorders, especially proteinuric diseases caused by injury of podocytes(Kitamura, 2008).UPR function was also strongly connected with cancer**.** In all solid tumors, cells respond to hypoxia and ER stress through the activation of the UPR. *XBP1*, an orthologue of Hac1p, was implicated as a critical transcriptional activator involved in UPR, which is required for tumor growth (Feldman et al., 2005). Pancreatic ER kinase (PKR-like ER kinase) regulates the translational branch of the UPR and is also important in the growth of tumors. Understanding the functional relationship between hypoxia, UPR, tumor growth, type II diabetes at the molecular level is crucial to shed light on the strategy of currently available cancer therapies.

1.7 Origin of the problem and Aims and objective of the work:

As described above, Unfolded Protein Response (UPR) is an intracellular signalling pathway that responds to stress causing a burden of unfolded proteins (ER stress) in the ER lumen.To mitigate this situation, baker's yeast *Saccharomyces cerevisiae* produce a huge burst of Hac1p, a transcription factor that activates genes encoding ER chaperones. Activation of these chaperones involves their rapid transcription of their genes induced by transcription factor Hac1p. Hac1p is encoded by*HAC1* pre-mRNA harbouring an intron and a bipartite

element (BE) that plays a crucial role in the targeting/transportation of the *HAC1* pre-mRNA from the site of transcription to that of Ire1p-mediated splicing, (Ire1p foci). Thus, BE lacking *HAC1* pre-mRNA fails to undergo transport to the Ire1p foci. Recent study from this lab established that, in the absence of the stress, the *HAC1* pre-mRNA undergoes a rapid and kinetic 3′→5′ decay by the nuclear exosome/CTEXT thus producing little or no Hac1p protein output. During ER stress, in contrast, the *HAC1* pre-mRNA undergoes diminished exosomal decay resulting in the production of an increased abundance of pre-*HAC1* mRNA population carrying an intact BE which facilitates its more efficient recruitment to Ire1p foci thus producing more Hac1p protein, which transcriptionally activates UPR. During this study, it was cleared that the pre-*HAC1* mRNA is regulated by CTEXT/Nuclear Exosome but how the UPR protein output is changed by the CTEXT/Nuclear Exosome remained obscure. Therefore, in the first chapter of this study we addressed the following questions

1. **To establish whether EXOSOME/CTEXT and UPR pathway are related or not.**
2. **To decipher the molecular mechanism of Nuclear Exosome/CTEXT dependent regulation of UPR.**

Meanwhile, a report from another investigation suggests that a Rab-GTPaseYpt1p, previously implicated in the ER to Golgi shuttling of proteins was also involved in the regulation of UPR signalling dynamics by promoting the decay of *HAC1* mRNA(Tsvetanova et al., 2012).This finding suggests a potential involvement of Ypt1p in the regulatory mechanism for selection and recognition of the *HAC1* pre-mRNA by the nuclear exosome/CTEXT. They also showed that the stability of the *HAC1* precursor message was altered by a physical association of the RNA with the Rab-GTPase-Ypt1p. However, neither the molecular consequence of binding of Ypt1p to the unspliced pre-mRNA nor the mechanistic insight of the alteration of the stability of the *HAC1* message during ER stress is clear.

This finding thus poses an interesting question about the connection between the selection/recognition of the pre-*HAC1* message and its subsequent decay. An attractive model suggests that the association of the pre-*HAC1* message with Ypt1p probably facilitates the recognition of this pre-mRNA by the exosome/CTEXT and its subsequent regulation. In the second chapter of this study, we attempt to answer the following questions.

1. To evaluate the role of RabGTPaseYpt1p in regulation of unfolded protein response.

2. To investigate the functional relationship between Ypt1p and the nuclear exosome/CTEXT/NNS.

3. To establish the mechanistic regulation of Ypt1p dependent mRNA surveillance by Nuclear Exosome/CTEXT/NNS.

4. To delineate the molecular determinants(s) present in *HAC1* mRNA which permit its recognition by Ypt1p and subsequent recruitment to CTEXT/Nuclear Exosome.

The methodologies used in this study are detailed in **Chapter 2 (Materials and Methods)**. The findings and results of our first study with the detailed observation is presented in **Chapter 3 (Results I)** and second chapter of this study is presented in **Chapter 4 (Results II).** In the **Chapter 5 (Discussion),** the interpretation of the observed results, unanswered questions, and possible future extensions of this study are discussed thoroughly.

CHAPTER 2

Materials and Methods

2. Materials and Methods:

2.1. Materials:

2.1.1. Yeast Strains:

The genotypes of all the *Saccharomyces cerevisiae* strains used in this study consists of those, which were either constructed in the laboratory or those received as gifts from other investigators. All of the strains are shown in Table. 2.1.

Table 2.1: List and Genotypes of yeast strain used in this study

Strain	Genotype	Reference
yBD 117	**Mat a leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801**	Mori et al.(1996)
yBD118	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801hac1D::TRP1**	Mori et al.(1996)
yBD129	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801 rrp6::URA3**	Sarkar et al.(2018)
yBD131	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801 cbc1:: HisG**	Sarkar et al.(2018)
yBD148	**MATa; ura3-52; leu2-3,112; trp1-1; his3-11,15; nrd1Δ::HIS3; lys2Δ2; ade2-1; met2Δ1, can1-100 [pRS316NRD1 (NRD1, URA3, CEN)]**	Buratowoski et al.(2006)
yBD157	**MATα; ura3Δ0; his3Δ1; leu2Δ0; met15Δ0; LYS2; nrd1Δ::KAN; RRP6-TAP::HIS3; [pJC580 (NRD1-HA, LEU2, CEN)]**	Buratowoski et al.(2006)
yBD158	**MATα; ura3Δ0; his3Δ1; leu2Δ0; met15Δ0; LYS2; nrd1Δ::KAN; RRP6-TAP::HIS3; [pJC720 (nrd1-102-HA, LEU2, CEN)]**	Buratowoski et al.(2006)
yBD177	**MATa ura3-52 leu2-3,112 trp1-1 his3-11,15 nrd1::HIS3 lys2Δ2 ade2-1 met2Δ1 can1-100, (pRS 424nrd1-1(nrd1-1, TRP1, 2μ ori))**	Buratowoski et al.(2006)
yBD276	**Mat a leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801 HIS3-GAL::protA-CBC1 (pGAL-cbc1)**	Sarkar et al.(2018)
yBD433	**MATa ura3-52 leu2-3,112**	(Tsvetanova et al., 2012
yBD434	**MATa ura3-52 ypt1-3**	(Tsvetanova et al., 2012
yBD457	**MATa ura3-52 leu2-3,112 YPT1-TAP:: URA3**	This Study
yBD549	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801hac1D::TRP1 pHAC1**	This Study
yBD550	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801hac1D::TRP1 pRS316 HAC1-ΔR1**	This Study
yBD551	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-**	This Study

Strain	Genotype	Reference
	801hac1D::TRP1 pRS316 HAC1-ΔR2	
yBD552	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801hac1D::TRP1pRS316 HAC1 ΔBE**	This Study
yBD553	**Mata leu2-3,-112 ura3-52 his3-D 200 trp1-D901 lys2-801hac1D::TRP1 pRS316pHAC1 Δ3'UTR**	This Study
yBD565	**MATa ura3-52 ypt1-3-TAP::URA3**	This Study
yBD573	***MATa ura3-52 leu2-3,112 trp1-1 his3-11,15 nrd1::HIS3 lys2Δ2 ade2-1 met2Δ1 can1-100, (pRS 424nrd1-1(nrd1-1, TRP1, 2μ ori)) pRS 315 YPT1-TAP***	This Study
yBD574	**MATa ura3-52 leu2-3,112 pBD217,pBD218, pBD219**	This Study
yBD575	**MATa ura3-52 leu2-3,ypt1-3 pBD217, pBD218, pBD219**	This Study
yBD579	**MATa ura3-52 ypt1-3 hac1 :: HisG pRS316 ΔR1-*HAC1***	This Study
yBD580	**MATa ura3-52 ypt1-3 hac1 :: HisG pRS316 ΔR2-*HAC1***	This Study
yBD581	**MATa ura3-52 ypt1-3 hac1 :: HisG pRS316 Δ3'UTR-*HAC1***	This Study
yBD582	**MATa ura3-52 ypt1-3 hac1 :: HisG pRS316 ΔBE-*HAC1***	This Study

2.1.2 Plasmid Strains:

All plasmids used in this study were either constructed in our laboratory as described (Sambrook et al. 1989**)** or received from various invstigators as kind gifts. The plasmids used in this study are listed in Table 2.2.

Table 2.2: List and Description of Plasmids used in this study

Plasmid	Genotype	Reference
pBD 42	*CBC1* gene disruptor	Das et al. (2000)
pBD 45	*RRP6* gene disruptor	Das et al. (2000)
pBD 217	*ND-GFP2* in low-copy *LEU2*	Anshu et al.(2015)
pBD 218	*Ire1-RFP* in high-copy *LEU2* vector	Anshu et al.(2015)
pBD219	*HAC1-NRE* in low-copy *URA3* vector	Anshu et al.(2015)
pBD85	A 2183 bp long PCR amplified fragment containing 634 bp upstream from ATG codon and 578 bp downstream from stop codon of *HAC1* gene was cloned in pJET1.2 blunt vector (Fermentas Inc.). Host: DH5a	This study
pBD93	A 3853 bp BamHI-BglII fragment containing containing	This study

Plasmid	Genotype	Reference
	hisG-URA3-hisG sequence was incorporated into pBD 85 by replacing a 450 bp SalI fragment of *HAC1* gene to create a hac1::hisG-URA3-hisG null allele. *HAC1* disrupter	
pBD95	Hac1 inseretd in pRS 316 via digestion by BamHI (clone in 5'-3' direction) Host: DH5a	This study
pBD220	HAC1-NRE-Delta BE pRS 316 CEN	Anshu et al.(2015)
pBD333	154 bp of 3'UTR HAC1 has been deleted from pRS 316 HAC1 by reverse PCR using primer pair OBD 842 & OBD843. Host Cell : DH5α	This study
pBD334	103 bp of 3'UTR HAC1 has been deleted from pRS 316 HAC1 by reverse PCR using primer pair OBD 844 & OBD845. Host Cell : DH5α	This study

2.1.3 Oigonucleotides:

All of the oligonucleotides used in this study were purchased from IDT(Integrated DNA Technology,USA), are listed in Table 2.3

Table 2.3 Oigonucleotides used in this study

Primer	Oligonucleotide Sequence	Gene to be amplified
oBD 321	Forward 5'-GCGGGAAACAGTCTACCCTTT-3'	Pre-*HAC1* mRNA
oBD322	Reverse 5'-CACTGTAGTTTCCTGGTCATCGTAA-3'	Pre-*HAC1* mRNA
oBD325	Forward 5'-ATTGACACTGCTCGTTTGTGTGT-3'	*IRE1* mRNA
oBD326	Reverse 5'-CGAGACGACAATGGGATTGA-3'	*IRE1* mRNA
oBD323	Forward 5'-TTTCCTGAACAAATAGAGCCATTCT-3'	*HAC1* Intron-Exon 2
oBD324	3' Reverse 5'-TGCGCTTCGGACAGTACAAG-3'	*HAC1* Intron-Exon 2
oBD 522	Forward 5'-ACATCGTACCGAGTGATGAACG-3'	*HAC1 Promoter*
oBD523	Reverse 5'-GGCGGTTGTTGTCGTAGGTG-3'	*HAC1 Promoter*
oBD184	Forward 5′-AAAGGGTGGCCCACATAAGG- 3′	*CYC1*
oBD185	Reverse5′- CTTCAACCCACCAAAGGCCA-3′	*CYC1*

Primer	Oligonucleotide Sequence	Gene to be amplified
OBD272	Reverse 5′-GTGTGGATTTGATGGTATGTGTGA-3′	*LYS2*
OBD273	Forward 5′-GATATGCAGGGTCGATAACTGAAA-3′	*LYS2*
OBD276	Reverse 5′-AGCGTCCGTAATGTCCAATTCT-3′	*YLR194c*
oBD277	Forward 5′-CCCGCACCATAAGCTATGTGA-3′	*YLR194c*
oBD842	SP_HAC1_3'DEL1 Rev-5'-TTATACCCTCTTGCGATTGTCT-3'	*Construction of HAC1 deletion R1*
oBD843	SP_HAC1_3'DEL1 For-5'-CTTCAACCGAAGAAGAAGAGG-3'	*Construction of HAC1 deletionR1*
oBD844	SP_HAC1_3'DEL2 REv-5'-GCCAAAAAAGGGTCTTGTTC-3'	*Construction of HAC1 deletionR2*
oBD845	SP_HAC1_3'DEL2 Rev-5'TGTTTGTGTCATATCTATCTGTC-3'	*Construction of HAC1 deletionR2*

2.1.4 Genetic Nomenclature:

Here *CBC1*, *RRP6*, *NRD1*, *HAC1*, *YPT1* and IRE1 etc. denote the wild type allele, corresponding mutant/knocked out alleles that produce defective or non-functional proteins of these genes are designated as *cbc1*-Δ, *rrp6*-Δ, *nrd1-1*, *hac1*-Δ, *ypt1-3 or cbc1::hisG*, *rrp6::URA3* respectively. The protein encoded by a specific gene is denoted, for example, Cbc1p, Hac1p, Ire1p, Ypt1p etc which are encoded by *CBC1*, *HAC1*, *IRE1* and *YPT1* respectively.

2.2. METHODS:

2.2.1 DNA Manipulations:

2.2.1.1 Plasmid DNA Isolation:

5 ml sterile LB broth containing 100 mg/ml ampicillin (or other antibiotic) was inoculated with a single bacterial colony harbouring the plasmid and was allowed to grow to saturation

with vigorous shaking (150 to 200 rpm) overnight (16-24 hours). The cells were harvested by centrifuging for 5mins in a microcentrifuge at 5000g speed. The pellet was resuspended in 100 µl GTE solution (50 mM Glucose, 25 mM Tris-Hcl, pH-8, 10 mM EDTA) or Solution I followed by the addition of 200 µl of 0.2(N) NaOH-1% SDS solution (Solution II) to the tube. The solution was mixed quickly but gently by inverting the tube 4-5 times and left at room temperature for not more than 5 minutes. 150 µl chilled 5M potassium acetate (pH-4.8-5.2) solution was added to it and mixed promptly and vigorously by inverting the tube for 4-5 times. A white flocculent precipitate appeared right after the addition of the potassium acetate solution. The suspension was then centrifuged at 12000g for 5 min at 4ºC. The supernatant was taken out and transferred to a fresh tube and 500 µl isopropanol (room temperature) was added and mixed to it and let stand for 30 min at room temperature. The content was centrifuged at 12000g for 10 minutes at 4ºC. The pellet was washed with 250 µl of 70 % ethanol and again centrifuged at 12000g for 10 minutes at 4ºC followed by drying at 37ºC for 20 minutes and resuspension in 50 µl TE (10 mM Tris-HCl pH-8.0, 1 mM EDTA) buffer and stored at 4ºC.

To the crude plasmid DNA preparation thus obtained 2 µl of 1 mg/ml RNase was added after adjusting the volume of the plasmid preparation to 100 µl by TE. The reaction was kept at 37ºC for 30 minutes followed by incubation at 55-60ºC for 15 minutes. The final volume was made up to 400 µl with TE buffer and the suspension was extracted twice with 400 µl Phenol-Chloroform-Isoamyl alcohol (25:24:1) and once with 400 µl chloroform. Finally the aqueous phase was transferred to a fresh tube and was mixed with 1/10th volume of 3M sodium acetate pH-5.3 and 2 volume of absolute ethanol and left at - 70ºC for 30 min. The DNA was recovered by centrifugation at 12000 rpm for 10 min followed by washing with 70% ethanol, dried at 37^{o}C and dissolved in 50 µl TE buffer and stored at 4ºC.

2.2.1.2 Cloning Foreign DNA into Plasmid:

The insert and vector DNA was prepared by digestion with appropriate restriction endonuclease or by PCR amplification using gene specific primer (only for insert preparation). When the ends of the insert and vector are not compatible they were made blunt by T4 DNA polymerase or Klenow polymerase (Fermentas Inc.) by following manufacturer's recommended procedure. Both of the insert and vector DNA were then purified by excising from the agarose gel followed by ligation with T4 DNA ligase. The ligated DNA was transformed as described below followed by screening of 12-24 colonies by isolating the plasmid DNA, digestion with appropriate restriction endonucleases and sequencing of specific segment.

2.2.1.3 Transformation of Plasmid or recombinant DNA into *E.coli* cells:

50 ml of LB broth were inoculated with overnight culture of *E. coli* cells and grown at 37ºC till the OD600 reaches 0.4-0.6. The cells were harvested in sterile polypropylene tubes by centrifuging the culture at 5000 rpm for 3 min and the pellet was resuspended in 10 ml ice cold 100 mM CaCl2. Resuspension should be performed very gently by keeping the cells on ice. The suspension was re-centrifuged and resuspended in 1 ml 100 mM ice cold CaCl2 by gentle pipetting up and down and incubated on ice for 30 min. At this stage part of the cell can be stored frozen by mixing sterile glycerol to 20% final concentration. 100 µl CaCl2 treated competent cells were then gently mixed with 2-10 µl of either plasmid or ligated DNA was let stand on ice for 15 to 30 minutes followed by sudden heat shock at 42ºC for 2 minutes. After heat shock the transformation mix was stored on ice for 2 minutes. 1 ml of fresh LB broth was added to the suspension and was shaken vigorously (150 to 200 rpm) at 37ºC for 45 minutes. About 50-250 µl of the culture was spreaded on LB plate supplemented

with appropriate antibiotic which was followed by incubation at 37ºC overnight until the colonies appear on plate.

2.2.1.4 Genomic DNA isolation by Spheroplast Method:

Overnight grown yeast Cells were harvested and pelleted by centrifugation at 6000 rpm. The pellet was then suspended in 10mM EDTA and pelleted again. The pellet is then resuspended in 0.3ml solution of 1M sorbitol, 100mM EDTA, 50mM DTT with pH 7.5. To this solution 2397 unit of Lyticase was added and whole solution was incubated in 37°C for 2hours. After the incubation is over solution is centrifuged at 3500 rpm for 1minute. After that the supernatant was dissolved in 0.3 ml of 50mM Tris and 50mM EDTA by pipetting. Next 30ul of 10% SDS was added and again incubated at 65°C for 30minutes. After the incubation 100µl of 5M potassium acetate was added and incubated on ice for 1hour.Then the solution was centrifuged 15000 rpm for 10min at 4°C. The pellet is thus formed washed with 400µl of ice cold ethyl alcohol and 70% ethyl alcohol respectively at 4°C by centrifugation at 15000 rpm. The final pellet was dried and dissolved in TE buffer.

2.2.1.5: Polymerase Chain Reaction (PCR)

The following components were added in a micro-centrifuge tube in accordance with manufacturer's protocol

2x Taq Buffer	5µl
2.5 mM dNTPs	2 µl
Mixture of Forward And Reverse Primer	1.25 µl
Taq Polymerease	0.25 µl
Nuclease free water	14.5 µl
DNA template	2 µl
Total	25 µl

The reaction mixtures were placed in Thermal Cycler and sets following the program in below;

a. Initial Denaturation -98ºC for 30 seconds

b. Denaturation -98ºC for 30 seconds

c. Annealing -48ºC - 65ºC 30 sec (depending on the which primer is used)

d. Elongation -72ºC depending on the size of the amplified product

e. From step a to d were repeated 20 to 40 cycles

f. Final Extension -72ºC 10 min

g. Final hold - 4ºC - ∞

After the polymerase chain reactions, the amplified DNA was loaded in 1-3% Agarose gel depending on the size of amplified product.

2.2.1.6. Construction of Knock out yeast strain by One Step Gene Replacement

Yeast genes can easily be characterized by disrupting it from yeast genome and it is the most widely used method in this purpose. The one step gene disruption method is preferred due to its simplicity and easy to handle. The whole procedure of one step gene disruption is outlined below. This method utilizes a linear fragment of DNA containing a selectable marker flanked by 5' and 3' homologous regions of the gene to be disrupted. In this study the free end of the fragment (disruptor fragment) is either prepared by suitable restriction endonucleases or by polymerase chain reaction (PCR) using gene specific overhang primer pair. In both of the cases an auxotrophic marker is present between the flanking sequences. The transformation was carried out in haploid strain since our genes of interest are not essential for survival. The

transformation must be carried out in a diploid strain if the gene encodes an essential function. The disruption of each gene was verified by PCR using genomic DNA as template.

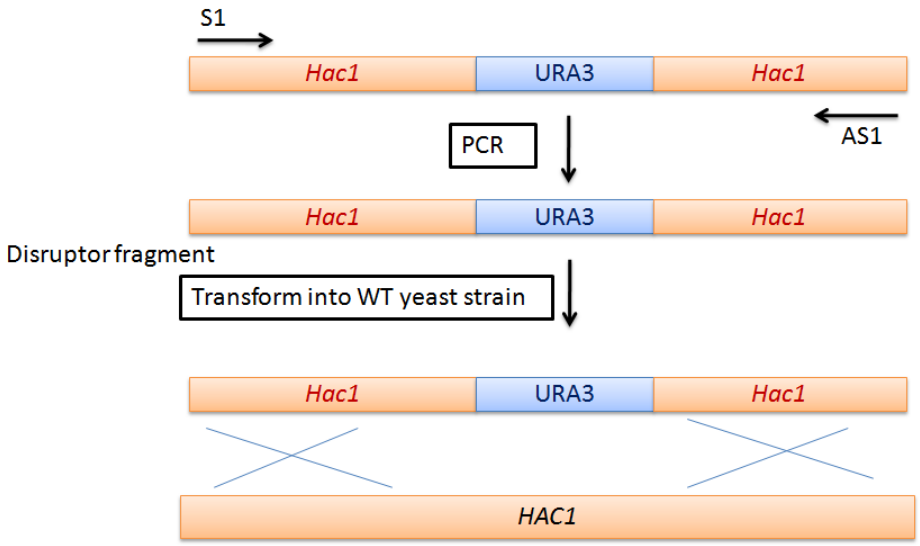

Fig.2.1 One step gene replacement: the basic outline for gene disruption using one step gene replacement. *The disruptor fragment containing URA3 selection marker is PCR amplified using S1 and AS1 primer.*

2.2.1.7: Reverse PCR for the construction of various deleted versions *HAC1* allele

For the construction of various deleted versions of 3'UTR of *HAC1* allele, specific primers flanking the region of interest are oriented facing away from each other such that the entire fragment of DNA excepting the specific region gets amiplified. A *CEN* plasmid harbouring a full length *HAC1* is used as template and Q5® High-Fidelity DNA Polymerase (NEB) is used. The reaction is set according to manufacturer's protocol. The amplicon thus obtained is gel purified and ligated using T4 DNA Ligase and maintained in *HAC1*-Δ background.

2.2.1.8: Transformation in Yeast by Lithium Acetate Method

1 ml of overnight *S. cerevisiae* culture was inoculated in 50 ml YPD (Yeast extract 1%, Peptone 2%, Dextrose 2%) for starting O.D to reach around 0.13 to 0.14. This was incubated at 30°C under shaking condition until O.D reached around 0.4 to 0.6. After the desired O.D was reached 25 ml of the culture was centrifuged for 5 mins at 4°C at 5000rpm followed by

resuspension of the pellet (in 20 ml sterile water) and re centrifugation under same condition. Pellet obtained was resuspended in 1.5 ml 1X T.E Lithium Acetate (Li Ac^-) and spun at top speed for 15 seconds followed by resuspension of the pellet in 0.4 ml 1X T.E Li Ac and followed by incubation at 30°C for 30 mins. The transformation mixture were prepared for control as well as test

Cell in 1X TE LiAc	50 µl
50% PEG (Poly Ethyl Glycol)	240 µl
1 M 10X LiAc	36 µl
10 mg/ml sonicated boiled Salmon sperm DNA	25 µl
Transforming Plasmid In TE(5-50ug of DNA)	50 µl

After preparation the transformation mix was incubated at 37°C for 30 mins followed by centrifugation at 5000 rpm for 10 mins. Supernatant was removed and pellet was resuspended in 200 µl sterile H2O and plated on selection medium plate (since our Transformants have got URA marker) and incubated at 30°C. After 2 days transformed colonies were observed on -URA plates.

2.2.2: Induction of UPR and Cell Viability Assay:

There are several available chemicals to induce ER stress and activate the UPR in yeast including tunicamycin, dithiothreitol (DTT), and 2-deoxy –D glucose. The concentration and duration of treatment in which ER stress is induced by these compounds should be determined for each particular system. Typically only a few hours are required to induce ER stress and long exposures often induce ER stress-mediated cell death. Tunicamycin is an inhibitor of the UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT), therefore blocking the initial step of glycoprotein biosynthesis in the ER. Thus, treatment of tunicamycin causes accumulation of unfolded glycoproteins in

the ER, leading to ER stress. DMSO (Dimethyl Sulfoxide) is the solvent for tunicamycin DTT is a strong reducing agent and blocks disulfide-bond formation, quickly leading to ER stress within minutes. Because DTT also blocks disulfide-bond formation of newly synthesized proteins in the cytosol, it is not a specific ER stress inducer.

Cell Viability Assay was performed according to the protocol Back et al, 2005. Individual strains of S. *Cerevisiae* were grown overnight and cell density of each culture was estimated by haemocytometer. The culture was then subsequently diluted to$1x10^7$ cells/ml in sterile water. This stock was further diluted 10 fold in serial manner in three successive steps to prepare 10-3, 10-4 and 10-5. 3 µl of each dilution including stock was then spotted onto the surface of YPD plate (Yeast Extract 1%, Peptone 2%, Dextrose 2%, Agar 2%) without and with Tunicamycin at 1µg/mL final concentration respectively. Plates were then incubated for 48-72 hours at 30°C. For liquid growth assay specific strains of *S. cerevisiae* were grown in triplicate until the OD reaches 0.6 when either tunicamycin was added to one of the culture to the final concentration of 1µg/mL final concentration or DMSO (solvent of Tunicamycin) was added for the mock treated culture. DTT was added in similar manner at a concentration of 5mM. Aliquots of each of these cultures were collected at different time points after tunicamycin or DTT addition for each strain and subsequently harvested and resuspended in 100 µl of sterile water. Tenfold serial dilution was then made in 100µL volume. Finally, 100µL of 10-5 & 10-6 fold diluted stocks were plated in YPD. Plates are incubated for 48-72 hours at 30°C and colonies were counted.

2.2.3 RNA Manipulation:

2.2.3.1: Extraction and purification of Total RNA:

Appropriately grown yeast cells in specific medium were harvested at 4ºC and subsequently washed with washing buffer (100mM LiCl, 100 mM Tris-HCl pH-7.5, 100 mM EDTA). The

pellet was further resuspended in RNA extraction buffer (100mM LiCl, 100 mM Tris-HCl pH-7.5, 100 mM EDTA, 0.5% SDS). The total cellular RNA was isolated by extracting the cell suspension in presence of phenol-chloroform-IAA (25:24:1) and glass bead for once followed by extraction with phenol-chloroform-IAA for two more times. Following the extraction, the RNA was purified and recovered by precipitation with RNAase free Ethanol following drying of the pellet and final resuspension in RNase free TE of water. The total RNA was quantified by recording its absorption at 260 nm.

2.2.3.2: cDNA Synthesis

Total RNA samples were first treated with 1 µg RNAase free DNase I (Fermentas Inc.) at 37ºC for 30 minutes by following the instruction of the manufacturer to remove the genomic DNA from each sample. DNA free RNA was then subjected to first strand cDNA synthesis in a 20 µL reaction mixture with PrimeScript RT reagent kit (TAKARA) using Random Primer by incubating the reaction mixture at 37ºC for 15 minutes followed by 85ºC for 5 sec.

2.2.3.3: Endpoint PCR

Appropriate amount of first strand cDNA thus prepared was amplified in standard 25 µL PCR reaction using Taq DNA polymerase as described previously and gene specific primer sets for various mRNAs such as *SCR1, HAC1, LYS2*. The amount of cDNA template, cycling parameters and cycle number are different for each specific primer set which was optimized during this work. Following the PCR reaction10 µL of the PCR product was analyzed in 2-3% agarose gel by quantifying each band in UV Tech Gel Doc System (Germany) using UVI Band software analysis program.

2.2.3.4: Real Time PCR:

2 to 4 ng of cDNA samples corresponding to each RNA sample was usually used for Real Time PCR using specific primers to quantify the levels of specific mRNAs such as *HAC1*, *IMP3*, *CYC1*, *LYS2*, *IRE1* and *SCR1* etc. using standard Syber Green Technology using iTaq^{TM} Universal SYBR® Green Super Mix (BIORAD). Q-PCR assays were run in triplicate and conducted using an Applied Biosystems StepOne real time PCR system to determine the absolute quantification of individual target mRNAs. The ΔCt of individual target mRNAs across the samples was determined from fluorescence by the StepOne^{TM} software version 2.2.2 (Applied Biosystems). The final fold or extent of stabilisation was calculated by determining the ΔΔCt value for each sample and using standard fold calculation formula.

2.2.3.5: Analysis of Transcription rate by Chromatin Immunoprecipitation (ChIP)

Chromaton preparation was essentially performed following the procedure described by Buratowski Lab (modified by Philip Komarnitsky from the Struhl lab version). The *YPT1* and *ypt1-3* strains or *NRD1* and *nrd1-1* strains in different background were used for this study.150 millilitre of cells grown till OD600 ≈ 0.5 (≈10^7cells/ml) and were fixed with 1% formaldehyde for 20min. After glycine addition to stop the reaction, the cells were washed and lysed with glass beads to isolate chromatin. The cross-linked chromatin was sheared by sonication to reduce average fragment size to≈500bp. Immunoprecipitation of chromatin was carried using ChIP assay kit (EZ-ChIP ™; 17-295) from Merck Millipore. Immunoprecipitation of 120 ug Chromatin fractions (≈100µl) from each strain was performed with protein G agarose and specific Antibody overnight at 4◦C. After washing beads and chromatin elution, the eluted supernatants and the input controls were incubated with Proteinase K for 1h at 37◦C followed by 5h at 65◦C to reverse cross-linked DNA complexes. DNA was extracted using the DNA-clean up column provided with the kit. The

immunoprecipitated DNAs (output) were quantitated by real time PCR (as mentionin section 2.2.3.4) using specific primers located along a specific gene coding sequence (described in Result section) and normalized to a half dilution of input DNA. Amplifications were done in duplicate for each sample, averages and standard deviations were calculated based on three independent experiments.

2.2.3.6: Analysis of the steady state levels and stability of specific mRNAs:

The stabilities of the various mRNAs were determined by the inhibition of global transcription with transcription inhibitor 1, 10 phenanthroline (Sigma-Aldrich) at 30°C, as described by Parker et al, 1991. Briefly, specific yeast strain was grown at 30°C till mid-logarithmic phase and then 1,10 phenanthroline was added to the growing culture at 20µg/ml final concentration followed by withdrawal of a 25 ml of aliquot of culture at various times after transcription shut off. Messenger RNA levels were quantified from cDNA by Real time PCR analysis and the signals associated with specific message was normalized against *SCR1* signals. The decay rates and half-lives of specific mRNAs were estimated with the regression analysis program (Origin 8) using a single exponential decay formula (assuming mRNA decay follows a first order kinetics), y =100 e-bx was used.

2.2.3.7. Galactose Induction of *CBC1* gene

Previously in the laboratory a plasmid is already constructed in which Protein A is placed under *GAL1/ GAL10* promoter.HIS3 sequence is also present upstream of *GAL1*/*GAL10* sequence for selection of positive strain. We used a chimeric-primer set which contains *CBC1* sequence in the two extreme ends and HIS3, *GAL1/GAL10* sequences present downstream and upstream of *CBC1* sequences respectively. If we amplify the construct, it will harbor *HIS3-GAL10* sequence flanked by *CBC1* sequences. This amplified product was transformed into wild type yeast cells which contains native *CBC1* promoter. After transformation, native

CBC1 promoter was supposed to be replaced by *GAL1/GAL10* promoter. *GAL1/GAL10* promoter should be placed in such a manner that it will be "in-frame" with *CBC1* ORF. Positive colonies which contain CBC1 gene under *GAL1/GAL10* promoter was selected in histidine drop out medium. Replacement of native *CBC1* promoter was confirmed by PCR using primers, which encompass *CBC1-HIS3* and *GAL1/GAL10-CBC1* sequences respectively. After successful insertion we want to check whether CBC1's expression is modulated by GAL10 promoter or not. So, we first grew the positive yeast cells in Yeast Raffinose Sucrose medium which is non-inducing and non-repressing medium up to mid log phase (0.6 O.D at 600nm).Then 2% galactose was added and cells are harvested after every one hour. Total RNA and protein were isolated and cDNA prepared. Then concentration of cDNA was measured and cDNA concentration was adjusted to 1 ng/µL. Afterwards PCR was done by using CBC1 specific primer. The isolated protein profiles were measured by immunobloting with specific antibody (Anti-Cbc1p and Anti-Hac1p).

2.2.3.8: Analysis of Binding Profile of mRNA by RNA Immunoprecipitation:

Yeast strains containing TAP-tagged *YPT1* cells or other cells were grown to $O.D._{600}$ 0.6 to 0.8. Prior to cell lysis intact yeast cells were Cross-linked by UV-irradiation in a petri dish on ice. Cells were washed once with Buffer A(50 mM Tris pH 7.4, 140 mM NaCl, 1.8 mM MgCl2, 0.1% NP-40) then cells were resuspended in Buffer B (Buffer A supplemented with 0.5 mM DTT, 40 U/ml RNase Inhibitor, 1 mM PMSF, 0.2 mg/mL heparin, protease inhibitor and lysed using glass beads and vortex. Lysates were cleared by centrifugation for 10 min at 8,000 rpm/4ºC. The lysate was quantified and pre-cleared for 30 mins at 4ºC. Protein A sepharose beads (Santacruz Biotechnology) were coated for overnight at 4ºC using TAP antibody (Thermo Fisher Scientific,Cat#CAB1001) in Buffer B. 1 mg pre-cleared cell lysate was incubated with antibody coated beads for 4hours at 4ºC on a rotator. Beads were washed

twice in Buffer B for 5 min/4ºC/rotator each, and once in Buffer C (50 mM Tris pH 7.4, 140 mM NaCl, 1.8 mM MgCl2, 0.01% NP-40, 10% glycerol, 1 mM PMSF, 0.5 mM DTT, 40 U/ml RNase Inhibitor, protease inhibitor) for 5 min/4ºC/rotator each. After washing, beads were resuspended in 200 ul Buffer C supplemented with 1% final SDS and heated at 70ºC for 45 min with constant mixing to de-crosslink samples and elute antibody-bound protein-RNA complexes from the beads. Cells for ‘‘Mock IPs’’ from isogenic parental untagged strain were grown, crosslinked, lysed, and incubated with anti-TAP antibody coated beads following the same procedure. Finally RNA was isolated from the eluates by phenol-chloroform-Isoamyl alcohol (25:24:1) pH 4.5 extraction and cDNA was prepared using 1st strand cDNA synthesis kit(Takara Bio) as described section 2.2.3.1&2 .

2.2.4 Protein Manipulation:

2.2.4.1 Preparation of Cell Lysate from Yeast:

50ml of YPD medium is seeded with sufficient amount of pre-inoculum and incubated at 37°C with shaking until OD_{600} 2.7-3.0 is reached. The cells are then pelleted, washed and resuspended in 1ml of Buffer A (50mM Tris-HCl pH= 7.5, 150mM NaCl, 5mM EDTA, 1mM DTT, 1mM Pmsf, 0.15% NP-40, 1.5mM $MgCl_2$, 1x Protease Inhibitor Cocktail (Abcam Cat No: ab201111)). The cells are lysed by vortexing with 20second pulses using glass beads (Unigenetics Instruments Pvt. Ltd Cat No: 11079105). After cell extraction the mixture is centrifuged to get rid of cell debris and glass beads. The supernatant is collected into a fresh microcentrifuge tube and centrifuged again at 2500rpm at 4°C for 10 minutes to get rid of any carried over cell debris and glass beads. The supernatant is collected again into a fresh microcentrifuge tube and centrifuged again at 15300rpm at 4°C for 45 minutes. The supernatant now obtained is clear cell lysate from *Saccharomyces cerevisiae*ready for downstream processing. The cell lysate is quantified using Coomassie Plus (Bradford) Assay

Kit (Thermo Scientific) according to standard Bradford assay protocol (Bradford, M.M, 1976).

2.2.4.2 Protein analysis by Immunoblotting: Total protein was isolated from specific yeast strains grown overnight at 30°C in YPD broth. Following the harvesting by centrifugation at 5000 rpm for 7 minutes the cell pellets were quickly frozen in liquid nitrogen and stored at -70C. Frozen pellets were thawed on ice and resuspended in 1 ml of Buffer A (50 mM Tris-cl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1mM DTT, 1mM PMSF) supplemented with Protease inhibitor (Invitrogen Inc. Carlsbad, CA, USA) and the cells were lysed by vortexing 10-15 times with glass beads followed by clarification of the particulate fraction by centrifugation. Supernatants were collected by centrifugation at 10,000 rpm for 20 minutes and saved as the total soluble protein fraction for further analysis. Protein concentration was determined by Bradford reagent assay kit (Bio-Rad Inc., Valencia, CA, USA). For Western Analysis, 30-50 µg of total protein was used, which was resolved either in a 8% or a 10% SDS-polyacrylamide gel. The separated proteins were transferred to PVDF membrane at 100mA for 16 hrs for immunobloting. Blots were blocked with 5% milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl, 0.1% Tween 20) and incubated with primary antibodies for specific proteins for 1hr at room temperature in following dilutions: rabbit polyconal anti-TAP(cat.#CAB1001) (1:1000), rabbit polyclonal anti-Hac1 (1:2000), rabbit polyclonal anti-Kar2 (1:2000), rabbit polyclonal anti-Cbc1 (1:1000), rabbit polyclonal anti-Rrp6 (1:1000), rabbit polyclonal anti-Tif4631(1:2000), mouse monoclonal anti-Tubulin (1:3000). Blots were further washed in TBS with 0.1% Tween 20 and incubated in HRP conjugated secondary anti-rabbit or anti-mouse antibody diluted at 1:5000 in wash buffer for 1hr. Immunoreactive bands were developed and detected by chemiluminescence (ECL imager kit,

Abcam) and the images were captured either by X-ray film or myECL Chemidoc Imager (Thermo Scientific, USA).

2.2.4.3 Co-Immunoprecipitation:

Cells were grown in 50ml YPD until OD_{600} 2.7-3.0 is reached. Cell lysate was prepared as described previously in 1ml of Buffer A. Protein A ^{PLUS}Agarose Beads (BioBharatiLifeSciences Pvt. Ltd.) ~50ul bed volume per reaction were equilibrated twice with ten volumes of Buffer A. The beads for each washing was resuspended in Buffer A and kept on a rotator wheel for 5mins at 4°C. The beads were centrifuged each time to remove residual Buffer A. The extract was then pre-cleared by adding the equilibrated beads and incubation on a rotator wheel at 4°C for 30 minutes followed by centrifugation to get rid of the beads. To the pre-cleared protein extract 2.5ug of anti-TAP Ab (Thermo Fisher Scientific, CAB1001) was added and incubated for 4 hours on rotator wheel at 4°C. To this extract 100ul bed volume of pre-equilibrated Protein A^{PLUS}Agarose Beads was added and incubated at 4°C on rotator wheel overnight. The beads are washed thrice with ten volumes of Buffer A by rotating on rotator wheel at 4°C for ten minutes each. Finally elution is performed by boiling the beads in 40ul of SDS loading dye for 5 minutes. Samples are now ready to be analysed on SDS-PAGE gel followed by Western blot analysis.

2.2.4.4 Immunofluorescence

Cells were grown in 10ml culture tubes up to mid-log phase. Cells were now fixed in 4% paraformaldehyde solution for 30 minutes after getting rid of the residual culture media. Cells are next washed twice in 0.1M KPO4 and once in 0.1M KPO4/1.2M Sorbitol and finally resuspended in 1ml 0.1M KPO4/1.2M Sorbitol. Following this cells are spheroplasted for permeabilisation. To about 0.5ml cells 5ul of 10mg/ml zymolase is added and incubated for 10-20mins as per cell density for spheroplasting. The reaction tube is supplemented with 10ul

of beta-mercaptoethanol for optimal reaction. After adequate spheroplasting, cells are pelleted at 5000rpm for 1minute and washed twice gently with 0.1M KPO4/1.2M Sorbitol and finally resuspended in 200ul of the same. Cells are now ready to be adhered onto poly-lysine coated cover-slips (regular glass-slips are appropriately poly-lysine coated according to Manufacturer's protocol). About 100ul of spheroplasted cells are added onto the poly-lysine coated cover-slips and allowed to sit for 15-20 minutes. After aspirating out the supernatant cells are blocked with 400ul of 10% PBS-BSA for 30 minutes followed by the addition of primary antibody (anti-TAP antibody at 1:200). Cells are incubated overnight at 4°C in humid chamber. The cells were then washed five times with PBS-BSA before incubation with secondary antibody (Goat anti-rabbit secondary antibody, FITC, Cat #F-2765 at 1:1000)at room-temperature in the dark for 1hour. Again cells were washed four times with PBS-BSA followed by incubating the cells with 1.5µg/ml Hoechst for Nucleas Staining. The cells were then washed extensively with PBS-BSA and finally mounted onto a clean glass slide using 10mg/ml p-phenylenediamine (Sigma) in 90% glycerol. The cells were observed in Confocal Laser Scanning Microscope (Leica) and the localization of YPT1was analyzed.

2.2.5 Confocal microscopy and image processing for targeting of pre-*HAC1* mRNA:

2.2.5.1 Construction of appropriate yeast strains and cell growth

The plasmids containing the $HAC1^{NRE}$allele, Nucleolin-GFP and Ire1p-RFP fusion genes was transformed using the standard LiCl procedure into the appropriate yeast strain in a sequential manner and select appropriate transformed colony in the single, double or triple omission SD medium. An appropriate colony was grew in liquid medium at 30°C till OD_{600nm}until the O.D. of the culture reaches0.6. 1µg/mL of Tunicamycin was added to the medium and incubated for another 1hour in 30°C. An aliquot of grown cells was used for *in vivo* imaging studies.

2.2.5.2 Confocal microscopy imaging and processing:

1ml cells were spun in the 1.5 ml microfuge tube and remove the supernatant. Cells were fixed with100 microliters of 4%paraformaldehyde incubating RT for 30 mins. Then cells were washed once in KPO_4/sorbitol (0.5-1 ml) and resuspended in small volume of KPO_4/sorbitol. One to three microliters of cells were taken onto coverslip and images were captured using confocal microscope (Leica). The GFP was excited at 458 nm, and emissions were recorded at 466-526 nm. The RFP was excited at 514 nm and emissions were recorded at 530-650 nm. Colocalization Index is measured by Image J software (NIH). The intensities of GFP signal and RFP signal was determined independently using the software and then GFP: RFP ratio was estimated in various strains. Pearson's correlation coefficient (PCC) (Dunn et al., 2011) was determined from the relationship given below for a typical image consisting of red and green channels.

$$\mathbf{PCC} = \frac{(\boldsymbol{R_i} - \bar{\boldsymbol{R}})\boldsymbol{X}\ (\boldsymbol{G_i} - \bar{\boldsymbol{G}})}{\sqrt{\{(\boldsymbol{R_i} - \bar{\boldsymbol{R}})^{\boldsymbol{2}}\boldsymbol{X}\ (\boldsymbol{G_i} - \bar{\boldsymbol{G}})^{\boldsymbol{2}}\}}}$$

Where, R_i&$G_i$refer to the fluorescence pixel intensity of the red and green channels of sample 'i', $\overline{R}$ & $\overline{G}$ mean fluorescence intensities of the red and green channels, respectively. PCC values range from +1 to -1 for two images. +1value indicates fluorescence intensities are linearly related to each other and perfect image registration. Whereas -1 indicates total lack of overlap between pixels from the images and fluorescence intensities are perfectly, but inversely, related to one another. Values near zero reflect distributions of probes that are uncorrelated with one another.

CHAPTER 3

Results I

3. RESULTS:

3.0 PREAMBLE

As described in Introduction, nuclear exosome/CTEXT pathway acts on several normal messages (known as special mRNAs), which lack any aberration (Kuai et al., 2005). These observations led to the hypothesis that exosome/CTEXT regulates their physiological abundance via the selective mRNA decay and thereby controls the respective biological processes in which the genes corresponding to these special messages are involved. *HAC1* is one such special message, whose abundance and stability was shown to be regulated by exosome/CTEXT. Since *HAC1* constitutes the major transcription activator of Unfolded Protein Response (UPR) pathway in *Saccharomyces cerevisiae*, our previous study indicated that that nuclear exosome/CTEXT may control the activity of UPR via the selective degradation of*HAC1* mRNA. Thus, the clue that UPR pathway may be physiologically linked to the nuclear exosome/CTEXT pathway prompted me to revalidate this hypothesis on a solid foundation. To confirm our hypothesis, I designed a series of experiments (i) to establish the hypothesis that CTEXT/Nuclear Exosome players have a functional role on the UPR activity and (ii) to deciphering the molecular mechanism of CTEXT dependent regulation of UPR. I personally joined in the later part of the study and carried out a series of experiments from diverse perspectives to strengthenour previous hypothesis on a solid foundation, which include cell viability assays, genetic & molecular biological techniques, and western blots as elaborated below.

3.1. To revalidate whether EXOSOME/CTEXT and UPR pathway are related or not?

Our previous investigations suggested that that the steady state levels of *HAC1* mRNA is affected by any of the mutations in CTEXT/Exosome players. The abundance of total *HAC1* mRNA was determined by Northern Blot and Real time PCR in WT, exosome deficient *rrp6*-

Δ, and CTEXT defective *cbc1*-Δstrains without any stress. This observations were initially identified in the previous microarray data and strongly indicates that the *HAC1* message may undergo an active degradation in a Cbc1p and Rrp6p dependent manner.Consequently, the steady-state levels and stability of the *HAC1* pre-mRNA were found to be significantly higher in yeast strains harbouring the null alleles of these two genes, *cbc1*-Δ and *rrp6*-Δ (Sarkar et al., 2018). However, it was also necessary to demonstrate thatHac1p protein levels also went up. To strengthen the view that the nuclear exosome/CTEXT governs the UPR response by dictating the Hac1p protein levels, I carried out immunoblot experiments in WT and CTEXT/Exosome deficient *cbc1*-Δ and *rrp6*-Δ strains both in the absence andin presence of ER stressinducer tunicamycin.We evaluated the Hac1p protein level and ER chaperone marker Bip/Kar2p level in WT, *cbc1*-Δ and *rrp6*-Δ strains by immunoblot analysis. As shown in Fig. 3.1A, in presence of stress, Hac1p level was enhanced in yeast strains deficient inCTEXT/Exosome componentsin presence of ER stress, whereas the steady-state levels ofα-tubulin did not alter. We also checked the ER chaperon marker Bip/kar2p levels in CTEXT/Exosome deficient strains and the data (Fig. 3.1 B), which revealed that Bip/Kar2 level was enhanced in CTEXT/Exosome deficient strains compared to the WT strain. These data, therefore, strongly supports our previous observation that UPR activity was enhanced in CTEXT/Exosome knocked out strain due to diminished degradation of *HAC1*pre-mRNA.

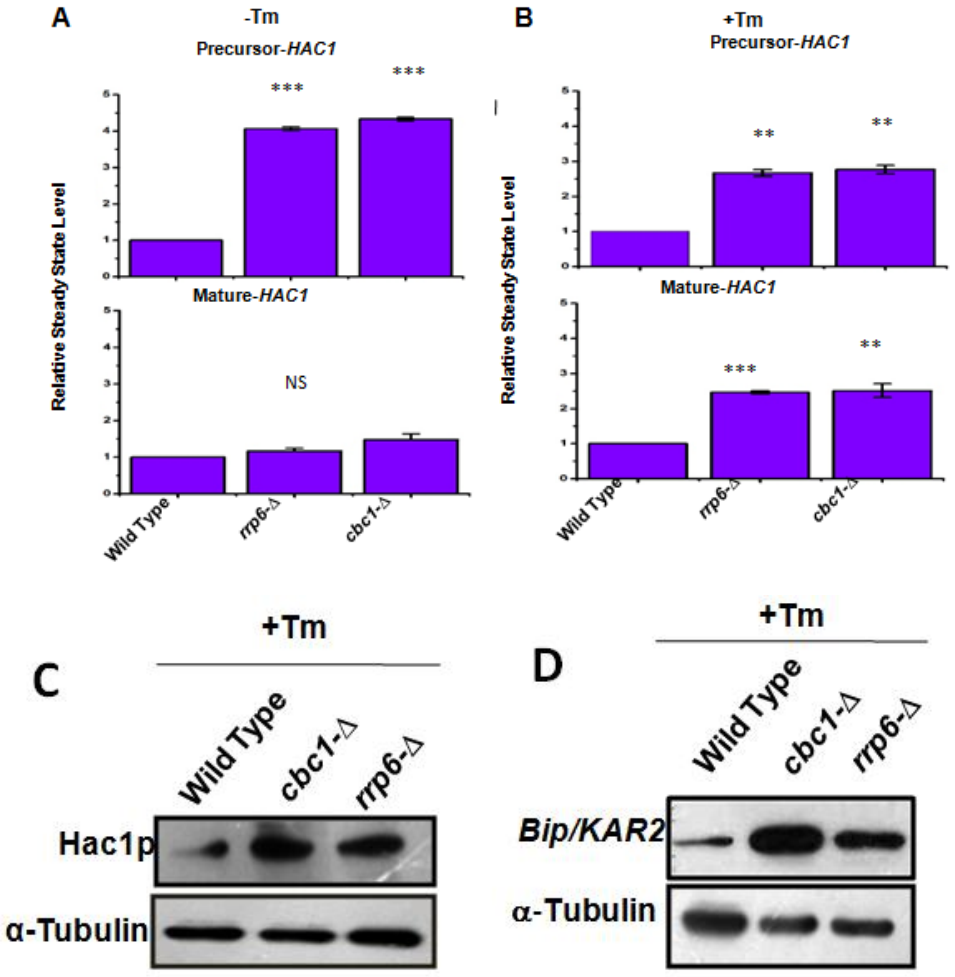

Fig. 3.1:A-B: Elevated levels of UPR activity in *rrp6*-Δ and *cbc1*-Δ strains is accomplished by the selective and targeted decay of pre-*HAC1* mRNA by nuclear exosome and CTEXT C-D:CTEXT/Exosome deficient strain produces more ER chaperones as well as Hac1p. Histogram showing the steady state levels of pre-*HAC1* (A) and mature *HAC1* (B) transcripts in the absence (-Tm) and the presence of 1 μg/ml tunicamycin (+Tm) in a normal, *cbc1*-Δ, and *rrp6*-Δ strains (n=3). Three independent cDNA preparations (biological replicates, n=3) were used to determine the levels of these mRNAs. Normalized value of individual mRNA from normal samples was set to 1

C. Relative steady state levels of Hac1p protein in wild type (yBD-117), *cbc1*-Δ (yBD-131), *rrp6*-Δ (yBD-129) strains in absence (-Tm) and in presence of 1 μg/ml tunicamycin (+Tm). α-tubulin (lower panel) was used as the loading control. Cells of indicated strains were grown in absence and in the presence of tunicamycin, harvested and total protein extract were prepared as described in Materials and Methods. 50μg of total cellular protein was loaded in each lane, resolved in 12% SDS-PAGE and probed with anti-Hac1p and anti-tubulin antibodies to detect these proteins as described in Materials and Methods. **D.** Relative steady state levels of *Bip/KAR2* protein in wild type (yBD-117), *cbc1*-Δ (yBD-131), *rrp6*-Δ (yBD-129) strains in the presence of 1 μg/ml tunicamycin (+Tm)at 30ºC. α-tubulin (lower panel) was used as the loading control. Cells of indicated strains were grown in the absence and in the presence of tunicamycin, harvested and total protein extract were prepared as described in Materials and Methods. 50μg of total cellular protein was loaded in each lane, resolved in 12% SDS-PAGE and probed with anti-*Bip* and anti-tubulin antibodies to detect these proteins as described in Materials and Methods.

One important speculation that followed from our previous findings indicated that sincethe exosome/CTEXT deficient strains accumulate a higher level of *HAC1* mRNA and Hac1p,this increased Hac1p protein level should lead to induction of a stronger UPR response in *cbc1*-Δ and *rrp6*-Δstrains in presence of ER stress. Therefore, it is anticipated that the exosome/CTEXT compromised strains should be able to grow and survive better than yeast strain either with the functional Exosome or CTEXT machinery. I therefore verify this predictionby evaluating the growth of an isogenic WT, *rrp6*-Δ and *rrp6-3*yeast strains in presence and absence of tunicamycin in solid medium. The drug, tunicamycin inhibits protein folding in ER and thus triggers UPR activity by mimicking ER stress (Bernales et al., 2006). Thus,theability of the growth of a given strain in presence of ER stress (Tunicamycin) is a strong and potent indicator of the level of the UPR activity. As shown in Fig. 3.2, both the *rrp6*-Δ and *rrp6-3* mutantyeast strains grew much better compared to wild type strains (WT and p*RRP6*) in solid YPD medium in presence of tunicamycin (1µg/ml). This finding, thus, clearly indicates that both the nuclear exosome deficient strain is viable in presence of tunicamycin, whereas normal wild type cells failed to grow. Notably, the *rrp6-3* allele (a gift from Prof. J. Scott Butler, the University of Rochester, USA) harbours a catalytically inactive point mutation in the exonuclease I domain (D238A) of the Rrp6p protein (Burkard & Butler, 2000). As shown in Supplementary Figure 3.2, the *rrp6-3* was unable to complement the growth defect phenotype of the *rrp6*-Δ strain,and the strain grew like an *rrp6*-Δ strain in the presence of ER stress-inducer tunicamycin. This result strongly suggests that in the presence of tunicamycin the enhanced growth of the yeast strains deficient in functional nuclear exosome is attributable to the exonucleolytic activity of the nuclear exosome. Collective evidence thus strongly favours our hypothesis that the nuclear exosome/CTEXT potentially influence the activity of the UPR of the nuclear exosome component, Rrp6.

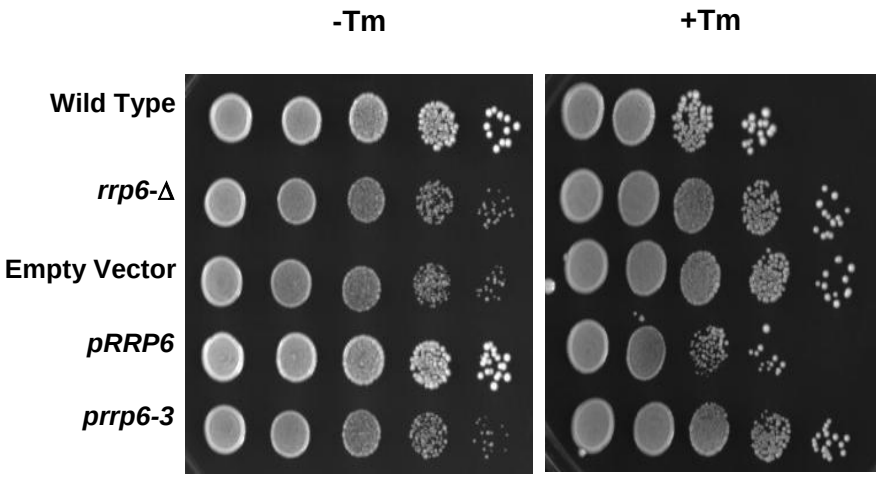

Fig.3.2A. Relative growth of a wild type (yBD-117), *cbc1*-Δ (yBD-131), *rrp6*-Δ (yBD-129) strains in YPD solid growth media in the absence and presence of 1 µg/ml tunicamycin (+TU).

3.2 Deciphering the molecular mechanism of CTEXT dependent regulation of UPR:

The observed enhancement of *HAC1* mRNA in CTEXT deficient *cbc1*-Δ or*rrp6*-Δ strains strongly suggests that *HAC1* pre-mRNA is subjected to a preferential decay by the nuclear exosome and CTEXT. Interestingly, the steady state level of *HAC1* pre-mRNA was also found to bear a reciprocal relationship with the steady state levels of *CBC1* mRNA. To further support our view, we evaluated this finding by investigating the levels of Hac1p as a function of differentially expressed Cbc1p proteins in an engineered yeast strain by immunoblotting experiment. In this experiment, the increasing levels of Cbc1p were accomplished by expressing *CBC1* gene under p*GAL1*/*GAL10* promoter followed by its induction with 2% galactose. As shown in Fig. 3.3A, the levels of Cbc1p expressed under the control of *GAL* promoter gradually enhanced due to the increased activity of p*GAL1*/*GAL10* promoter due to galactose induction. Interestingly, the levels of Hac1p protein gradually decreased under the same condition at different time post-induction with 2% galactose. Under the same conditions, there was no significant alteration in the level of α-tubulin protein level. Most significantly, the relative profile of steady state levels of these proteins indeed reflected a reciprocal relationship as revealed by their levels under identical conditions. Here we also

analyzed the protein level of individual CTEXT players under stressed and unstressed condition. As shown in Fig. 3.3B, the Cbc1p and Rrp6p protein levels decreased under ER stress condition, whereas the α-tubulin protein level remained unchanged. Taken together, we firmly concluded that the Nuclear Exosome/CTEXT inversely regulate the function of the UPR in baker's yeast by maintaining an optimum supply line of precursor *HAC1* mRNA under both the unstressed and ER-stressed conditions, which is mediated by a preferential degradation of pre-*HAC1* mRNA.

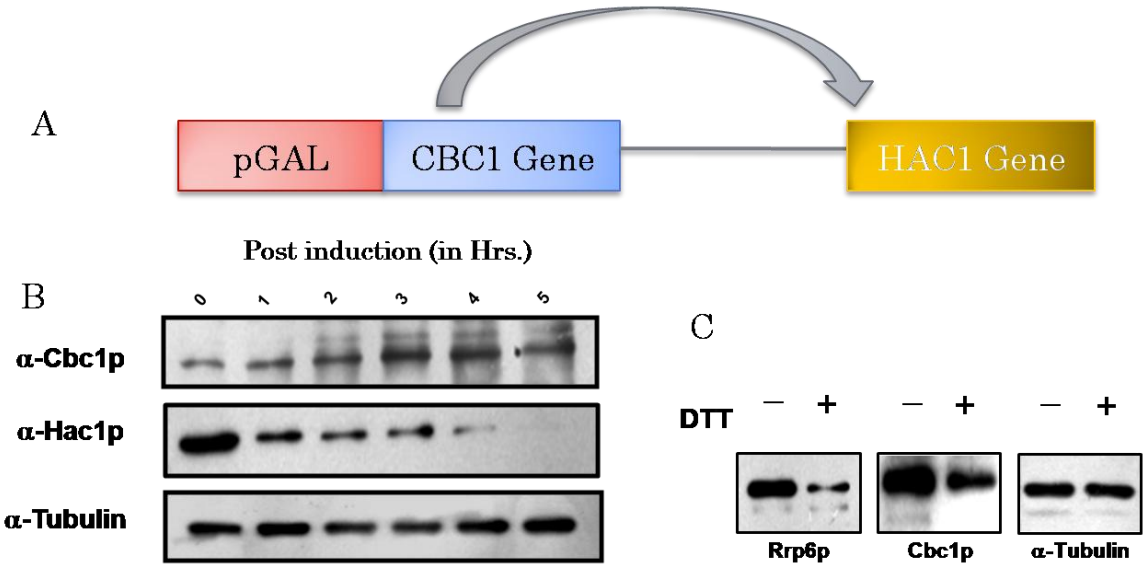

Fig.3.3A.Relative Levels of Cbc1p protein and Hac1pprotein bears an inverse relationship. B.Levels of Cbc1p and Hac1pproteins expressed in wild type yeast cells expressing a *CBC1* allele under the control of p*GAL1-GAL10*promoter as determined by western blot analyses using total cellular proteins harvested from a normal strain harbouring the engineered *CBC1* allele at different times after the induction with 2% galactose. Cells were harvested at different times post-induction as indicated above the panel and total cellular protein extracts were prepared as described and resolved at 12% SDS-PAGE followed by the immunodetection using anti-Cbc1p and anti-Hac1p antibodies. As before, the α-tubulin served as the loading control. **C.**Relative steady state levels of Cbc1p and Rrp6p proteins in wild type (yBD-117)strains in the absence (-) and in the presence of5mM DTT (+). α-Tubulinwas used as the loading control. Cells were grown in the absence and in the presence of DTT, harvested and total protein extract were prepared as described in Materials and Methods. 50µg of total cellular protein was loaded in each lane, resolved in 12% SDS-PAGE and probed with anti-Cbc1p, anti-Rrp6p, and anti-tubulin antibodies as described in Materials and Methods.

3.3 Conclusion:

In this chapter, several experimental evidence were presented that suggest that in baker's yeast, *Saccharomyces cerevisiae*the nuclear exosome and its co-factor CTEXT selectively targets pre-*HAC1* mRNA to exertits preferential 3′→5′ decay. As mentioned in the introduction, these experiments were originally addressed to validate the query if the nuclear mRNA surveillance mechanism plays any functional role in controlling the unfolded protein response (UPR) signalling in baker's yeast. Estimation of cellular level of pre-*HAC1* and mature *HAC1* mRNAs in WT, *cbc1*-Δ and *rrp6*-Δ strains and the comparison between their steady-state levels in these strains demonstrated that the levels of pre-*HAC1* (but not the mature *HAC1*) message becomes dramatically up-regulated in both *cbc1*-Δ and *rrp6*-Δ strains relative to WT (Fig. 3.1A-B).Interestingly, a point mutation in the exonuclease domain of Rrp6p (*rrp6-3*) displayed a similar growth phenotype as that displayed by *rrp6*-Δstrain in presence of ER-stress inducer tunicamycin thus suggesting that the exonuclease activity is involved in the decay of pre-*HAC1* mRNA. Collectively, these observations are consistent with the idea that the pre-*HAC1* mRNA is undergoing a selective nuclear decay by the nuclear exosome and its co-factor CTEXT (Sarkar at al., 2018). To further confirm the impact of this differential decay, the survival ability of the WT , *cbc1*-Δ and *rrp6*-Δ strains were also tested in a growth medium challenged with the ER-stress inducer tunicamycin to see whether the strain lacking the functional components of the nuclear degradation machinery can handle this challenge in more efficient manner. Consistent with our prediction, *cbc1*-Δ and *rrp6*-Δ strains indeed exhibited an ability to grow much better when they encounter the ER-stress. We further verified this view by showing that indeed the levels of both the Hac1p and ER-marker Bip/Kar2p proteins showed significantly elevated levels in the *cbc1*-Δ and *rrp6*-Δ strains (Fig. 3.1). Thus, the impact of the differential pre-*HAC1*

mRNA decay event initially detected at the molecular level is finally transmitted at the cellular as well as phenotypic level of the enhanced growth in presence of ER-stress of the cells lacking the functional exosome/CEXT components.

These initial experiments led to the speculation that the Cbc1p and Hac1p protein and RNA levels would display an inverse relationship between themselves, since Cbc1p is acting as the negative regulator of the *HAC1* mRNA expression. Consistently, validation of this prediction, suggests that indeed the RNA and protein levels of Cbc1p and Hac1p levels (Fig. 3.3A) remain inversely proportional to one another thus establishing a causal relationship between these genes. Furthermore, the ability of growth in presence of tunicamycin also slowly decreases, as the level of expression of Cbc1p is gradually increases (Fig. 3.3A). Consistently, imposing the ER stress by DTT led to the lower expression levels of the CTEXT component Cbc1p (Fig. 3.3B) thereby strengthening the relative inverse relationship between the functions associated with nuclear mRNA decay and UPR signalling mechanism in yeast cell.All these findings clearly suggest that the nuclearexosome/CTEXT plays a pivotal role intuning the output of theUPR signalling in *S. cerevisiae*.

One shortcoming from these experiments, however, that remained involved how the pre-HAC1 mRNA was selectively degraded by the nuclear exosome/CTEXT? What feature(s)/determinant(s) in the *HAC1* pre-mRNA qualify it as a privileged substrate forthe nuclear exosome/CTEXT. In this context, it is worth mentioning that a Rab-GTPase, Ypt1p was recently reported to be associated with the unspliced *HAC1* mRNA and thereby altered its decay (Svetanova et al., 2012). Although no detailed mechanism of how possibly the Ypt1p could bring about the alteration of decay of this precursor message was provided by this workers. Moreover, no insight into the associationof *HAC1* pre-mRNA with the Rab-GTPase Ytp1p so far was explored to reveal further novel mechanistic insight intothis

regulatory decay mechanism. We therefore made a systematic attempt to explore into the physical association between Ypt1p and *HAC1* pre-mRNA and thereby show that indeed Ypt1p is a component of the machinery that collectively brings about the decay of pre-*HAC1* mRNA and participates in the regulation of the UPR signalling dynamics (see next Chapter).

CHAPTER 4

Results II

4. RESULTS II

4.0. PREAMBLE:

As mentioned in the Introduction (Chapter 1), previous study from our lab revealed that an additional layer of regulation exists in the UPR pathway in *Saccharomyces cerevisiae,* that involves a preferential nuclear degradation of *HAC1* pre-mRNA in a 3′→5′ direction that requires the nuclear exosome and its co-factor CTEXT (Sarkar et al., 2018). Therefore, CTEXT/nuclear exosome finely tunes the output of UPR signalling in *S. cerevisiae*. However, the mechanism of recognition and selection of the pre-*HAC1* transcripts among a plethora of other aberrant and special mRNAs is accomplished by the nuclear exosome and CTEXT still not clear. Furthermore, a report from another independent investigation suggests that the Rab-GTPase-Ypt1p modulates the decay of pre-*HAC1* mRNA by physically associating with the unspliced *HAC1* transcript(N G Tsvetanova et al., 2012). However, they didnot provide any explanation about how the Rab-GTPase Ypt1p could bring about the rapid decay of *HAC1*-pre mRNA and which decay factors are involved. This finding thus poses an interesting question about the connection between the selection/recognition of the pre-*HAC1* message and its subsequent decay by the nuclear exosome/CTEXT. These two independent findings let us hypothesize that the association of the pre-*HAC1* message with Ypt1p facilitates the recognition of this pre-mRNA by the exosome/CTEXT and its subsequent regulation.

To validate my hypothesis, I designed a series of experiments to establish that the association between Rab-GTPase Ypt1p and pre-*HAC1* mRNA leads to its selective recognition and degradation by the Nuclear Exosome and NNS/CTEXT. Moreover, I also investigated the molecular determinants (such as a *cis*-element) present in pre-*HAC1*transcript, which permits its recognition and subsequent recruitment to thenuclear exosome/CTEXT and the molecular

mechanism involved inthis process. Consequently, I carried out a series of experiments from diverse perspectives to establish my hypothesis, which include cell viability assays, genetic & molecular biological techniques, confocal imaging as elaborated below.

This chapter depicts our analysis by evaluating the role of Ypt1p in UPR activity and decay of *HAC1*-pre mRNA. The collective results of these experiments revealed that yeast strain deficient in Ypt1p could grow better in presence of ER-stressand displayed a diminished decay rate of pre-*HAC1*mRNA. Once this preliminary observation is validated, I extended my preliminary findings to investigate the functional relationship between Ypt1p and Nuclear Exosome/CTEXT/NNS. In particular, we address the physical association between Ypt1p and the nuclear decay machinery. To investigate this particular association, I carried out Co-immunoprecipitation of Ypt1p to show quite convincingly that the association of Ypt1p with the decay machinery reduced in presence of ER-stress. Next, I went ahead to dissect out the mechanistic insight into this relationship and consequently a thorough analysis was carried out to confirm the specific and selective association between the Ypt1p and pre-*HAC1* mRNA.The physical association (as detected by RNA-immunoprecipitation) ofRab-GTPase Ypt1p with *HAC1*-pre mRNA facilitates the recruitment of nuclear exosome/CTEXT/NNS on to the pre-*HAC1* mRNA and stimulates its nuclear decay in absence of stress. This increased population of BE containing pre-*HAC1* mRNA results in the increased targeting to the Ire1p foci followed by an enhanced Ire1p/Rlg1P mediated splicing and production of a large burst of Hac1p. Then we went further deep inside this story by addressing another interesting issue. We postulated that a *cis*-acting element within the *HAC1* mRNA is responsible for recognition by Ypt1p and for subsequent recruitment of the nuclear exosome/CTEXT/NNS. Accordingly, an effort was made to delineate the *cis*-acting molecular determinant on *HAC1* mRNA for its recognition and subsequent degradation. These findings, together, will help to

gain an insight into the additional layer of regulatory mechanism that dictates the output ofUPR that is accomplished by differential degradation of pre-*HAC1* mRNAat the post-transcriptional level.

4.1. Revalidating the role of Rab-GTPase Ypt1p in UPR activity:

In order to validate the findings from the previous study, we first analyzed the growth rate of WT and *ypt1-3* mutant yeast strains in liquid YPD medium in the absence and presence of 5 mM DTT and 1µg/ml Tunicamycin. The drug DTT/tunicamycin inhibits protein folding in endoplasmic reticulum(ER) thereby triggering UPR response via accumulation of unfolded proteins within ER lumen(Bernales et al., 2006). After the yeast cells grew for the indicated times following UPR induction in mock and experimental cultures, small aliquots were collected, washed thoroughly to remove DTT/Tunicamycin, harvested and 1000 cells were plated on normal YPD plates without DTT. The viable yeast colonies appeared on each plates were counted and presented in Fig. 3.1A that revealed that in absence of Tunicamycin (no ER stress) the *ypt1-3* cells grew much slower relative WT cells, whereas their growth in presence of Tunicamycin is much better than WT (Fig. 3.1A). Similar finding was observed when the growth experiment was done (Fig 3.1B) in presence of 5mM DTT. Notably, the rate of mortality of the WT strain in presence of both tunicamycin and DTT are much sharper with time compared to that of *ypt1-3* cells so that they could maintain their viability for a much longer period (Fig. 3.1A &3.1B). This data suggests that depletion of Ypt1p promotes a better survival efficiency index of the *ypt1-3* cells in presence of the ER stress (Fig. 3.1A and 3.1B) thereby indicating that Ypt1p is certainly playing a role in regulating the UPR function.As shown in Fig. 3.1C, the normal WTcells grew well in absence of Tunicamycin whereas mutant cells grew better in presence of these drugs.Taken together, these data supports strongly that Rab-GTPase Ypt1p exerts a regulatory influence on the functioning of

UPR in *Saccharomyces cerevisiae.*

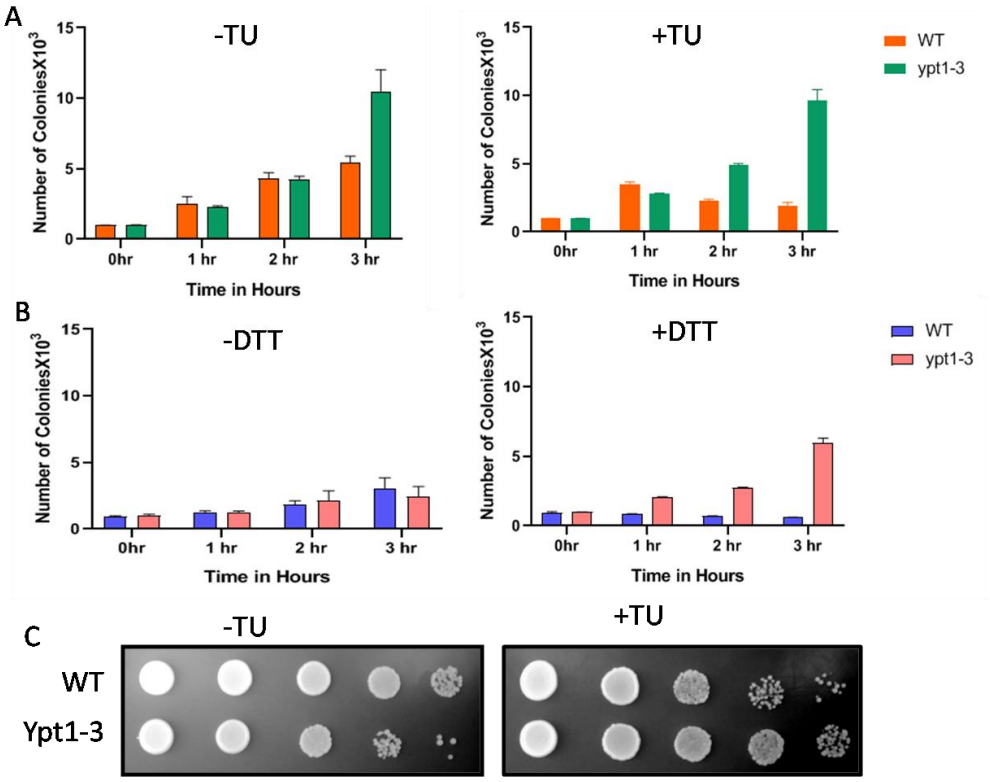

Fig.4.1Ypt1p may play a functional role in the regulation of Induction of UPR in baker's yeast: *A. Relative growth of WT (yBD-433) (orange Bar), ypt1-3(yBD-434) (green Bar) strains in YPD liquid growth media in absence (-TU) and in presence of 1 µg/ml tunicamycin (+TU). Each strain was grown in liquid YPD medium in absence of tunicamycin to and optical density of 0.6 and divided into two parts. In one-half of the culture of each strain, tunicamycin was added to a final concentration to 1 µg/ml (+TU) whereas equal volume of DMSO was added to other of the culture of each strain (-TU). Cultures were continued to grow for 3 hrs and each time point 100 µl of each culture were spreaded onto solid YPD plate at indicated time after tunicamycin (or DMSO) addition. Plates were incubated for 72 hours at 30ºC and the colony numbers were scored and plotted as bars. The graph is the mean of three independent experiments (n=3) and the standard error of means were plotted as error bars.B. Relative growth of WT (yBD-433) (orange Bar), ypt1-3(yBD-434) (green Bar) strains in YPD liquid growth media in absence (-DTT) and in presence of 5mM Dithiothreitol (+DTT). Cultures were continued to grow for 3 hrs and each time point 100 µl of each culture were spreaded onto solid YPD plate at indicated time after tunicamycin (or DMSO) addition. Plates were incubated for 72 hours at 30ºC and the colony numbers were scored and plotted as bars.**4.1C** Relative growth of wild type (yBD-433), ypt1-3(yBD-434), strains in YPD solid growth media in absence (-TU) and in presence of 1µg/ml tunicamycin (+Tm). Equal no. of cells of normal, ypt1-3 yeast strains grown in YPD liquid medium in absence of tunicamycin were spotted with ten-fold dilution on medium with or without tunicamycin and grown at 30°C for 72 hours and photographed.*

To further confirm this finding, we extended our investigation to examine if the cellular abundance of pre-*HAC1* mRNAs is also enhanced in absenceof Rab-GTPase Ypt1p. As reported previously, in presence of stress the level of *HAC1* mRNA is typically up-regulated during UPR activation in order to maximize the protein folding in the ER(Sarkar et al., 2017).

Examination of the steady state levels of pre-*HAC1*mRNA revealed a 2 to 2.5 folds up-regulation in *ypt1-3* strain (Fig.4.1D).As shown in the same figure, a higher steady state level of pre-*HAC1*mRNA, (almost 3 to 4 fold up regulation)was observed in presence of stress both in WT and *ypt1-3* mutant strain.

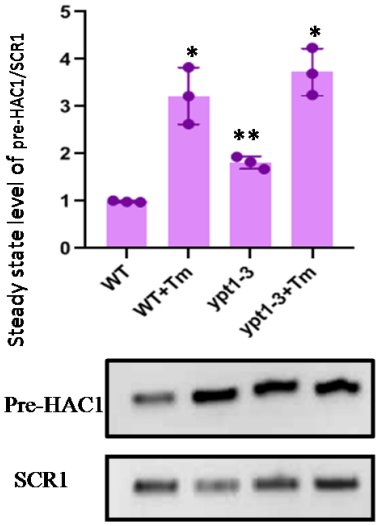

***Fig.4.1 D**. Histogram depicting the steady state levels of total cellular HAC1 mRNA levels in wild type (yBD-433), ypt1-3 (yBD-434), strains in absence of tunicamycin using qPCR analysis using a primer pair specific to HAC1 exon 1. Transcript copy numbers/2ng cDNA of each strain was normalized to SCR1 RNA levels in respective strains and are presented as means ± SE (n=3 for each strain). Normalized value of individual mRNA from normal samples was set to 1. The statistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, ∗<0.05, ∗∗<0.005 and ∗∗∗<0.001, NS, not significant.*

To corroborate if the ability of better growth and survival of the *ypt1-3* strains was accompanied bythe possible diminutionin the decay rate of *HAC1* pre-mRNA, we determined the decay rates of *HAC1* pre-RNA in the WTand *ypt1-3* strains after shutting off their transcription by 1,10-Phinanthroline. As shown in Fig.4.1E, the decay rate of this RNA diminished significantly in *ypt1-3* strain compared toWT strain. Collective findings thus strongly reveal that Rab-GTPaseYpt1p influences the UPR signalling dynamics by altering the decay of pre-*HAC1* mRNA.

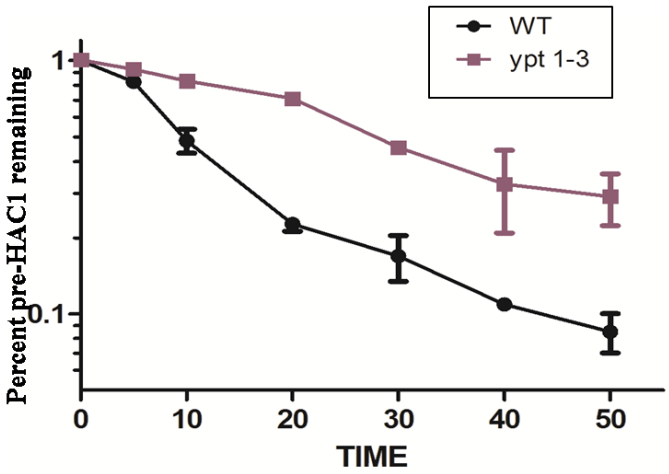

Figure4.1E *Decay rates of pre-HAC1 mRNA in wild type (black line), ypt1-3 (violet line). Decay rates were determined from three independent experiments (biological replicates) by q-PCR analysis (using primer sets of HAC1 intronic sequences) and the intronic signals were normalized to SCR1 RNA and normalized signals (mean values ± SD were presented as the fraction of remaining RNA (with respect to normalized signals at 0 min) vs. time of incubation in the presence of 1, 10-phenanthroline.*

4.2. Investigating the functional relationship between the Rab-GTPase Ypt1p and Nuclear Decay Machinery

As stated above, it is not known how the *HAC1* pre-mRNA is specifically selected, targeted and degraded by the nuclear exosome/CTEXT in absence of ER stress (Sarkar et al., 2018). During the activation of UPR, this decay becomes diminished and thereby resulting in the formation of a population of *HAC1* pre-mRNA most of which carry the BE element. This situation causes an increased delivery of the *HAC1* pre-mRNA to the Ire1p foci causing a more efficient splicing, translation and huge burst of Hac1p protein (Sarkar et al., 2018). Our recent finding from a different study suggested that Nrd1p-Nab3p-Sen1p (NNS) complex acts as the exosome-specificity-factor (ESF) and the differential recruitment of trimeric NNS onto aberrant and special messages marks them as the exosomal target for decay(Singh et al., 2021). Based on our preliminary data (shown in 4.1E), that Ypt1p promotes the decay of pre-*HAC1* mRNA, we hypothesized that Ypt1p might execute its decay by recruiting the nuclear exosome and CTEXT to the *HAC1* pre-mRNA. We dissected this possibility out in two

different steps. In the first step, we investigated the protein-protein interaction between Ypt1p, and components of exosome (such as Rrp6p), and its co-factor CTEXT (such as Cbc1p/Tif4631p) and NNS (such as Nrd1p/Nab3p) in WTand *ypt1-3* mutant cells in the absence and presence of ER stress. Later, we investigated the binding profile of the Ypt1p with the pre-*HAC1* mRNA as well as query if Ypt1p is recruited to the *HAC1* pre-mRNA co-transcriptionally.

To gain an insight into this protein-protein interaction between Ypt1p and the decay machinery, we carried out experiments in which WT cells expressing Ypt1p-TAP were grown in the absence and presence of stress(1µg/ml tunicamycin) followed by the co-immunoprecipitation using anti-TAP antibody. The immunoprecipitate (IP)from both the samples were further analyzed by western blotting for the presence of the CTEXT component Cbc1p/Tif4631p and Exosome component Rrp6p/Rrp4p using their respective antibodies (Fig. 3.2 B). As shown in this figure, the data displays quite convincingly, that in presence of ER stress, the interaction between Ypt1p and both the exosome and CTEXT components become significantly reduced,which is suggestive of the fact that Ypt1p binds significantly less to these decay factors. If we assume that interaction of Cbc1p and Rrp6p is the initial step of their recruitment onto *HAC1* pre-mRNA, these exciting finding therefore strongly support our model that possibly Ypt1p interacts and further recruits these decay factors onto *HAC1* pre-mRNA.

Later, we also analysed the protein-protein interactions between the *ypt1-3*(TAP tagged) and decay machinery in both presence and absence of stress(1µg/ml Tunicamycine).To investigate the interaction between mutant *ypt1-3* and the decay factors in both stressed and unstressed conditions, we further immunoprecipitated with anti-TAP antibody using the cell extract prepared from the mutant strain expressing mutant Ypt1-3-TAPfollowed by western

blotting for the presence of CTEXT and exosome components using their respective antibodies. As shown in Fig. 4.2 C, the interaction of Ypt1-3p with the Cbc1p/Tif4631p and Rrrp6p/Rrp4p were almost absent in presence of ER-stress.These collective data thus clearly suggests that the interaction profiles of Ypt1p with the components of exosome, CTEXT is consistent with our hypothesis that Ypt1p is playing a role in the recruitment of these decay factors on the *HAC1* pre-mRNA.

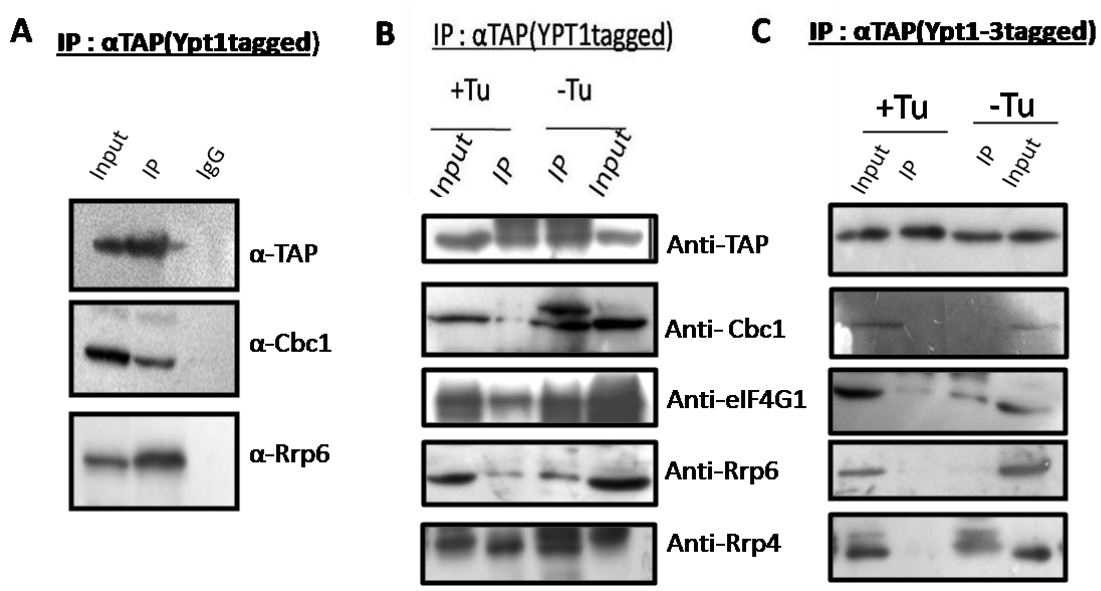

Fig. 4.2 A-C Ypt1p interacts with the exosome and CTEXT components during pre-activation of UPR in baker's yeast that is diminished during the activation stage:4.2A*Ypt1p strongly interacts with the CTEXT component Cbc1p and exosome component Rrp6p in the absence of ER stress. Lane1 detection of Ypt1p, Cbc1p and Rrp6p, using their respective antibodies from their input samples Lane 2 detection of Ypt1p, Cbc1p and Rrp6p from their immunoprecipitate samples and Lane 3 Mock IP using IgG****4.2B** *ER stress leads to the diminution of physical association between Ypt1p and Cbc1p/eIF4G1p/Rrp6p andRrp4p. For this experiment, the wild-type cells were grown in the absence and presence of tunicamycin (1 μg/ml final concentration) and the culture were grown for one more hour to saturate the induction. Samples were collected and Ypt1p was precipitated by standard immunoprecipitation followed by the detection of Cbc1p, eIF4G1, Rrp6p and Rrp4 using their respective antibodies. Lane 1and Lane 4 detection of Ypt1p, Cbc1p, Rrp6p and Rrp4p, using their respective antibodies from the Input samples of stressed and unstressed condition respectively, Lanes 2 and 3, detection of Ypt1p, Cbc1p, Rrp6p and Rrp4p using their respective antibodies from the immunoprecipitate samples of stressed and unstressed condition respectively.* ***4.2C*** *Physical association between ypt1-3 and Cbc1p, eIF4G1p, Rrp6p and Rrp4p. Lane 1and Lane 4 detection of ypt1-3p, Cbc1p, Rrp6p and Rrp4p using their respective antibodies from the Input samples of stressed and unstressed condition respectively, Lanes 2 and 3, detection of ypt1-3p, Cbc1p, Rrp6p and Rrp4p using their respective antibodies from the immunoprecipitate samples of stressed and unstressed condition respectively.*

Our previous observation of differential interaction of Ypt1p with the decay factors Cbc1p/Rrp6p in presence and absence of stress prompted us to investigate the mechanism of Ypt1p dependent nuclear mRNA surveillance.In order to gain an insight into this

phenomenon, it is necessary to determine the binding and recruitment profiles of Ypt1p onto the pre-*HAC1* and other mRNAs(substrates of CTEXT/Exosome).We addressed this issue first by studying the binding profiles of Ypt1p to the *HAC1* pre-mRNA and other mRNAs, which are substrates of CTEXT/Exosome by RNA-immunoprecipitation (RIP) in both stressed and unstressed condition. In this procedure, the stressed and unstressed yeast cells were harvested and collected. However, 10 minutes before harvesting, each culture was incubated with 1% formaldehyde that induces the formation of the crosslink between RNA and proteins. Cells were subsequently harvested, lysed, and the cell extract wasprecipitated by the anti-TAP antibody against Tap-tagged Ypt1p followed by the detection of Ypt1p by western blotting (using the same antibody) from half of the immunoprecipitate. The rest half of the immunoprecipitate is used to extract total RNA followed by the determination of the level of *HAC1* pre-mRNA and a negative control mRNAs not directly associated with UPR response (using the qRT-PCR assay). The level of the estimated *HAC1* pre-mRNA retrieved from the immunoprecipitate shouldserve as a precise indicator of the extent of binding of Ypt1p with *HAC1* pre-mRNA.

To address the first query, we first carried out an RNA-immuno-precipitation (RIP) experiment from the UV-cross-linked extracts prepared from yeast strains expressing Ypt1p-TAP with anti-TAP antibody followed by the estimation of the recovered pre-*HAC1* mRNA in the immunoprecipitate (IP) using qRT-PCR (Fig. 4.2 D). As shown in this figure, the abundance of the pre-*HAC1* mRNA recovered from the Ypt1p-TAP IP displayed a ≈ 5-6 folds enrichment relative to three non-specific mRNAs,*CYC1*, *LYS2* and *NCW2* that could be retrieved from the same IP sample (Fig. 4.2 E) thereby demonstrating the specificity of Ypt1p-*HAC1* pre-mRNA interaction.Furthermore, a comparison of pre-*HAC1* mRNA bound to Ypt1p in absence and presence of ER-stress showed thatthe binding affinity is significantly

lowered/reduced when ER stress was induced with tunicamycin(Fig. 4.2D and E). In contrast, binding of the other mRNA (negative control) like *CYC1*, *NCW2*, *LYS2* did not alter during these conditions. Together, these data presented in Fig. 4.2D, 4.2Esuggest that Ypt1p may play a significant functional role in selection and recognition of pre-*HAC1* mRNA in absence of ER stress possibly by recruiting the decay machinery, the exosome and CTEXT during UPR activation.

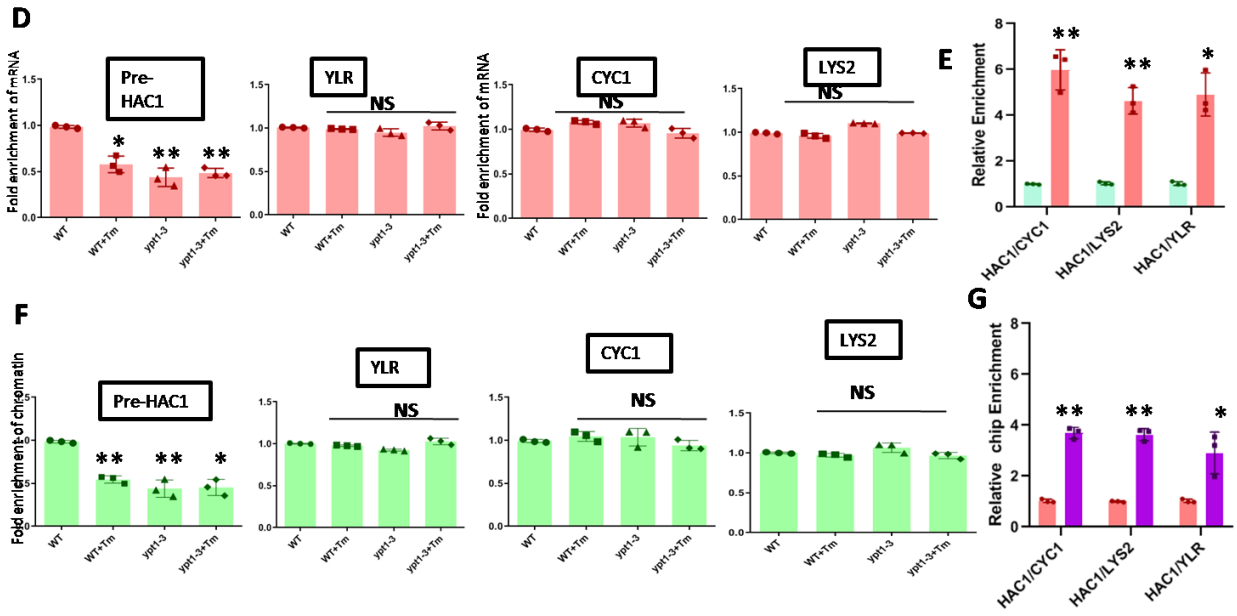

Fig. 4.2 D-G: Ypt1p binds to the HAC1 pre-mRNA during pre-activation of UPR in baker's yeast that is reversed during the activation stage: 4.2 D-E *Ypt1p strongly and selectively binds to the HAC1 pre-mRNA in the absence of ER stress. ER stress weakens association between Ypt1p and HAC1 pre-mRNA. For this experiment, the wild-type (yBD-457) cells and ypt1-3(yBD-565) cells were grown in the absence or presence of tunicamycin (1µg/ml final concentration) followed by crosslinking the culture to induce RNA-protein cross-linking. After harvesting, the Ypt1p and ypt1-3p was precipitated by anti-TAP antibody that was followed by the extraction of RNA and detection of either pre-HAC1 mRNA or a non-specific YLR, CYC1 and LYS2 mRNA (not related to UPR) by qRT-PCR. Their abundance was presented as scattered plot showing the mean and distribution of individual data points.* ***Fig. 4.2 F-G****Ypt1p strongly and selectively binds to the HAC1 chromatin in the absence of ER stress. ER stress weakens association between Ypt1p and HAC1 chromatin. For this experiment, the wild-type (yBD-457) cells and ypt1-3(yBD-565) cells were grown in the absence or presence of tunicamycin (1µg/ml final concentration) followed by crosslinking the culture to induce DNA-protein cross-linking. After harvesting, the Ypt1p and ypt1-3p was precipitated by anti-TAP antibody that was followed by the decrosslinking of DNA and detection of either intronic HAC1 chromatin or a non-specific YLR, CYC1 and LYS2 chromatin (not related to UPR) by qRT-PCR. Their abundance was presented as scattered plot showing the mean and distribution of individual data point. The average of three independent experiments is shown where immunoprecipitated (output) samples were normalized to input following quantification by real-time PCR. The error bars in the graph represent standard deviations (S.D.). The statistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, * <0.05,**<0.005 and ***<0.001, NS, not significant*

Previously it was demonstrated that the co-transcriptional recruitment/binding of the decay machinery exosome/TRAMP onto specific target aberrant mRNAs is crucial for the specific targeting and subsequent decay of the given mRNA(Honorine et al., 2011)(Singh et al., 2021). Furthermore, any protein factor, which promotes the selective recruitment of the decay machinery onto a specific RNA was demonstrated to be recruited first on to the target mRNA prior to the recruitment of the decay machinery (Singh et al., 2021). As stated earlier, these finding prompted us to build a model that Ypt1p itself undergoes co-transcriptional recruitment onto the pre-*HAC1* mRNA and further recruitsof the exosome/CTEXT onto the *HAC1* pre-mRNA. Consequently, we planned to extend our previous findings of differential binding of Ypt1p to *HAC1* pre-mRNA and decay factors by determining the profile of co-transcriptional recruitment of Ypt1ponto *HAC1* pre-mRNA by Chromatin-IP (ChIP) assay in WT and *ypt1-3* mutant cells in both stressed and unstressed condition.It should be noted here that, although the ChIP assay was believed to crosslink chromatin/DNA with the DNA-binding proteins, a vast majority of RNA binding proteins also remain associated to the transcribing messenger RNAs, which remain physically associated with the chromatin. RNA-ChIP, adapted from the classical DNA-ChIP, is now widely used to study and analyze the binding profile of transcription, splicing, export, and mRNA decay factors, which are recruited co-transcriptionally and remained strongly associated with the transcribing/maturing nascent mRNAs (D & D, 2012; Obrdlik & Percipalle, 2009). Before carrying out the actual comparison of Ypt1p occupancy profiles on pre-*HAC1* mRNA between various yeast strains and stress conditions by RNA-ChIP, we first determined if the ChIP signal generated from our assay procedure is highly specific. In our assay, we used the fragmented chromatin preparation prepared from yeast cells expressing Ypt1p-TAP that is precipitated with the anti-TAP antibody followed by quantification of the abundance of chromatin bound pre-

*HAC1*mRNA by qRT-PCR (Fig 4.2 D). As shown in Fig.4.2E, the abundance of the pre-*HAC1* mRNA recovered from the Ypt1p-TAP chromatin-IP samples exhibited a ≈3-4 folds enrichment relative to the abundance of the three other non-specific mRNAs, *CYC1*, *LYS2* and *NCW2* that could be recovered from the same chromatin-IP sample

Next, we determined the transcriptional activity of pre-*HAC1* locus in real time in WT and *ypt1-3* mutant cells in presence and absence of tunicamycin. Genomic occupancies of Rab-GTPase Ypt1pand Ypt1-3pproteins on *HAC1* locus was measured by the ChIP technique as an indicator of co-transcriptional recruitment of these factors activity. We also determined the occupancies of Ypt1p on others substrate mRNAs of CTEXT/exosome in different conditionsof UPR activity. As shown in Fig. 4.2 F, occupancy of Rab-GTPase Ypt1p on pre-*HAC1* locus displayed a dramatic difference in WT and *ypt1-3*mutant strains in the absence and presence of ER stress. However, no alteration in the occupancy of Ypt1p on other mRNAs like *LYS2*, *CYC1* and *IRE1*were observed. This data therefore clearly indicates that the recruitment of Ypt1p on pre-*HAC1* message is selective specific and reversible during different phases of UPR signalling response.

4.3 Investigating the functional relationship between the Rab-GTPase Ypt1p and Nuclear Decay Machinery

Next, we went further deep inside this story by addressing another interesting issue.It was previously postulated that the NNS complex acts as a primary Exosome Specificity Factor (ESF)(Kilchert et al., 2016; Vasiljeva & Buratowski, 2006)that was recently confirmed by our recent finding that indeed NNS complex was recruited upstream to the exosome/CTEXT components (Singh et al., 2021).Consequently, we query if NNS complex is involved in the differential nuclear decay of the pre-*HAC1* message and if involved, what functional role it plays. To this end, we first analysed the steady state levels of pre-*HAC1* mRNA in WT and

nrd1-1 mutant yeast strains.As shown in Fig. 4.3A, the pre-*HAC1* mRNA level was found to dramatically increase almost 6 to 7 fold in *nrd1-1*mutant strain compared to WT strainthereby suggesting that indeed NNS component, Nrd1p plays an important role in the rapid nuclear degradation of pre-HAC1 mRNA in absence of stress.

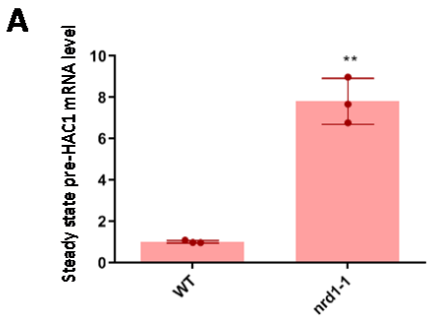

Fig. 4.3A*: Histogram depicting the steady state levels of total cellular HAC1 mRNA levels in wild type (yBD-148), nrd1-1 (yBD-177), strains using qPCR analysis using a primer pair specific to HAC1 exon 1. Transcript copy numbers/2 ng cDNA of each strain was normalized to SCR1 RNA levels in respective strains and are presented as means ± SE (n=3 for each strain). Normalized value of individual mRNA from normal samples was set to 1. The statistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, ∗<0.05, ∗∗<0.005 and ∗∗∗<0.001, NS, not significant.*

The significant enhancement of steady state level of pre-*HAC1* mRNA in *nrd1-1* mutant strain relative to WT strain posed an interesting question whether the binding and recruitment of Ypt1p onto *HAC1* mRNA is Nrd1p dependent or not. We addressed this interesting issue in two different sequential steps. In the first step, we investigated the protein-protein interaction between Ypt1p and NNS components during the stressed and unstressed conditions. Further, we also investigated the binding and recruitment profiles of Nrd1p on pre-*HAC1* mRNA during different phases of UPR signalling. As shown in Fig.4.3B, Ypt1p was found to associate strongly with the NNS component Nrd1p in absence of ER stress, which become significantly reduced when ER stress was employed. More interestingly, this interaction was abolished in the mutant Ypt1-3 protein (Fig.4.3C). Therefore, these data

support our hypothesis that recruitment/binding of Ypt1p on to pre-*HAC1* mRNA is NNS complex dependent.

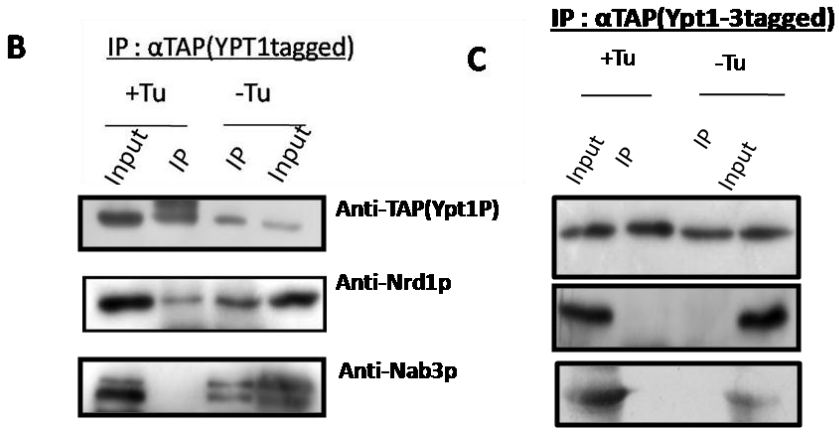

Fig. 4.3 B-CYpt1p interacts with the NNS components during pre-activation of UPR in baker's yeast that is diminished during the activation stage:4.3B *Ypt1p strongly interacts NNS component, Nrd1p and Nab3p in the absence of ER stress. ER stress leads to the diminution of physical association between Ypt1p and Nrd1p/Nab3p. For this experiment, the wild-type(yBD-457) and ypt1-3(yBD-565) cells were grown in the absence and presence of tunicamycin (1 µg/ml final concentration) and the culture were grown for one more hour to saturate the induction. Samples were collected and Ypt1p was precipitated by standard immunoprecipitation followed by the detection of Nrd1p and Nab3p using their respective antibodies. Lane 1and Lane 4 detection of Ypt1p, Nrd1p and Nab3p, using their respective antibodies from the Input samples of stressed and unstressed condition respectively, Lanes 2 and 3, detection of Ypt1p, Nrd1p and Nab3p using their respective antibodies from the immunoprecipitate samples of stressed and unstressed condition respectively.**4.3C** Interaction between ypt1-3p and NNS components abolished in the both presence (Lane 2) and absence (Lane3) of ER stress.*

To confirm this hypothesis further, we investigated the binding and recruitment profiles of Nrd1p on pre-*HAC1*mRNA and other three non-specific mRNAs as a negative control by RIP and ChIP experiment respectively. Notably, the study of differential recruitment of Nrd1p on to pre-*HAC1* mRNA is necessary to explain the possible mechanism of recruitment of Nrd1p on to pre-*HAC1*, which in turn may selectively recruit cofactor like CTEXT/Exosome. Most importantly, the data obtained from this systematic analysis will help us to determine if NNS complex acts as an ESF. As shown in Fig 4.3D and E, the binding and recruitment of Nrd1p on pre-*HAC1* mRNA decreased almost half in *ypt1-3*(mutant) strain (yBD-434) compared to WT (yBD-433) strain. Interestingly, no alteration was displayed in binding/recruitment profiles of Nrd1p on other Non-Specific mRNAs like *CYC1* and *IRE1*.So, these collective

data clearly concludes that therecruitment of Ypt1p on pre-*HAC1* mRNA takes place inspecific and Nrd1p dependent manner.

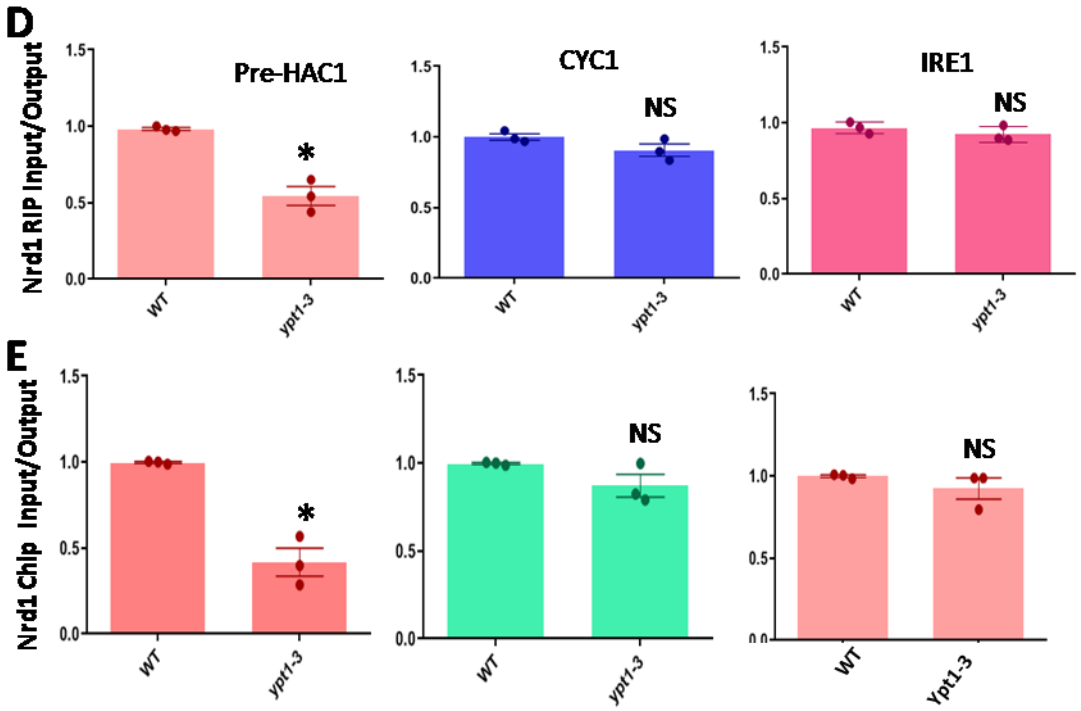

Fig.4.3 D-E: Nrd1p binds to the HAC1 pre-mRNA in WT cells that is diminished in absence of Ypt1p:4.3 D *Nrd1p strongly and selectively binds to the HAC1 pre-mRNA in presence of Ypt1p. For this experiment, the wild-type (yBD-433) cells and ypt1-3(yBD-434) cells were grown followed by crosslinking the culture to induce RNA-protein cross-linking. After harvesting, Nrd1p was precipitated by anti-Nrd1 antibody that was followed by the extraction of RNA and detection of either pre-HAC1 mRNA or a non-specific CYC1 and IRE1 mRNA by qRT-PCR. Their abundance was presented as scattered plot showing the mean and distribution of individual data points.* ***Fig. 4.3E****Nrd1p strongly and selectively binds to the HAC1 chromatin in presence of functional Ypt1p. For this experiment, the wild-type (yBD-433) cells and ypt1-3(yBD-434) cells were grown at 30ºC followed by crosslinking the culture to induce DNA-protein cross-linking. After harvesting, the Nrd1p was precipitated by anti-Nrd1 antibody that was followed by the decrosslinking of DNA and detection of either intronic HAC1 chromatin or a non-specific CYC1 and IRE1 chromatin by qRT-PCR. Their abundance was presented as scattered plot showing the mean and distribution of individual data point. The average of three independent experiments is shown where immunoprecipitated (output) samples were normalized to input following quantification by real-time PCR. The error bars in the graph represent standard deviations (S.D.). The statistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, * <0.05,**<0.005 and ***<0.001, NS, not significant*

Finally, to confirm our hypothesis further that Ypt1p facilitates/promotes the selective recruitment of the nuclear decay machinery via Nrd1p, we have further analysed the recruitment and binding profile of Cbc1p(CTEXT component) and Rrp6p(Exosome component) on pre-*HAC1* mRNA in WT and *nrd1-1*yeast strain by ChIP and RIP

experiments. The analysis of differential recruitment of Cbc1p and Rrp6p on to pre-*HAC1* mRNA is crucial to explain the possible mechanism of recruitment of Nrd1p onto pre-*HAC1*mRNA, which in turn may selectively recruit the decay machinery, CTEXT/Exosome onto it. As shown in Fig.4.3F, the recruitment of both Cbc1p and Rrp6p on to pre-*HAC1* mRNA found to decrease significantly in *nrd1-1* strain compared to WT strain. The binding profile of pre-*HAC1* mRNA to Cbc1p and Rrp6p also decreased in*nrd1-1* strain as shown in Fig 4.3G, H, whereas no significant change observed in others non-specific mRNAs like *CYC1* and *IRE1* mRNA. These collective data strongly concluded that Nrd1p is selectively recruited by the Ypt1p on the premature *HAC1* mRNA, which in turn recruits the nuclear exosome and the CTEXT Complex.

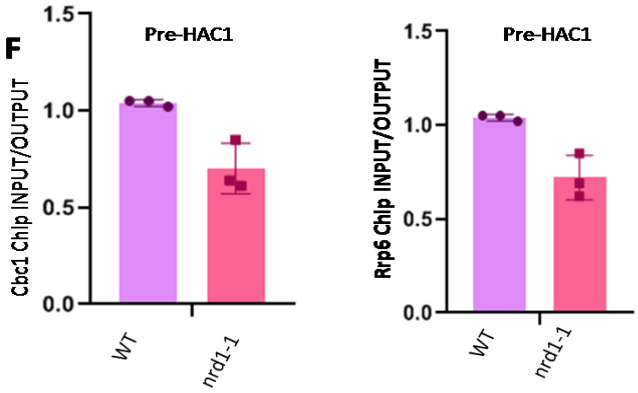

Fig.4.3 FRecruitment of Cbc1p and Rrp6p drastically decreases on pre-HAC1 messages in absence of functional Nrd1p*Quantification by real-time PCR of ChIP samples obtained from WT (yBD-157) and nrd1-1(yBD-158) strain Immunoprecipitation was performed using specific Cbc1 and TAP antibody(Rrp6-TAP tagged) and qRT-PCR analyses using oligonucleotide for the intronicHAC1 target.The average of three independentexperiments is shown where immunoprecipitated (output) samples were normalized to input followingquantification by real-time PCR. The error bars in the graph represent standard deviations (S.D.). Thestatistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, *<0.05,**<0.005 and ***<0.001, NS, not significant.*

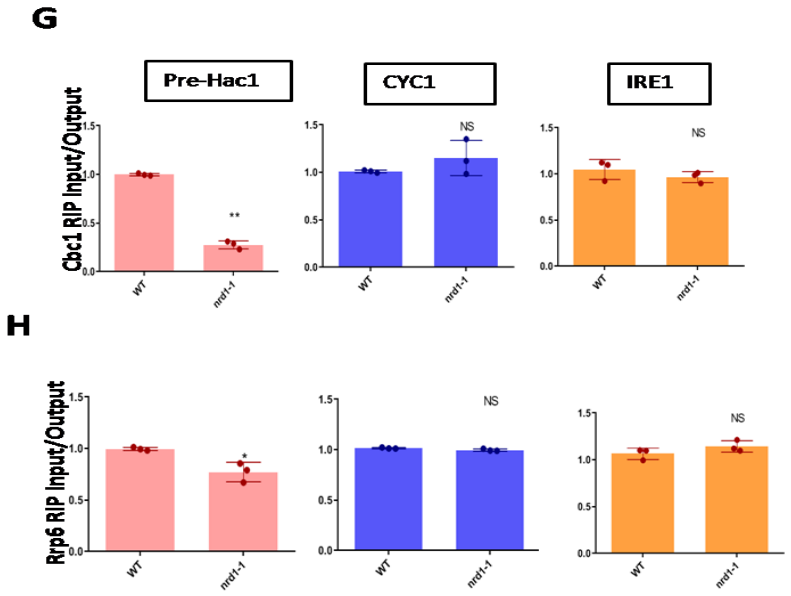

Figure.4.3Binding of Cbc1p and Rrp6p drastically decreases on pre-HAC1 messages in absence of functional Nrd1p*Quantification by real-time PCR of RNA-Immunoprecipitation samples obtained from WT (yBD-157) and nrd1-1(yBD-158) strain. Immunoprecipitation was performed using specific Cbc1****(Fig.4.3G)*** *and TAP antibody(Rrp6-TAP tagged)****(Fig.4.3H)*** *and qRT-PCR analyses using oligonucleotide for the intronicHAC1 target and non specific target CYC1 and IRE1 .The average of three independent experiments is shown where immunoprecipitated (output) samples were normalized to input following quantification by real-time PCR. The error bars in the graph represent standard deviations (S.D.). The statistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, * <0.05, **<0.005 and ***<0.001, NS, not significant.*

4.4 Ypt1p binds to a specific segment within the 3′-UTR of *HAC1* mRNA which permits its recognition and subsequent recruitment to Decay Machinery

In order to find out the molecular determinants present in *HAC1*pre-mRNA, which regulates its degradability via putative binding of Ypt1p; we asked whether the elimination of specific sequences in the *HAC1* transcript could prevent its recognition and subsequent recruitment to decay machinery. From our previous data obtained in this study, we concluded that Ypt1p by binding to pre-*HAC1* messages facilitates the recruitment of Nrd1p to pre-*HAC1* mRNA thereby targeting it to Nuclear Exosome/CTEXT. Previous study revealed that,*HAC1* 3′-UTR is necessary for Ypt1-*HAC1* interaction(Nikoleta G. Tsvetanova et al., 2012). We postulated that if any specific segment present in the 3′-UTR of pre-*HAC1* mRNA provides a binding-

site for Ypt1p, then elimination of this segment would abolish its rapid decay and prevent its participation in UPR response. Therefore, a series of deletion constructs (Fig. 4.4A) in 3′-UTR of this *HAC1* mRNA was constructed using a combination of restriction endonuclease-digestion and reverse PCR. In order to compare the steady state levels of the resulting deleted transcripts, with respect to the WT(full-length) message qRT-PCR assay was employed using primer pair OBD 321/322 (that encompasses pre-*HAC1*exon1-intron sequence).Final steady state levels of pre-*HAC1* mRNA were determined by normalizing their raw signals with respect to the *SCR1* signal. When the steady state levels of WT (FL) *HAC1* allele and its various deletion constructs were analyzed, *HAC1*-ΔR2 and *HAC1*-ΔBE mRNA levels were found to be very low, which was similar to the cellular level of WT *HAC1*(FL) mRNA (Fig 4.4B). In contrast, the abundance of *HAC1*-Δ3′-UTR and *HAC1*-ΔR1mRNAs were found to be almost 2 and 5 to 5.5 folds higher as compared to that WT *HAC1* (FL) mRNA level respectively (as shown in Fig 4.4B). Next, we also analyzed the decay rate of *HAC1* pre-mRNA in the WT *HAC1*(FL) and *HAC1*-ΔR1 strains after shutting their transcription off by 1, 10-Phenanthroline. As shown in Fig.4.4J, the decay rate of this mRNA was much diminished in *HAC1*-ΔR1 strain compared to WT *HAC1* (FL) strain thereby suggesting that region R1 harbours a putative.Ypt1p-binding/recognition element,which permits its recognition and recruitment to nuclear decay machinery.Collective findings thus revealed that Ypt1p binds to a region spanning the residues 1-154 (assuming the TGA of the *HAC1* ORF as 0 and the very next nucleotide in the 3′-UTR as +1) of the 3′-UTR of HAC1 pre-mRNA.

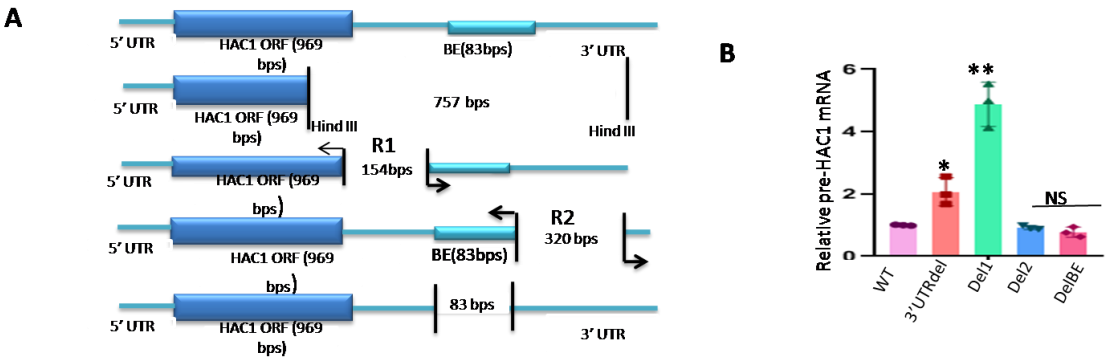

Figure4.4*The cellular abundance of pre-HAC1 message is governed by its putative Ypt1p binding element.****Fig.4.4A****the schematic representation of HAC1 mRNA along with the various deletions construct of the HAC1mRNA that were used in this study.* ***Fig.4.4B****The relative steady state levels of full length HAC1 mRNA and its various 3'UTR deleted version strains using qPCR analysis using a primer pair specific to HAC1 exon 1. Transcript copy numbers/2 ng cDNA of each strain was normalized to SCR1 RNA levels in respective strains and are presented as means ± SE (n=3 for each strain). Normalized value of individual mRNA from normal samples was set to 1. The statistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, ∗<0.05, ∗∗<0.005 and ∗∗∗<0.001, NS, not significant.*

The finding of the Ypt1p-dependent regulation of the abundance of pre-*HAC1*messages by a putative Ypt1p-binding element raised an interesting question: whether the elimination of this binding element from *HAC1* mRNA would lead to an alteration of its recognition and recruitment to the Ypt1p mediated nuclear decay machinery. To test this hypothesis, we determined whether the steady state levels of any of the deletion construct would display a similar type of abundance when expressed in those strain in which Rab-GTPase Ypt1p and CTEXT were inactivated by a mutation of the *YPT1*(*ypt1-3*) gene and by deletion of the *CBC1*(*cbc1*-Δ) gene relative to WT strain. As shown in Fig 4.4E &F, the steady state levels of *HAC1*-ΔR2 and *HAC1*-ΔBE mRNAs were enhanced in *ypt1-3* and *cbc1*-Δ strains as compared to WT strain. The abundance of the *HAC1*-Δ3′-UTR (Fig 4.4C) and *HAC1*-ΔR1 (Fig 4.4D) messages did not alter any further in absence of Ypt1p- and Cbc1p-deficient strain relative to other deleted messages. This finding firmly confirmed our hypothesis that both *HAC1*-ΔR2and *HAC1*-ΔBE mRNAs were recognized by Ypt1p, which in turn recruited to

nuclear decay machinery leading to its rapid degradation in Ypt1p/NNS dependent manner.Consistently, Ypt1p mutation resulted in the diminution of nuclear Exosome/CTEXT dependent degradation resulting in their stabilization of steady state levels. In contrast, *HAC1*-Δ3′-UTR and *HAC1*-ΔR1 messages displayed a very high steady state levels in all the WT, *ypt1-3* and *cbc1*-Δ strains because these mRNAs are not subjected to Ypt1p dependent Nuclear Exosome/CTEXT action. Therefore, the binding element is present within the first 154 bp region of *HAC1* 3′-UTR mRNA, which is responsible for binding to the Ypt1p and subsequent nuclear decay of *HAC1* mRNA.We call this region as the YBS (**Y**pt1p **B**inding **S**ite).

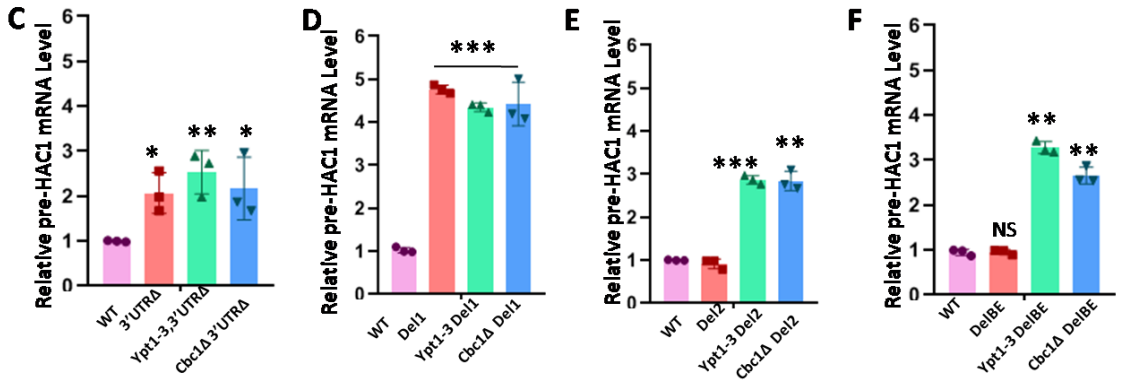

Figure.4.4 *The relative steady state levels of full length HAC1 mRNA and its various3'UTR deleted versions in wild type ypt1-3, cbc1Δ strains using qPCR analysis with a primer pair specific to HAC1 exon 1.* ***Fig.4.4C****. The relative steady state levels of FL HAC1 mRNA and 3'UTR deleted HAC1 mRNAin WT, ypt1-3 and cbc1Δ strains.****Fig.4.4D****.The relative steady state levels of FL HAC1 mRNA and ΔR1-HAC1 mRNA in WT, ypt1-3 and cbc1Δ strains.* ***Fig.4.4E****The relative steady state levels of FL HAC1 mRNA and ΔR2-HAC1 mRNA in WT, ypt1-3 and cbc1Δ strains.****Fig.4.4F****The relative steady state levels of FL HAC1 mRNA and ΔBE-HAC1 mRNA in WT, ypt1-3 and cbc1Δ strains. Transcript copy numbers/2 ng cDNA of each strain was normalized to SCR1 RNA levels in respective strains and are presented as means ± SE (n=3 for each strain). Normalized value of individual mRNA from normal samples was set to 1. The statistical significance of difference as reflected in the ranges of p-values estimated from Student's two-tailed t-tests for a given pair of test strains for each message are presented with following symbols, ⁎<0.05, ⁎⁎<0.005 and ⁎⁎⁎<0.001, NS, not significant.*

To strengthen the above finding, we have used Streptotag-based affinity purification system(Windbichler & Schroeder, 2006) to confirm that the (Ypt1p) is the putative *trans*-acting factor that binds to the YBS in the *HAC1* mRNA. For this experiment, a sense primer

consisting of a T7 promoter and complimentary region of *HAC1*gene sequence and an anti-sense primer consisting of a complementary region of *HAC1* gene and Streptotag aptamer sequence flanking the 154 nt region of *HAC1*-3′-UTR and 83 nt of BE region were used in the PCR reaction (Fig.4.4G). The PCR reaction used a cloned version of *HAC1* gene as template to yield a linear PCR DNA product. This PCR amplicon consists of an upstream T7 promoter followed by the Ypt1p-bindingelement-streptotagaptamer. This DNA sequencewas further usedas the template for *in vitro* transcription reactionusing T7 RNA polymerase.1 mM dihydrostreptomycin was then coupled to epoxy-activated Sepharose 6B resin followed by the formation of an *in-vitro* hybrid RNA aptamer-protein complex by mixing hybrid Ypt1p binding element-aptamer RNA with 1 mg of protein extract prepared from WT strain expressing Ypt1p-TAP protein. This complex was further loaded onto streptomycin Sepharose 6B column, washed with tRNA and finally the bound protein complex was eluted with streptomycin. The total proteins were characterized by SDS-PAGE electrophoresis followed by silver staining of the gel. To evaluate whether the Ypt1p was present in the eluate, the input and two eluate samples from the streptotag affinity chromatography was subjected to western blot analysis using anti-TAP antibody (Ypt1p TAP tagged). As shown in Fig. 4.4I, both the input and 1steluate samples, in which the154 bp *cis*-acting region. YBS was used as bait in the Streptotag procedure exhibited the signals specific for the Ypt1p protein (TAP tagged Ypt1p, MW: 45 kDa). Interestingly, however, 2ndeluate samples, in which the BE region was used as bait, did not display any western signal, which confirmed that the protein is present in only the eluate from the 154 *cis*-acting region thus confirming that it binds *in-vitro* to the 154 nt *cis*-acting YBS element.

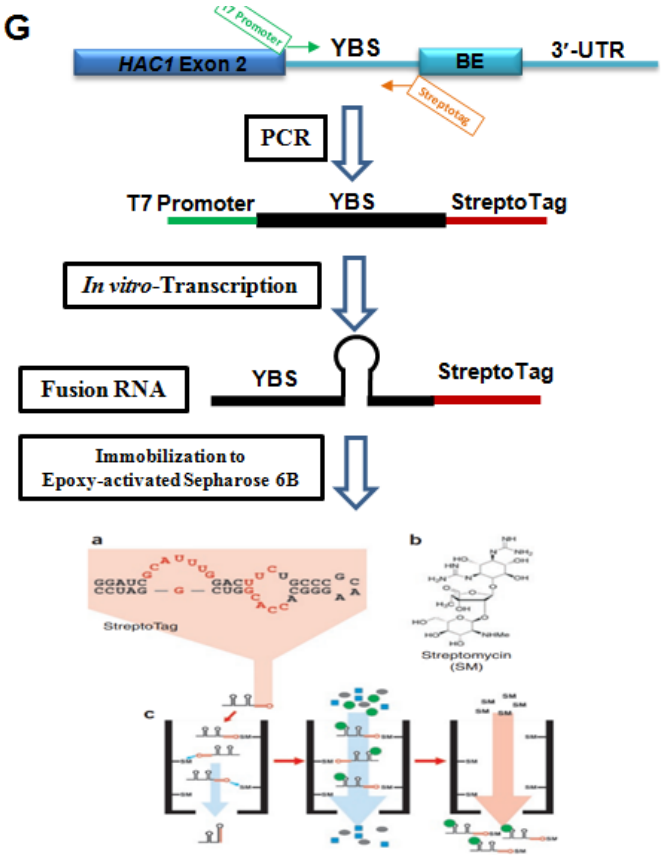

Fig. 4.4G *Rationale of Strepto-tag affinity purification System*

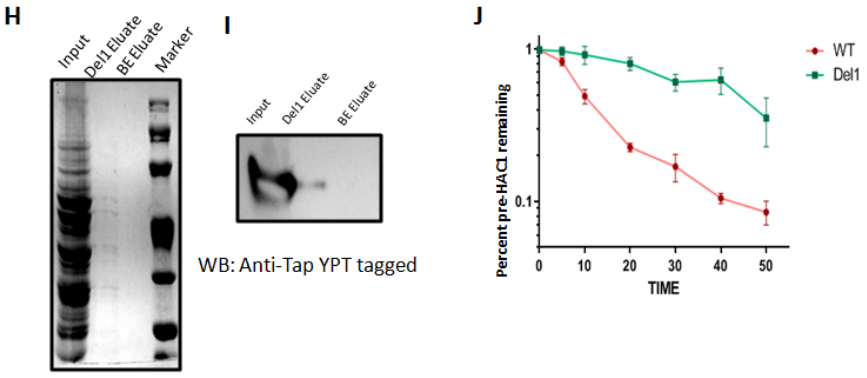

Figure.4.4*The putative trans-acting factor (Ypt1p) binding to the specific region of 3'UTR of HAC1 mRNA.. Fig.4.4 H Proteins bound to the 154 nt Del1 region of 3'UTR of HAC1 mRNA was eluted from dihydrostreptomycin coupled sepharose 6B column followed by electrophoresis in a 10% SDS PAGE gel and immunoblotting* ***(Fig.4.4I)*** *with Anti-TAP antibody(Ypt1-TAP tagged).* ***Fig.4.4J*** *Decay rates of pre-HAC1 mRNA in wild type (red line), ΔR1 HAC1(geen line). Decay rates were determined from three independent experiments (biological replicates) by q-PCR analysis (using primer sets of HAC1 intronic sequences) and the intronic signals were normalized to SCR1 RNA and normalized signals (mean values ± SD were presented as the fraction of remaining RNA (with respect to normalized signals at 0 min) vs. time of incubation in the presence of 1, 10-phenanthroline.*

4.5.Intra-Cellular distribution of *HAC1* mRNA and RabGTPase Ypt1p

Ourpreviousfindings,thus, established that the nuclear exosome/CTEXT while attacking the precursor-*HAC1* mRNA from its 3′-termini encounters the 3′-BE element on the very first hand before it hits any of the more 5′-part of the transcript body. Thus, by maintaining a critical concentration of 3′-BE of pre-*HAC1* mRNA, CTEXT potentially control the targeting and recruitment of the *HAC1* pre-mRNA to the Ire1p foci (Sarkar et al., 2018). It was demonstrated in our previous study that higher and more efficient targeting and recruitment of the precursor-*HAC1* mRNA to the Ire1p foci in*cbc1*-Δ strain as compared to the WT strain (Sarkar et al., 2018). In this study, we established that Rab-GTPase Ypt1p facilitates the recognition and recruitment of pre-*HAC1* mRNA to nuclear decay machinery. As a result, a higher amount of intact *HAC1* pre-mRNA species carrying a functional 3′-BE is predicted to exist in a *ypt1-3* strain as compared to the WT strain leading to the more efficient recruitment of precursor *HAC1* mRNA to Ire1p foci is expected to take place in this strain.

We, therefore, investigated this hypothesis by determining the intracellular distribution of *HAC1* mRNA in a WT and *ypt1-3* mutant strains, both of which co-express *HAC1-NRE* mRNA (an engineered version of *HAC1* construct carrying an RNA aptamer with a 22 nt RNA module identical to nucleolin recognition element, NRE), a GFP-Nucleolin fusion protein (Anshu et al., 2015) and Ire1p-RFP (Mannan et al., 2013) (Ire1p protein tagged with RFP) (Fig.4.5A) using confocal imaging.The *in situ* distribution/localization of $HAC1^{NRE}$-NRE-GFP mRNA and Ire1p-RFP (Fig.4.5A) clearly shows that in WT cells, where Rab-GTPase Ypt1p is functioning optimally, GFP signal representing the *HAC1* precursor/mature mRNA appears to be dispersed and extent of co-localization is less ,which is depicted by less number of yellow spots (see merged panel of Fig. 4.5A). Interestingly, the level of *HAC1-NRE* mRNA is lower in WTstrain as compared to *ypt1-3* strain since pre-*HAC1* mRNA

undergoes rapid degradation the nuclear exosome/CTEXT, which is in agreement with our previous genetic and molecular data. Under the same condition, the *HAC1*^{NRE}-NRE-GFP signal in the *ypt1-3* strain is stronger due to diminished degradation of the *HAC1* mRNA in this strain. Most importantly, as shown in Fig. 4.5A, the intense and frequent co-localization of *HAC1*^{NRE}-NRE-GFP with the Ire1p-YFP was observed in *ypt1-3* strain.This finding, thus clearly indicates higher and more efficient targeting of pre-*HAC1* mRNA to Ire1p focioccurs in absence of functional Ypt1p.

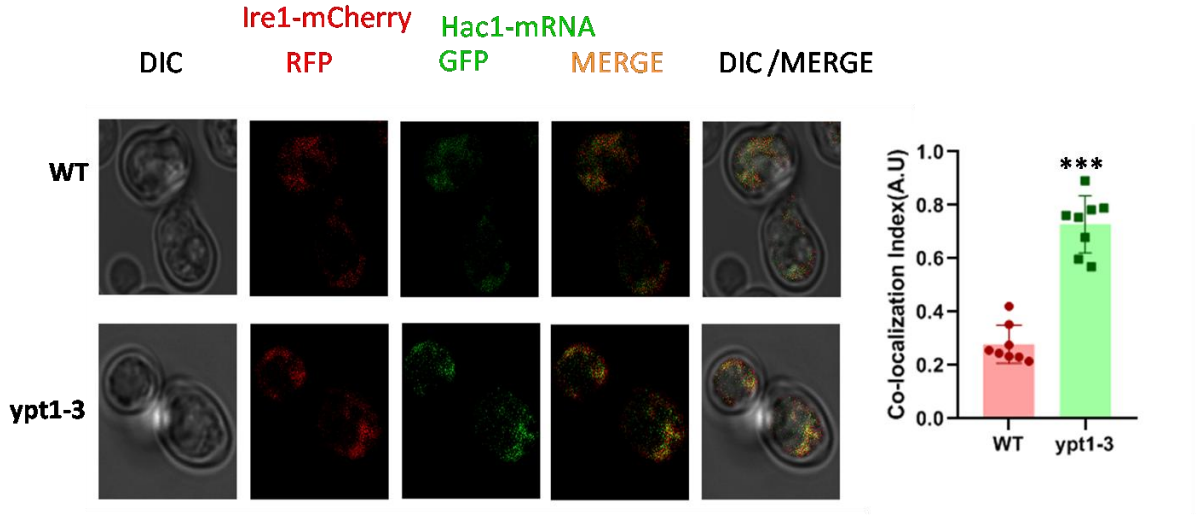

Figure4.5. Higher recruitment of pre-HAC1 mRNA to Ire1p foci in ypt1-3 cells. Localization of HAC1NRE mRNA decorated with NRE-GFP2 and Ire1-RFP following the induction of ER stress by 1 µg/ml tunicamycin in wild type and ypt1-3 strain. The images were captured and processed as described in Materials and methods. Histogram depicting the co-localization of HAC1^{NRE}*-GFP-nucleolinm RNA into Ire1p-RFP foci at 10 minutes after ER stress by 1 mg/ml tunicamycin in a WT and ypt1-3strain. Co-localization index for HAC1*^{NRE}*-GFP-nucleolin recruitment into Ire1 foci was expressed in arbitrary units. Co-localizationindex (Pearson correlation coefficient, PCC) were determined as described in the method section.*

Next, We also determined the cellular distribution of Rab-GTPase Ypt1p in both ER stressed and unstressed condition. To determine whether Ypt1p localizes to the nucleus, the protein was expressed from their native promoter as C-terminally TAP tagged fusion proteins and their intra-cellular localization was observed by indirect immunofluorescence followed by confocal imaging. As shown in Fig.4.5B, the degree of nuclear localization of Ypt1p (TAP

tagged) in unstressed condition is significantly higher to that of stressed condition. In stressed condition, more dispersed cytoplasmic localization of Ypt1p with dramatically less

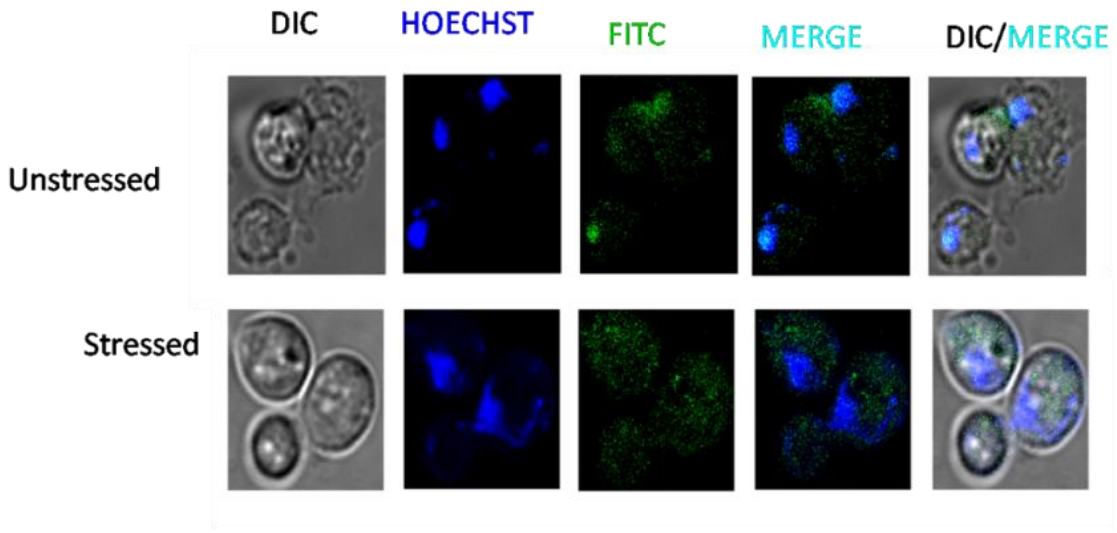

Figure 4.5 B*Ypt1p relocalizes to the cytoplasm during the ER stress. Localization of Ypt1p expressed from their native promoter as C-terminally TAP tagged by immunofluorescence using Rabbit Anti-TAP antibody as primary and anti-rabbit secondary FITC conjugated as secondary antibody in both unstressed(upper panel) and stressed (1µg/ml Tunicamycin)(lower panel) condition.*

localization in the nucleus was noted in all visible fields. This interesting finding suggests that in absence of ER-stress, Ypt1p is primarily localized in the nucleus whereas ER stress promotes its redistribution to cytoplasm leading to significant exclusion from the nuclear territory (Fig. 4.5B, Merge Panel). This novel finding not only supports our hypothesis but also provides a mechanism by which Ypt1p-association with pre-*HAC1* mRNA could be reversed when the ER stress was employed.

4.6. Conclusion

Studies presented in this chapter depict the role of Ypt1p in controlling the activity and the signaling dynamics of the unfolded protein response in baker's yeast *S. cerevisiae*. Our findings revealed that inactivation of the *YPT1* gene caused the mutant cells to be able to grow and survive more efficiently when they were challenged with the ER-stress inducer

tunicamycin. Further analysis showed that Ypt1p stimulates the exosomal decay of the pre-*HAC1* mRNA in absence of ER-stress by recruiting the NNS complex followed by the CTEXT and the nuclear exosome on to this pre-mRNA. Interestingly, our observation suggests that Ypt1p is able to sense the ER stress independently on the Ire1p. Notably, our findings also indicated that Ypt1p is a shuttling protein, which is primarily localized in the nucleus although about 25-40 percent of the protein is also distributed in the cytoplasm. ER-stress induces the transport of this protein from the nucleus to cytoplasm resulting in the loss of binding of Ypt1p from the pre-*HAC1* mRNA. Ypt1p dissociation leads to the lack of recruitment of the decay factors on to the pre-*HAC1* mRNA resulting in its reduced degradation when cells encounter ER-stress. Our observation thus provides a strong mechanism of the reversible signaling dynamics of UPR pathway in baker's yeast.

More interestingly, our data indicates that Ypt1p may act as another independent sensor of the UPR system. Notably the kinase-endoribonuclease Ire1p acts as the other well-known sensor of the unfolded proteins in the ER lumen and subsequently transduces the signal to splice-out the pre-*HAC1* message via the induction of its auto-phosphorylation, oligomerization, and formation of the endoribonuclease active site. Ypt1p upon sensing the ER-stress signal promptly redistributes from the nucleus. Although, it is not clear how the nuclear Ypt1p can sense the protein unfolding signal in ER lumen, it is conceivable that accumulation of massive protein unfolding in ER lumen may trigger a rapid redistribution kinetics in Ypt1p leading to its rapid movement from nucleus to cytoplasm. Nonetheless, it is really interesting to note that tow independent ER-stress sensors and response circuits exist in baker's yeast. One involves the Ire1p circuit that results in the formation of the machinery (*trans*-acting component) of the non-canonical splicing while the other involves the Ypt1p circuit that leads to the optimum delivery of the *HAC1* pre-mRNA substrate (*cis*-acting

component) of the non-spliceosomal splicing. Most importantly, both of these independent circuits converge to the key event of the regulation of UPR –the non-canonical splicing. Although, currently we are unclear about the benefit of having two independent circuits to induce UPR, but triggering two independent circuits simultaneously and independently help in causing a massive amplification of two outputs together at the same time thereby generating a robust and strong output response in a stepwise fashion within a very narrow window of time. This rapid generation of the signal may help the cells to respond to restore the protein folding homeostasis within the ER-lumen following a huge protein unfolding. Future research will be directed to gain a thorough understanding about how the Ypt1p sensor functions and undergoes fine regulation.

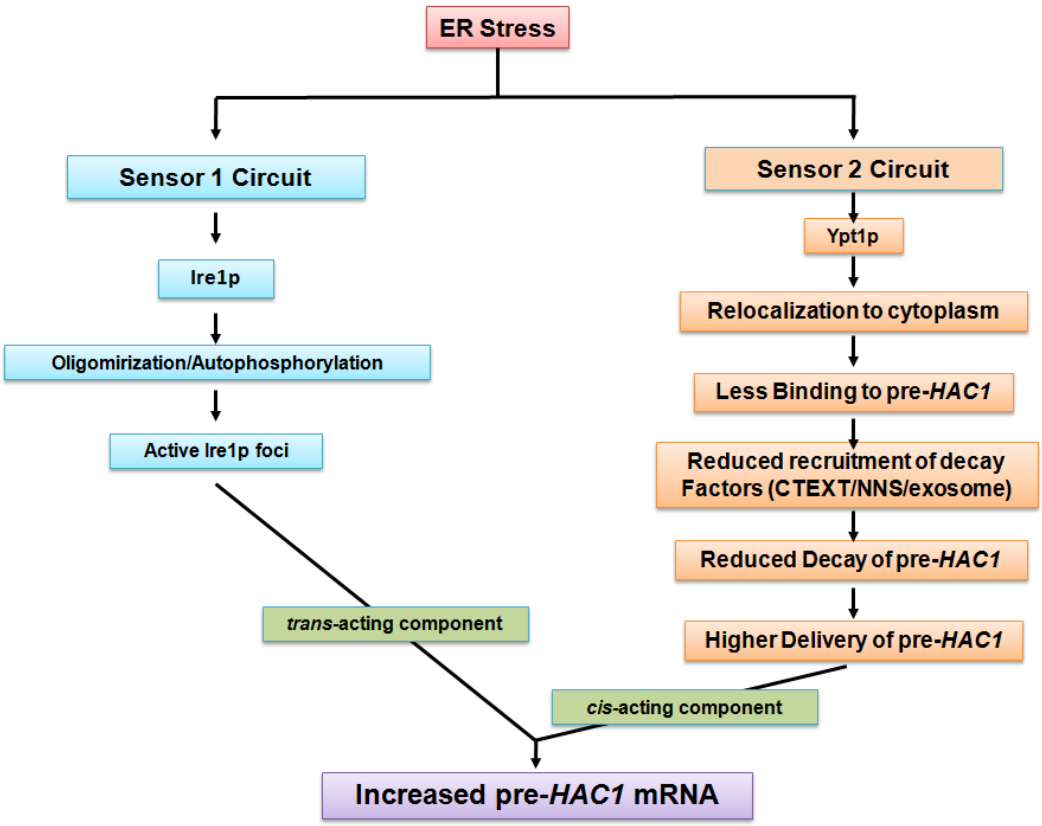

Fig.4.5 Two independent ER sensor circuit

CHAPTER 5

Discussions

Discussion:

The endoplasmic reticulum (ER) is a major depot of folding and maturation of numerous proteins in eukaryotic cells. This organelle has an incredible ability to adapt its folding capacity to match with the cellular requirement to synthesize proteins within the ER. However, diverse kinds of extracellular insults and aberrations in protein folding may result in a disparity in the normal protein folding activity of ER thereby leading to the accumulation of misfolded protein, dubbed "ER stress." The unfolded protein response (UPR) is an intracellular signaling system that readjusts ER folding capacity to restore protein homeostasis within the ER lumen. The key transcription activator of UPR signaling, Hac1p, responds to the ER stress by aiding in the massive transcription of genes encoding various ER chaperones and heat-shock proteins. The bulk amount of chaperones promptly refolds the unfolded proteins within the ER lumen and thereby restores the ER homeostasis. *HAC1* gene bears an intron and in absence of the ER-stress, the majority of the transcripts of this gene remain in the unspliced form as the pre-*HAC1* mRNA. Although, the ribosome initiates the translation of this pre-*HAC1* mRNA in absence of ER-stress, the translation elongation is typically halted by a secondary structure formed between the 5′-UTR and the intron of this pre-mRNA resulting in the formation of either very little or no Hac1p protein.

In baker's yeast, *Saccharomyces cerevisiae* unfolding of proteins within the ER lumen is primarily sensed by ER-membrane-resident protein, a kinase-endoribonuclease, Ire1p, which undergoes a prompt oligomerization on sensing the ER-stress. Oligomerization results in the formation of an active RNase domain (located on the cytoplasmic side of ER membrane) leading to the non-canonical splicing of the unspliced pre-*HAC1* mRNA that is aided by Ire1p and tRNA ligase Rlg1p. Translation of the mature spliced mRNA produces copious amount

of Hac1p protein, which is immediately brought to nucleus to trigger the transactivation of the genes encoding ER chaperones.

As stated in the introduction, previous work from our laboratory established that during normal condition in absence of ER-stress, pre-*HAC1* mRNA undergoes a dynamic and reversible decay in the nucleus by the nuclear exosome/CTEXT from its 3′-termini (Sarkar et al., 2018). This accelerated decay produces a population of *HAC1* pre-mRNA, majority of which lack a key *cis*-element in their 3′-UTR, dubbed BE (Aragon et al., 2010). Notably the BE prompts a rapid transport of the unspliced pre-*HAC1* mRNA from the site of transcription in the nucleus to the Ire1p oligomeric foci on the ER surface. Absence of the BE in most of the members in the population of unspliced pre-*HAC1* mRNA prevents its transport and recruitment to the Ire1p foci. ER-stress caused a diminished 3′→5′ decay of the pre-*HAC1* mRNA thereby causing the retention of the BE element in the transcript body of the most of the pre-*HAC1* mRNA in the resulting population. This situation leads to an increased and improved delivery of the pre-*HAC1* mRNA to the Ire1p foci resulting in the enhanced amount of Ire1p-dependent splicing of the delivered pre-*HAC1* mRNA (Sarkar et al., 2018). Increased splicing and subsequent translation finally caused a significantly higher abundance of Hac1p during the ER-stress, which further promotes the transcription of genes encoding ER-chaperones and heat-shock proteins to refold back the unfolded proteins and thereby restores ER-homeostasis.

Although the pre-*HAC1* mRNA was previously reported to undergo a selective and preferential nuclear decay by the nuclear exosome/CTEXT dependent manner, it remained unclear how *HAC1* pre-mRNA was specifically recognized from the vast majority of the other normal messages and targeted in a selective fashion by the nuclear decay machinery. Recent evidence that a Rab-GTPase Ypt1p by binding to the unspliced *HAC1* message

altered the stability prompted us to explore if Ypt1p plays any key role in the recognition and targeting of the *HAC1* mRNA. This RabGTPase Ypt1p was previously known to be an essential modulator of ER-to-Golgi transport in the secretory pathway (Jedd et al., 1995). Our data demonstrated that Ypt1p plays an important role in the initial recognition of pre-*HAC1* mRNA via its specific and selective recruitment onto pre-*HAC1* mRNA followed by subsequent recruitment of the exosome-specificity factor (ESF), NNS complex. The NNS complex in turn, loads the CTEXT and the nuclear exosome onto the pre-*HAC1* mRNA to bring about its preferential nuclear decay. Recruitment of Ypt1p onto pre-*HAC1* mRNA in preferential manner thus provides a mechanism by which this precursor message may be recognized by the decay machinery in a selective fashion in the vast majority of normal and other aberrant messages. Involvement of Rab-GTPase Ypt1p thus provides a means by which the initial recognition of the pre-*HAC1* mRNA may be accomplished.

Consistent with the directionality of the exosomal degradation of pre-*HAC1* mRNA in the 3′→5′ direction that typically initiates from the extreme 3′-termini of the precursor message, our data suggests that Ypt1p binds to the 5′-part (in a segment encompassing the residues 1-154 with respect to the TGA codon) of the 3′-UTR of pre-*HAC1* mRNA. Binding of the Ypt1p further recruits the NNS complex presumably in the vicinity of the *HAC1* pre-mRNA and thereby assists loading of the nuclear exosome and CTEXT for its dynamic and reversible decay. Our observation that Ypt1p actively and strongly interacts with the components of the NNS complex (Fig. 3A), CTEXT (Cbc1p and Tif4631p) and the nuclear exosome (Rrp6p) (Fig. 2A) therefore strongly supports this idea, which was reversed when ER stress was imposed.

In a good agreement of a nuclear role of Ypt1p, we found that it is a shuttling protein instead of primarily cytoplasmic. In absence of ER-stress, a majority of cellular Ypt1p is distributed

in the nucleus and upon imposition of ER-stress, it redistributes itself to the cytosol (Fig. 3). Although, the exact mechanism of reversible binding of Ypt1p to the pre-*HAC1* mRNA during various phases of ER-stress is currently unknown, our finding of the relocalization of the Ypt1p to the cytosol during the activation phase of the UPR suggests that this reversibility could be partly accomplished by its relocalization from the nucleus to cytoplasm. However, it is currently unclear what factor drives Ypt1p from the nucleus to the cytoplasm when ER-stress is enforced. Future research should unfold the mechanism of Ypt1p redistribution during various phases of UPR, such as activation and attenuation.

In this work, we provide evidence, the cellular Rab-GTPase Ypt1p that plays a vital role in the protein trafficking from ER-to-Golgi (Jedd et al., 1995), is also involved in the regulation of UPR signalling dynamics in baker's yeast *Saccharomyces cerevisiae*. Our data demonstrates that Ypt1p distributes principally to the nucleus in absence of ER stress thereby recruited efficiently onto the pre-*HAC1* mRNA and thereby recruiting the nuclear mRNA surveillance factors Nrd1p, Nab3p, CTEXT and exosome components onto it to bring about its kinetic decay to preferentially eliminates the BE element from the resulting population of pre-*HAC1* mRNA. The elimination of BE element results in the formation of a population of *HAC1* precursor message, majority of which lack the BE. Imposition of the ER-stress triggers a rapid relocalization of Ypt1p from the nucleus-to-cytoplasm that further results in the decreased recruitment of itself and the other mRNA surveillance factors onto the pre-*HAC1* mRNA leading to its diminished decay and thereby causing the production of a precursor RNA population, majority of which harbour the BE. The increased retention of BE in the resulting *HAC1* pre-mRNA population leads to its increased targeting and recruitment to the Ire1p foci onto ER surface causing its enhanced splicing.

Our finding of the existence of a separate layer of regulation in the control of cellular UPR dynamics suggests a possible mechanism by which a small extent of signalling cue is able to amplify the final output of this pathway in an optimal fashion. It is notable that the protein unfolding status within the ER lumen is sensed by two different sensors, which is eventually translated into the massive production of Hac1p. While one sensor of protein unfolding is Ire1p, the other sensor of ER-stress is probably the Ypt1p. As reported by numerous laboratories, the sensing of the unfolding status by Ire1p is followed by its oligomerization leading to the activation of its RNase domain to eventually splice out the *HAC1* intron (Credle et al., 2005). Sensing by Ypt1p is manifested by its quick relocalization from the nucleus to the cytoplasm thereby causing its decreased recruitment onto pre-*HAC1* mRNA resulting in its diminished decay and increased delivery to Ire1p foci. Remarkably, therefore, the discovery of the second regulatory circuit involving nuclear mRNA decay working upstream of the Ire1p sensing circuit thus indicates that during the activation of UPR in response to ER-stress the outputs of two independent circuits converge on the Ire1p interface on ER surface thereby magnifying the transduced output in a synergistic fashion. Ypt1p thus plays a vital role in sensing the ER-stress signal independently and transduce that signal to the Ire1p kinase-endoribonuclease via the increased delivery of pre-*HAC1* mRNA during the activation phase of ER-stress cycle.

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Nuclear mRNA degradation tunes the gain of the unfolded protein response in *Saccharomyces cerevisiae*

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ABSTRACT

Unfolded protein response (UPR) is triggered by the accumulation of unfolded proteins in the endoplasmic reticulum (ER), which is accomplished by a dramatic induction of genes encoding ER chaperones. Activation of these genes involves their rapid transcription by Hac1p, encoded by the *HAC1* precursor transcript harboring an intron and a bipartite element (3′-BE) in the 3′-UTR. ER stress facilitates intracellular targeting and recruitment of *HAC1* pre-mRNA to Ire1p foci (requiring 3′-BE), leading to its non-spliceosomal splicing mediated by Ire1p/Rlg1p. A critical concentration of the pre-*HAC1* harboring a functional 3′-BE element is governed by its 3′→5′ decay by the nuclear exosome/DRN. In the absence of stress, pre-*HAC1* mRNA undergoes a rapid and kinetic 3′→5′ decay leading to a precursor pool, the majority of which lack the BE element. Stress, in contrast, causes a diminished decay, thus resulting in the production of a population with an increased abundance of pre-*HAC1* mRNA carrying an intact BE, which facilitates its more efficient recruitment to Ire1p foci. This mechanism plays a crucial role in the timely activation of UPR and its prompt attenuation following the accomplishment of homeostasis. Thus, a kinetic mRNA decay provides a novel paradigm for mRNA targeting and regulation of gene expression.

INTRODUCTION

The nuclear exosome selectively degrades a diverse spectrum of aberrant messages in the nucleus of *Saccharomyces cerevisiae* and thereby safeguards the cells from their detrimental effects (1–6). These aberrant transcripts include transcription elongation assembly-defective transcripts (7–12), intron-containing splice-defective messages (13–17), transcription termination-defective 3′-extended read-through transcripts (18,19), and export-defective global and individual mRNAs (20,21). The exosome is a large multi-protein complex consisting of eleven 3′→5′ exoribonucleases and a few associated nuclear and cytoplasmic cofactors (4,5,22–24). Of these eleven subunits, nine core subunits are arranged in a barrel (known as EXO9), which consists of two stacked-ring structures—a 'trimeric' cap (composed of Rrp4p, Rrp40p and Csl4p) placed on the top of a 'hexameric' ring (consisting of Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p) with a central channel. Accordingly, EXO9 contacts two catalytically active subunits, a processive tenth subunit (Dis3p/Rrp44p, possessing both endo- and 3′→5′ exoribonuclease activity) and a distributive eleventh subunit (Rrp6p, 3′→5′ exoribonuclease) from opposite sides to form EXO11$^{Dis3p+Rrp6p}$ (24–26). Notably, EXO11$^{Dis3p+Rrp6p}$ requires ancillary cofactors to target and degrade a specific class of substrate RNA since it lacks the catalytic specificity. Consequently, a set of ancillary cofactors, referred to as the exosome-cofactors, collectively modulates and enhances exosomal function to target the appropriate substrate RNA class among the plethora of RNA substrates in a highly selective manner (27). The TRAMP complex in *S. cerevisiae* provides the best-characterized example of such exosome-cofactor, which consists of a noncanonical poly(A) polymerase, Trf4p (Pap2p)/Trf5p, a DExH box RNA helicase, Mtr4p (6,28,29); and the Zn-knuckle RNA binding proteins, Air1p/2p (6,29). Moreover, DRN (Decay of RNA in the nucleus) defines another nuclear mRNA decay apparatus, which consists of the nuclear mRNA cap-binding protein Cbc1p/2p (18,30), Rrp6p (20) and two nucleocytoplasmic shuttling proteins, Upf3p and Tif4631p (30). Previous genetic data and a body of recent findings indicate that DRN also acts as an additional exosomal cofactor by modulating the nuclear exosome function and by assisting the exosome to selectively target and degrade a few distinct classes of aberrant nuclear mRNA substrates (31).

A global transcriptomic analysis revealed an enhancement in the stability and the steady-state levels of nearly two hundred normal messages including *HAC1* mRNA in both *cbc1*-Δ and *rrp6*-Δ yeast strains, both of which are deficient in the nuclear exosome/DRN function (32). No-

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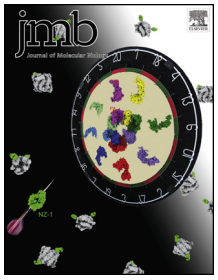

Nuclear mRNA Surveillance Mechanisms: Function and Links to Human Disease

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Abstract

Production of export-competent mRNAs involves transcription and a series of dynamic processing and modification events of pre-messenger RNAs in the nucleus. Mutations in the genes encoding the transcription and mRNP processing machinery and the complexities involved in the biogenesis events lead to the formation of aberrant messages. These faulty transcripts are promptly eliminated by the nuclear RNA exosome and its cofactors to safeguard the cells and organisms from genetic catastrophe. Mutations in the components of the core nuclear exosome and its cofactors lead to the tissue-specific dysfunction of exosomal activities, which are linked to diverse human diseases and disorders. In this article, we examine the structure and function of both the yeast and human RNA exosome complex and its cofactors, discuss the nature of the various altered amino acid residues implicated in these diseases with the speculative mechanisms of the mutation-induced disorders and project the frontier and prospective avenues of the future research in this field.

Introduction

Messenger RNA surveillance mechanisms limit the unwanted accumulation of the faulty messages produced due to error-prone mRNP biogenesis [1–9]. From *Saccharomyces cerevisiae* to human, the nuclear exosome and its various cofactors play a vital role in the elimination of these aberrant transcripts in the nucleus. Consistent with its importance, mutations in the nuclear exosome and its cofactors are implicated in various diseases in humans. Here, we review the structure and functions of the nuclear exosome and its cofactors in baker's yeast and humans and examine the links between the mutations in core subunits of the exosome and its co-factors and various human diseases.

mRNP biogenesis: the birth and the early life of mRNAs in the nucleus

In eukaryotes, mRNA biogenesis involves the transcription by RNA polymerase II, capping of the primary transcript at the 5′-end, splicing, and site-specific cleavage/polyadenylation event at the 3′-end of the message [10–20] (Fig. 1). During these events, each transcript interacts and associates with a vast repertoire of mRNA-binding factors and heterogeneous nuclear ribonucleoproteins. Association of the mRNA with these factors dictates the subsequent fates of maturing transcripts later in their life (such as the ability of their nuclear export and competence of their translation) [21–23]. Concerted and coordinated execution of these nuclear events ultimately leads to the formation of a mature export-competent mRNP followed by its nuclear export (Fig. 1) [10–18,23–26]. Remarkably, the transcription and nuclear pre-mRNA processing events are physically and functionally coupled *via* the C-terminal domain (CTD) of the Rpb1p, the largest subunit of RNA polymerase II [20,27–30], which lowers the risk of formation of unproductive and aberrant mRNPs [22,23,29,31–34].

The necessity for mRNP surveillance and quality control

Genetic suppressor analysis and subsequent molecular studies led to the consensus that despite

Chapter 9

Determination of the Stability and Intracellular (Intra-Nuclear) Targeting and Recruitment of Pre-*HAC1* mRNA in the *Saccharomyces cerevisiae* During the Activation of UPR

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Abstract

Nuclear degradation of pre-*HAC1* mRNA and its subsequent targeting plays a vital role in the activation as well as attenuation of Unfolded Protein Response (UPR) in *Saccharomyces cerevisiae*. Accurate measurement of the degradation of precursor *HAC1* mRNA therefore appears vital to determine the phase of activation or attenuation of this important intracellular signaling pathway. Typically, pre-*HAC1* mRNA degradation is measured by the transcription shut-off experiment in which RNA Polymerase II transcription is inhibited by a potent transcription inhibitor to prevent the de novo synthesis of all Polymerase II transcripts followed by the measurement of the steady-state levels of a specific (e.g., pre-*HAC1*) mRNA at different times after the inhibition of the transcription. The rate of the decay is subsequently determined from the slope of the decay curve and is expressed as half-life ($T_{1/2}$). Estimation of the half-life values and comparison of this parameter determined under different physiological cues (such as in absence or presence of redox/ER/heat stress) gives a good estimate of the stability of the mRNA under these conditions and helps gaining an insight into the mechanism of the biological process such as activation or attenuation of UPR.

Intra-nuclear targeting of the pre-*HAC1* mRNA from the site of its transcription to the site of non-canonical splicing, where the kinase-endonuclease Ire1p clusters into the oligomeric structures constitutes an important aspect of the activation of Unfolded Protein Response pathway. These oligomeric structures are detectable as the Ire1p foci/spot in distinct locations across the nuclear-ER membrane under confocal micrograph using immunofluorescence procedure. Extent of the targeting of the pre-*HAC1* mRNA is measurable in a quantified manner by co-expressing fluorescent-labeled pre-*HAC1* mRNA and Ire1p protein followed by estimating their co-localization using FACS (Fluorescence-Activated Cell Sorter) analysis. Here, we describe detailed protocol of both determination of intra-nuclear decay rate and targeting-frequency of pre-*HAC1* mRNA that were optimized in our laboratory.

Key words *HAC1*, mRNA degradation, Unfolded Protein Response, mRNA targeting, mRNA Recruitment

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