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Characterization of mRNA export and nuclear quality control under heat stress in the yeast *Saccharomyces cerevisiae*

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submitted by

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Göttingen, February 2017

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Affidavit

I hereby declare that this doctoral thesis entitled "Characterization of mRNA export and nuclear quality control under heat stress in the yeast *Saccharomyces cerevisiae*" has been written independently with no other sources and aids than quoted.

Göttingen, February 2017

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1 Abstract

Elevated temperatures and other changes of the environment a cell lives in, can have tremendous impact on cellular processes and integrity. To counteract these threats, cells have established various mechanisms to avoid severe damage and ensure survival. One of them is the highly-conserved heat shock response that results in expression of a special set of proteins among them chaperones, which aid denatured proteins in refolding and protect them from further destruction. This response to heat stress is accompanied by the disruption of normal cellular processes like general mRNA transcription, splicing and export in the nucleus and translation of housekeeping transcripts in the cytoplasm.

This study analyzed by what mechanisms the cell can distinguish between normal mRNAs that need to be blocked in the nucleus and stress responsive transcripts, which are required to be exported and translated. Several experiments show that while the normal mRNA export relies on the support of the shuttling adaptor proteins Npl3, Gbp2, Hrb1 and Nab2, a general dissociation of these proteins together with the export receptor Mex67 from mRNAs can be observed under stress, which explains the mRNA export block. Heat stress (HS) mRNAs in turn are exported by direct binding of Mex67 without the need of any known adaptor. This preferential binding of Mex67 to mRNAs expressed under heat stress is most likely facilitated through its early recruitment by the heat shock transcription factor Hsf1. Further experiments revealed that replacing the promoter of a normal mRNA with a stress responsive promoter and even artificially inserting an Hsf1 binding site can turn a housekeeping mRNA in a heat stress responsive transcript. All these mRNAs, strongly expressed under heat stress, strikingly bypass nuclear quality control and are quickly exported. Under normal conditions faulty mRNAs are labeled by the Mtr4-containing TRAMP complex and degraded by the nuclear Rrp6-containing exosome. Detection of errors in normal mRNAs is most likely coupled to the adaptor proteins, which might control correctness of every maturation step. As mRNA adaptor proteins are dispensable during HS mRNA transcription and export, this appears to be the mode of how heat stress transcripts evade to be quality controlled.

These mechanisms probably result in two different mRNA export ways: under normal conditions adaptor proteins facilitate a close quality control of an mRNA before Mex67 can associate and perform export, while upon heat stress direct binding by Mex67 results in a fast export of HS mRNAs that are not controlled for their quality. Thus, even though more faulty HS mRNA reach the cytoplasm, the fast response to stress appears to be more beneficial for survival.

2 Introduction

2.1 The life cycle of an mRNA

Adapting to changing environmental conditions demands cells to permanently adjust the composition of their proteome. While alterations in gene expression result in major changes, regulation of mRNA levels allows fine tuning of certain protein levels. Therefore, the place of mRNA transcription and its translation into proteins are physically separated. In the nucleus mRNAs are generated from their gene locus, mature and are packed with proteins into messenger ribonucleoprotein particles (mRNPs) that are exported to the cytoplasm. Here the mRNP undergoes compositional changes that enable translation of the mRNA into an amino acid chain at the ribosomes before it is finally degraded. At all points of its life the mRNA underlies control processes that either facilitate its further existence or promote its degradation. The following chapters will give a broad overview of these processes in the yeast *Saccharomyces cerevisiae*.

2.1.1 Transcription initiation and mRNA capping

In yeast and higher metazoans RNAs are transcribed by three RNA polymerases (RNAP). While RNAP I is responsible for transcription of ribosomal RNAs (rRNA) and RNAP III mainly synthesizes small RNAs like tRNAs or the 5S rRNA, the only polymerase performing mRNA transcription is RNAP II (Cramer et al., 2008). It consists of 12 proteins of which Rpb1 promotes its catalytic activity and contains a C-terminal domain (CTD) that represents a crucial platform for a plethora of regulating factors.

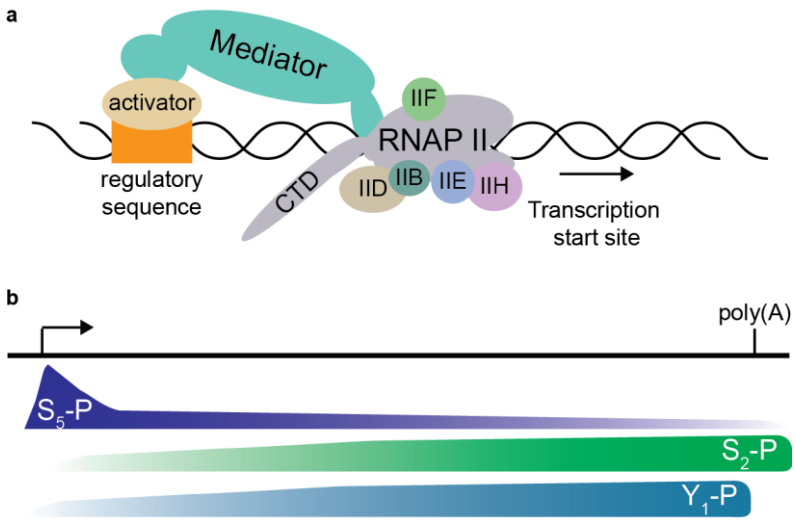

Figure 1: Transcription initiation requires a set of initiation factors and modification of the RNAP II CTD. a, The RNA polymerase II (grey) is recruited to the transcription start site by several initiation factors (IIB, IID, IIE, IIF, IIH). The Mediator complex transfers the information of regulating sequences to the transcription machinery. Adapted from (Björklund and Gustafsson, 2005). **b**, Schematic overview of the changes in the phosphorylation pattern of the Ser5, Ser2 and Thr1 of the RNAP II CTD during transcription. Adapted from (Meinel et al., 2013).

For transcription initiation the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIH, the Mediator complex and RNAP II binding to the genes promoter are needed (Figure 1a) (Björklund and Gustafsson, 2005; Hsin and Manley, 2012). Their recruitment and activity is in turn regulated by effectors specialized for a certain set of genes. Transcription initiation is closely linked to phosphorylation of the CTD. This C-terminal extension consists of multiple heptad repeats with the consensus sequence Thr_1-Ser_2-Pro_3-Thr_4-Ser_5-Pro_6-Ser_7 and is highly conserved (Heidemann et al., 2013; Hsin and Manley, 2012). Only their number changes as yeast's CTD consist of 26 heptads and vertebrates carry 52 copies (Corden, 1990). At the beginning of transcription Ser_5 and Ser_7 become phosphorylated and Ser_2 and Thr_4 are phosphorylated during elongation while earlier phosphorylations are removed. In general, Ser_5 is highly phosphorylated at the start and Ser_2 highly phosphorylated at the end of transcription (Figure 1b) (Hsin and Manley, 2012). These modifications seem to represent a universal CTD code that couples transcriptional progress with regulatory mechanisms irrespective of the gene's identity or length (Bataille et al., 2012; Bentley, 2005). Indeed, already the first co-transcriptional modification performed on the newly emerging mRNA, which is the synthesis of a 5' cap, is supported by Ser_5 phosphorylation of the CTD (Cho et al., 1997; Gu et al., 2010; Suh et al., 2010). The capping process adds a 5' guanine-N_7 cap over a triphosphate linker to the mRNA and requires the capping complex, which in yeast consists of the RNA triphosphatase Cet1 and the guanylyltransferase Ceg1 (Gu et al., 2010) (Figure 2).

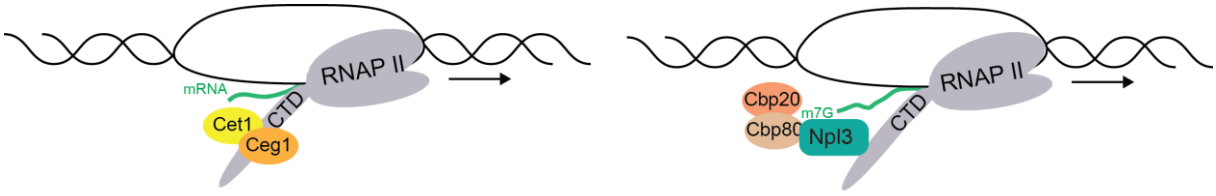

Figure 2: Capping and binding of the CBC is performed directly after transcription started. Once the mRNA leaves the polymerase, capping is carried out by Cet1/Ceg1. As soon as the m7G-cap is formed the cap binding complex composed of Cbp20 and Cbp80 assembles and the mRNA adaptor protein Npl3 binds to the newly emerging mRNA.

Methylation is carried out by the methyltransferase Abd1 (Furuichi and Shatkin, 2000). *In vitro* data identified that capping is occurring very early, already after synthesis of a 20 nt long RNA (Coppola et al., 1983). This is likely a mechanism to directly protect the new transcript as it was shown that unprocessed RNAs trigger their own capping by recruiting the capping apparatus (Martinez-Rucobo et al., 2015). Consistently, uncapped mRNAs are sensed and degraded (Jiao et al., 2010; Schwer et al., 1998).

After successful capping the heterodimeric cap-binding complex (CBC) consisting of Cbp20-Cbp80 binds to the cap structure and later supports export of the mature mRNA (Lewis and Izauralde, 1997). Export is among others facilitated by the serine/arginine (SR)-rich adaptor protein Npl3, which shuttles between nucleus and cytoplasm. Npl3 already interacts with RNAPII (Lei et al., 2001) as well as with the cap binding complex (Baejen et al., 2014; Shen et al., 2000) arguing for a very early recruitment of the adaptor protein Npl3 in the mRNA life.

2.1.2 Splicing of pre-mRNAs and recruitment of the THO/TREX complex

While the capping process and recruitment of interacting proteins is completed, the nascent mRNA grows as transcription continues. The second maturation step is the splicing process during that non-coding introns are excised from the pre-mRNA and the two flanking exons joined to a functional coding sequence. This conserved process is ubiquitous in higher eukaryotes while in yeast only very few mRNAs, mainly encoding ribosomal proteins, contain introns (Davis et al., 2000; Spingola et al., 1999). Recruitment of the spliceosome requires the presence of the CBC and is thus linked to successful 5' capping (Görnemann et al., 2005).

The spliceosome is a ribozyme machinery and consists of five small nuclear RNAs (snRNAs) termed U1, U2, U4, U5 and U6 that form complexes with numerous associated proteins (snRNPs). Basically, for splicing the U1 snRNP recognizes the 5' splice site in the intron and recruits the U2 snRNP, which binds the 3' splice site. Subsequently, the U4/5/6 tri snRNP associates and several catalytic steps result in cleavage of the 5' splice site and ligation of this end to the branch point forming a lariat structure. Finally, the 3' end of the intron is cleaved, the intron lariat released and the 3' and 5' ends of the two exons are ligated (Will and Lührmann, 2006).

The process of splicing is connected to the recruitment of the elongation complex THO, consisting of Hpr1, Tho2, Mft1 and Thp2. Association of the THO complex with the RNA is regulated by the sumoylation of Hpr1 (Bretes et al., 2014). THO forms the TREX complex by recruitment of the mRNA export factors Yra1 and Sub2 together with Tex1 during transcription elongation. Thus, the THO/TREX complex links transcription elongation with mRNA export (Abruzzi et al., 2004; Saguez et al., 2013; Strässer et al., 2002). The TREX complex can be recruited by the Prp19 splicing complex (Chanarat et al., 2011) or by direct interaction of the THO complex with the CTD of RNAP II, which explains its presence on both intron-containing and intronless genes (Abruzzi et al., 2004; Meinel et al., 2013). This process seems to be crucial

for general mRNA export as deletions of *HPR1*, *SUB2* or *YRA1* lead to accumulation of poly(A)$^+$-RNA in the nucleus (Jensen, Boulay, et al., 2001; Zenklusen et al., 2002).

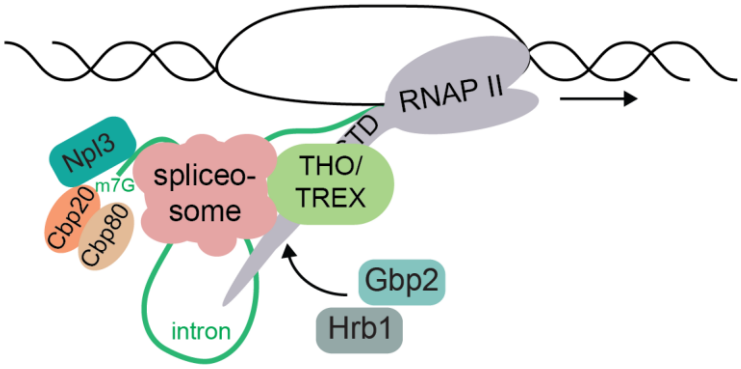

Figure 3: Splicing and recruitment of the THO/TREX complex. Recruitment of the multi protein spliceosome is supported by the CBC and leads to cleavage of the intron out of the nascent mRNA. The THO/TREX complex is recruited during or before splicing and correct processing of the mRNA leads to association of the mRNA adaptor proteins Gbp2 and Hrb1.

Furthermore, the two SR-rich mRNA adaptor proteins Gbp2 and Hrb1 interact with the THO/TREX complex and are thus loaded co-transcriptionally to the mRNA (Figure 3) (Hacker and Krebber, 2004; Hurt et al., 2004; Martínez-Lumbreras et al., 2016). Beside its role in promoting transcription elongation and simultaneous export-factor loading, the THO/TREX complex is involved in maintenance of genome integrity by controlling recombination events (García-Rubio et al., 2008) and preventing formation of DNA:RNA hybrids during transcription elongation (Huertas and Aguilera, 2003). Another factor that supports transcription elongation is the SR protein Npl3 that in its unphosphorylated form competes for a factor of the 3' processing machinery (Rna15) (Bucheli and Buratowski, 2005; Dermody et al., 2008) enabling a fine-tuned balance between elongation and cleavage of the 3' end.

2.1.3 3' end processing, polyadenylation

Once the entire mRNA is completely transcribed, sensing of a polyadenylation signal results in cleavage of the mRNA and synthesis of a poly(A)-tail. Again the CTD of the RNAP II supports efficient cleavage (Hsin and Manley, 2012; Ryan et al., 2002) and it is speculated that the 3' processing complex can join the transcription machinery already after transcription initiation (Chan et al., 2011), which would explain the importance of Npl3 to antagonize this process. Detection of the correct poly(A) site is supported by efficiency elements (EE), positioning

elements (PE) and U-rich elements located around the cleavage site (Tian and Graber, 2012). These elements are sensed by the 3' processing machinery consisting of CF1A, CF1B and CPF, which promotes endonucleolytic cleavage of the mRNA about 20 bp downstream of the poly(A) site. After cleavage the poly(A) polymerase Pap1 adds a 70-90 nt long adenosine tail to the 3' end of the RNA (Figure 4) (Bentley, 2005; Chan et al., 2011; Proudfoot, 2004).

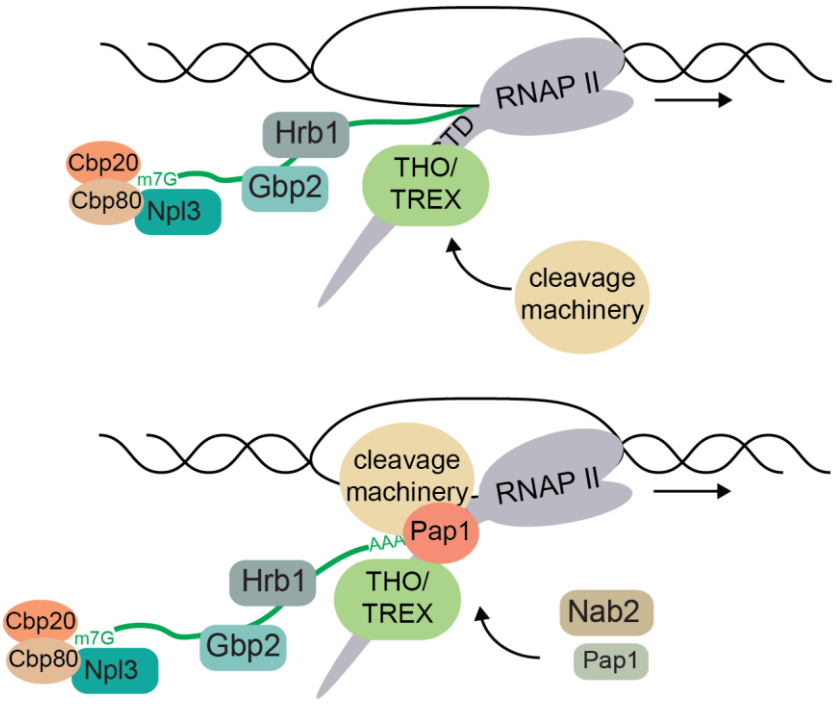

Figure 4: Recruitment of the cleavage machinery and 3' processing. Sensing of 3' processing elements leads to recruitment of the cleavage machinery (top). Upon 3' cleavage the polyadenylase Pap1 adds a poly(A) tail to the mRNA. This process is regulated by the poly(A)-binding proteins Nab2 and Pab1.

Length and quality of the poly(A) tail is controlled by the two poly(A)-binding proteins Nab2 and Pab1 (Dunn et al., 2005; Soucek et al., 2012).

The RNA downstream of the cleavage site is still associated with the RNAP II and is degraded by the 5' exonuclease Rat1 resulting in the release of the RNA polymerase (Kim et al., 2004). Formation of the mRNAs 3' end and the final export-competent messenger ribonucleoprotein particle (mRNP) is in turn closely regulated by the export factors Yra1 and Sub2 (Johnson et al., 2011; Rougemaille et al., 2008).

2.1.4 mRNA export adaptors and mRNP packaging

Export of mRNAs from the nucleus into the cytoplasm requires their covering with adaptor proteins that can be bound by the export receptor Mex67-Mtr2. The best characterized mRNA adaptors are the three SR-rich proteins Npl3, Gbp2 and Hrb1 together with the poly(A)-binding proteins Nab2 and Pab1. The *S. cerevisiae* SR proteins and Pab1 share a general structure with several RNA recognition motifs (RRM) crucial for their binding to RNAs (Figure 5).

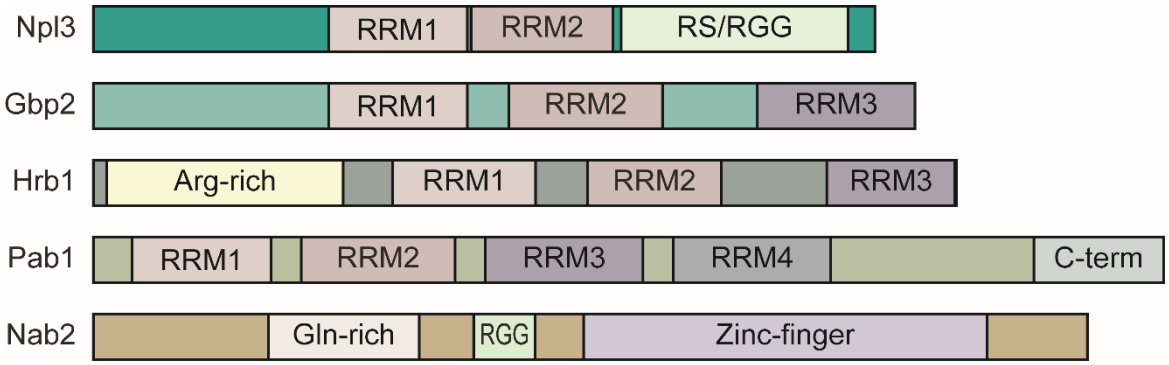

Figure 5: Schematic overview of the *Saccharomyces cerevisiae* RNA adaptor proteins. The three SR-proteins Npl3, Gbp2 and Hrb1 contain several RNA-recognition motifs (RRM), which also can be found in the poly(A)-binding protein Pab1. Nab2 in contrast has a different organization without RRM motifs. Some proteins contain regions enriched for certain amino acids: RS: Arg-Ser-rich; RGG: Arg-Gly-Gly-rich. Adapted from (Santos-Pereira et al., 2014).

The structure of Nab2 differs from this general organization as it lacks an RRM region. However, these proteins commonly bind RNA, whereas they have different functions on mRNA maturation and assemble with it at different time points. As presented above the first to bind to the newly emerging mRNA is Npl3 by interacting with the 5' cap structure (Lei et al., 2001; Shen et al., 2000). It further is among the factors required for efficient splicing (Kress et al., 2008) and elongation of the RNA (Dermody et al., 2008). Finally, correct 3' processing of the mRNA results in the dephosphorylation of Npl3 by the phosphatase Glc7 and recruitment of the export receptor Mex67 (Gilbert and Guthrie, 2004). Once the mRNA has matured completely, Npl3 shuttles with it into the cytoplasm where it becomes phosphorylated again by the kinase Sky1. This allows the nuclear importer Mtr10 to dissociate Npl3 from the mRNP and promote its re-import to the nucleus (Gilbert et al., 2001; Windgassen et al., 2004; Yun and Fu, 2000). Beside its role in mRNA export Npl3 is involved in transport of the large ribosomal subunit to the cytoplasm (Hackmann et al., 2011), translation initiation (Baierlein et al., 2013) and increasing evidence points to the importance of Npl3 in precise transcription and the export of most RNA species and not only mRNAs (Holmes et al., 2015; Santos-Pereira et al., 2014).

The broad involvement of Npl3 in gene expression is further supported by the finding that like the THO/TREX complex Npl3 stabilizes the genome by prevention of R-loop formation (Santos-Pereira et al., 2013).

The two other SR proteins Gbp2 and Hrb1 are also loaded early in the mRNAs life and accompany it into the cytoplasm. Unlike Npl3, they are loaded co-transcriptionally by the THO/TREX complex (Hacker and Krebber, 2004; Hurt et al., 2004), which is accomplished by interaction of the RRM3 domain of Gbp2 or Hrb1 with the THO/TREX complex (Martínez-Lumbreras et al., 2016). Unlike Npl3 they are not involved in efficient splicing itself , but rather control efficiency and correctness of this process (Hackmann et al., 2014). Once loaded they stay bound to the mRNA until having reached the cytoplasm and are recycled in a way comparable to Npl3 as most likely Gbp2 is phosphorylated by Sky1 and both, Gbp2 and Hrb1, are imported in the nucleus by Mtr10 (Hacker and Krebber, 2004; Windgassen and Krebber, 2003). Even though all three SR proteins are mainly nuclear and are actively reimported, some amounts can be found on translating mRNAs and thus may control translation (Windgassen et al., 2004).

The last processing step that results in association of RNA adaptor proteins is formation of the 3'end and its elongation with a poly(A) tail. The essential protein Nab2 is crucial for this process as it interacts with the polyadenosine sequence over its zinc finger domain (Figure 5) (Anderson et al., 1993; Kelly et al., 2007). Length and quality of the poly(A) tail are tightly regulated by an interplay of Nab2 and the nuclear exosome component Rrp6 (Schmid et al., 2012, 2015). Interestingly the mainly cytoplasmic poly(A) binding protein Pab1 can compensate for the deletion of *NAB2*, is required for proper release of the transcript from its site of transcription and seems to shuttle between nucleus and cytoplasm in mRNA export as well (Brune et al., 2005; Dunn et al., 2005; Soucek et al., 2012). However, the exact roles of these two similar proteins have not been elucidated yet. Beside its role in regulation and binding to the poly(A) tail, methylation of Nab2 by Hmt1 (Green et al., 2002) and docking the mRNA, to the nuclear-pore-associated protein Mlp1 (Fasken et al., 2008) are crucial components of mRNA export.

Another mRNA binding protein that joins the maturing mRNP is the recruiting factor Yra1. In contrast to the other adaptor proteins, Yra1 does not shuttle with the mRNA to the cytoplasm. Genome-wide analyses suggest loading of Yra1 early (5' end) and late (3' end) to the mRNA (Baejen et al., 2014), while earlier publications argue for a predominantly late recruitment (Lei et al., 2001; Lei and Silver, 2002). This would be consistent with the finding that Yra1 enhances

the interaction of Nab2 with the export receptor Mex67 (Iglesias et al., 2010). Loading of all three proteins, Yra1, Nab2 and Mex67, depends on the activity of the helicase Dbp2 and its interaction with Yra1 results in formation of a stable mRNP (Ma et al., 2013). Furthermore, the same domain of Yra1 is important for binding to either Sub2 or Mex67 (Strässer et al., 2002) arguing for a mutually exclusive interaction and a way of preventing premature mRNP export. Shortly before export of the matured mRNP, Yra1 is ubiquitinylated by the E3 ligase Tom1 resulting in its dissociation (Iglesias et al., 2010) leaving an export-competent particle with the shuttling adaptor proteins Npl3, Gbp2, Hrb1 and Nab2 and its interacting heterodimeric receptor Mex67-Mtr2 bound to it.

Formation of mRNPs is performed during the entire maturation of the mRNA and requires assembly of multiple factors. Some of them are replaced or dissociated once a process is completed, others accompany the mRNA until it reaches the cytoplasm. Detailed composition of these mRNPs has been studied and contribute more and more to the understanding of each of the maturation steps (Baejen et al., 2014; Mitchell and Parker, 2014). Thus, not only the coding sequence of the transcript, but also the 3' untranslated region of the mRNA and secondary structures therein, influence the mRNP composition (Freeberg et al., 2013; Liu et al., 2016).

2.1.5 The RNA export receptor Mex67-Mtr2

In *S. cerevisiae,* the essential factor for mRNA export is Mex67, which forms a heterodimeric complex with Mtr2. Together they contact the mRNP by interaction with the already bound adaptor proteins and thus facilitate its transport from the nucleus to the cytoplasm (Hobeika et al., 2009; Hurt et al., 2000). In general, Mex67 is mainly nuclear with enriched localization on the inner nuclear rim (Segref et al., 1997). Contrastingly, the well-studied temperature sensitive mutant *mex67-5* localizes to the cytoplasm (Segref et al., 1997). Together with its interaction partner Mtr2, Mex67 interacts with the nuclear pore complex (NPC) during export (Kadowaki et al., 1994; Santos-Rosa et al., 1998). Not only is Mex67 crucial for export of polymerase II transcripts during normal conditions, but also the export of stress-responsive transcripts is executed by Mex67 (Hurt et al., 2000). This role of Mex67-Mtr2 in mRNA export is highly conserved as its metazoan counterpart TAP-p15 (NXF1-NXT1) fulfills the same function in the cell (Cole and Scarcelli, 2006). In fact, expression of TAP-p15 can compensate for the loss of *MEX67/MTR2* in yeast (Katahira et al., 1999). The interaction of Mex67-Mtr2 with SR-rich adaptor proteins is conserved as well (Hargous et al., 2006; Müller-McNicoll et al., 2016),

arguing even more for the generality of this mechanism. Recent research in several organisms contributed to detailed knowledge about the structural setup of Mex67 and its interaction partner Mtr2. While Mtr2 is relatively small with only approximately 21 kDa in mass and only one domain, Mex67 is a protein of 67 kDa with four different domains (Figure 6).

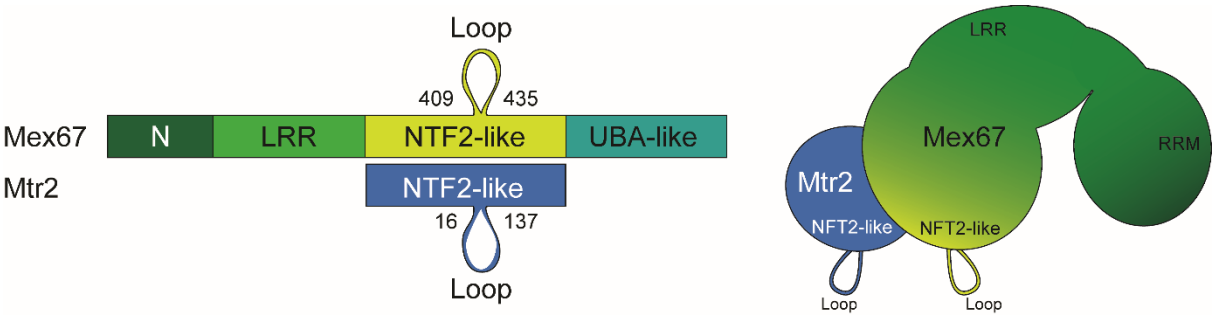

Figure 6: Schematic domain organization and globular structure of Mex67 and Mtr2. Mex67 consists of an N-terminus with an RNA-recognition motif (RRM), a leucine rich repeat (LRR), a nuclear transport factor 2-like (NTF2-like) domain and a ubiquitin-associated (UBA) domain. Mtr2 mainly consists of an NTF2-like domain. In both proteins, the NFT2-like domain contains a loop structure essential for binding to the 5S rRNA. Position of this structure is indicated by the number of the corresponding amino acids (left). Schematic domain organization according to crystallization experiments lacking the UBA-domain is shown on the right (adapted from (Aibara et al., 2015; Faza et al., 2012; Yao et al., 2007)).

The ubiquitin-associated (UBA) domain of Mex67 is important for interaction with the NPC during mRNA export. It binds to the phenylalanine-glycine (FG)-rich repeats of the nucleoporins that fill up the interior of the NPC, thus enabling transport of the mRNP through the hydrophobic meshwork. Additionally, the UBA domain links this export to transcription as it can interact with the ubiquitinylated component of the THO complex Hpr1 (Gwizdek et al., 2006). Interaction with either the loading complex THO or the NPC during export seems to be a mutually exclusive condition (Hobeika et al., 2009) indicating an organizational step for correct timing of transcription and export of mRNAs.

The nuclear transport factor 2-like (NTF2-like) domain is also able to bind the NPC as it contains several binding sites for FG-repeats, one of these sites however contributes to binding to Mtr2 (Senay et al., 2003). Interestingly, like the RNA-recognition motif (RRM) and the leucine rich repeat (LRR), the NFT2-like domain is able to bind to RNA as well (Aibara et al., 2015; Katahira et al., 2015). In fact, the NFT2-like domains of both, Mex67 and Mtr2 build a platform for interaction with binding partners. This way one site of the heterodimeric complex interacts with the mRNA while the other site contacts the NPC during export (Aibara et al., 2015; Yao et al., 2008).

Beside the role of Mex67-Mtr2 in general mRNA export, this complex additionally contributes to the nucleo-cytoplasmic transport of both ribosomal subunits (Yao et al., 2008). The two loop regions emerging from the NTF2-like domains of both receptors play an essential function in this process by directly contacting the ribosomal RNA (rRNA) (Faza et al., 2012; Yao et al., 2007). Consistent with that, mutations in the Mex67 loop lead to accumulation of the 5S rRNA as well as the entire 60S ribosomal subunit. Strikingly, mRNA is not retained under these normal conditions (Yao et al., 2007) arguing for different export modes for mRNPs and ribosomal subunits.

2.1.6 Nucleo-cytoplasmic export and its way through the NPC

While mRNA export from the nucleus to the cytoplasm is mainly carried out by the receptor dimer Mex67-Mtr2, another mode of export involving importin and exportin receptors called karyopherins is present in the cell. To promote this transport, karyopherins bind to proteins, which contain a nuclear export signal (NES) or nuclear localization signal (NLS) (Ström and Weis, 2001). For instance, the karyopherin Kap104 imports the adaptor protein Nab2 by binding to its NLS sequence (Lange et al., 2008). The entire nucleo-cytoplasmic transport by karyopherins relies on a gradient of RanGTP, whose concentration is high in the nucleus and low in the cytoplasm. For export the receptor binds to its cargo together with RanGTP in the nucleus. Upon arrival in the cytoplasm the cargo is released by hydrolysis of the GTP to GDP. The import process uses the same mechanism vice versa as the interaction of the importer with its cargo is released by binding of RanGTP to the receptor in the nucleus. Notably, unlike karyopherins, the transport process of Mex67-Mtr2 does not rely on a Ran gradient (Aitchison and Rout, 2012; Sloan et al., 2015). All karyopherins fulfill their role in transport by binding to proteins and contribute very little to RNA export. One factor involved here is Xpo1/CRM1 that exports snRNAs and the 60S ribosomal subunit by binding to adaptor proteins like Nmd3 (Sloan et al., 2015). While important for export of certain RNAs, Xpo1's involvement in transport of bulk mRNA is presumably neglectable (Neville and Rosbash, 1999).

Export of cargo from the nucleus to the cytoplasm requires passage through the nuclear envelope. To allow this, the lipid bilayer membrane around the nucleus is interspersed with high molecular nuclear pore complexes (NPC). This structure is an octagonally symmetrical cylinder build by nucleoporins (Nups) that form an inner and an outer ring. The inner channel is filled by phenylalanine-glycine (FG)-rich repeats of the nucleoporins localized there, building the hydrophobic meshwork that prevents diffusion of higher molecular particles.

On the nucleoplasmic site the complex consists of an octameric basket while on the face of the cytoplasm the NPC has filaments, both of which are made of FG-containing nucleoporins (Aitchison and Rout, 2012). The NPC itself and many associated complexes and factors contribute to an ordered expression of genes and control of the deriving transcripts prior to export. Thus, another complex involved in coupling transcription with export called TREX-2, composed of Sac3, Thp1, Sus1, Cdc31 and Sem1, interacts with the nuclear pore and the mRNP (Fischer et al., 2002; Niño et al., 2013; Stewart, 2010). The Sus1 subunit of TREX-2 is again part of the SAGA complex, which modifies histone complexes and regulates transcription activation (Niño et al., 2013). Both TREX-2 and SAGA are implicated in tightly coupling mRNA transcription to export in a process called gene gating. Here the chromosomal regions of highly transcribed genes are tethered to the NPC allowing for a direct export once transcription is completed (Oeffinger and Zenklusen, 2012). Even some of the Nups of the NPC themselve seem to regulate the process from transcription regulation to export (Burns and Wente, 2014). However, gene gating appears to happen only for very few transcripts that are highly expressed.

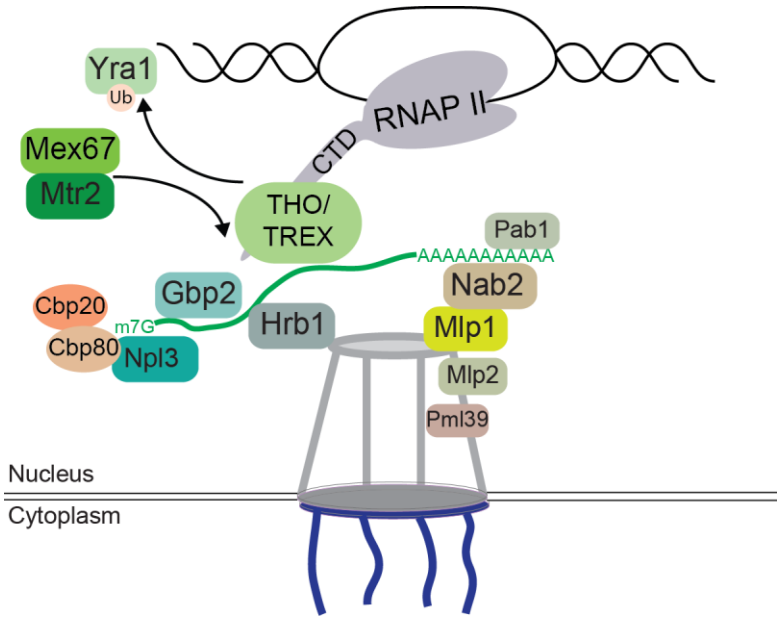

Figure 7: Formation of an export compatible mRNP requires dissociation of Yra1 and recruitment of Mex67-Mtr2. Ubiquitinylation of Yra1 dissociates it from the mRNP and enables binding of Mex67-Mtr2. Docking of the particle to the nuclear pore complex is facilitated by Nab2 binding to Mlp1, which controls the quality of the mRNA before its export.

The general mRNA export is facilitated by docking of the mRNP to the NPC. The myosin-like protein 1 (Mlp1) is located at the nuclear basket of the NPC and supports this docking by

interaction with the RNA adaptor protein Nab2 (Green et al., 2003). Together with its homolog Mlp2 and another associated factor, Pml39, Mlp1 resembles a final checkpoint for the correctness of the mRNP directly before it is exported (Figure 7) (Galy et al., 2004; Palancade et al., 2005).

Successful docking and transport of the mRNP through the NPC relies on several levels of regulation including modifications of the cargo and overall composition of the mRNP (Terry et al., 2007). This in turn influences the kinetics of how fast an mRNP finds a pore and is allowed to pass through it. This pore scanning termed process and disassembly of the cargo at the cytoplasmic site is rather the rate limiting step in export and not the passage through the NPC channel itself (Ben-Yishay et al., 2016). Translocation of both, karyopherin- and Mex67-Mtr2-transported cargo, through the NPC requires contact of the receptor with the FG-rich inner meshwork of the pore (Sloan et al., 2015; Ström and Weis, 2001). The amount and density of these FG repeats creates a barrier between both cellular compartments for larger particles, which needs the association of a stoichiometric number of transporters to overcome this barrier (Ribbeck and Görlich, 2002; Strawn et al., 2004).

At the cytoplasmic face of the NPC the exported mRNP undergoes several remodeling steps to finalize the export. These are supported by the cytoplasmic filament nucleoporins Nup159 and Nup42/Rip1 (Adams et al., 2014). Nup159 anchors the DEAD-box helicase Dbp5 to the NPC, which is activated by its Nup42-interacting cofactor Gle1 and the small molecule inositol hexakisphosphate. The helicase activity of Dbp5 leads to remodeling of the mRNA by ATP-hydrolysis and results in dissociation of export factors like Mex67 and Nab2 (Folkmann et al., 2011; Kelly and Corbett, 2009; Tieg and Krebber, 2013). Especially the release of Mex67 from the mRNP appears to be crucial to establish a directionality of the export process (Niño et al., 2013; Smith et al., 2015). The cap binding complex CBC is released by binding of importins to it, resulting in an exchange of CBC with the translation initiation factor eIF4E (Lewis and Izauralde, 1997). Consistent with that, the place of Nab2 at the poly(A) tail is taken over by the mainly cytoplasmic Pab1, which is important for translation as well (Kelly and Corbett, 2009). When the other adaptor proteins Npl3, Gbp2 and Hrb1 leave the exported mRNP is not completely elucidated yet, as they might accompany the mRNA during translation (Windgassen et al., 2004).

2.1.7 The cytoplasmic fate of an mRNA

Once the mRNA has reached the cytoplasm and cap structure and poly(A) tail are bound to their cytoplasmic interacting factors eIF4E and Pab1, recruitment of the initiation factors results in formation of a closed-loop structure in which the 5' end of the mRNA is connected to its 3' part. Then the 43S preinitiation complex containing the small ribosomal subunit binds to the mRNA and scans for the start codon, which in short leads to association of the 60S ribosomal subunit and initiation of translation. In the following the mRNA is translated into a polypeptide chain and translation is terminated upon sensing the stop codon by separation and recycling of the ribosomal subunits (Jackson et al., 2010). How long an mRNA is actively transcribed relies on certain factors. If the mRNA is erroneous in itself or cannot be properly translated for other reasons, surveillance mechanism like nonsense-mediated decay (NMD), non-stop decay (NSD) or no-go decay (NGD) assure cytoplasmic degradation of the mRNA (Houseley and Tollervey, 2009; Huch and Nissan, 2014). Transcripts that can be translated into functional proteins underlie degradation mechanism that are influenced by multiple factors. In general, translation initiation and degradation are two processes that permanently act on the mRNA until translation is no longer possible and the transcript is fully degraded (Huch and Nissan, 2014). Associated proteins and the overall composition of the mRNP can regulate this process allowing for mRNAs that are fast and others which are only slowly degraded. For instance, differences in the length and secondary structure of the 3' UTR and the poly(A) tail can have an impact on the mRNA's half-life (Moqtaderi et al., 2014).

Cytoplasmic degradation requires removal of the poly(A) tail, which is carried out by the deadenylase complexes Pan2/3 and Crr4/Not (Tucker et al., 2002). As soon as the poly(A) tail is degraded to a short oligoadenosine sequence, further degradation in the 3' to 5' direction is carried out by the cytoplasmic exosome. Like the poly(A) tail protects the 3' end from the degradation machinery, the 5' end of the mRNA is shielded by is 7-methyl-guanosine cap. Hence, degradation from the 5' end depends on the decapping complex Dcp1/Dcp2/Edc3 that cleaves off the cap structure and enables decay by the 5'-3' exonuclease Xrn1 (Parker, 2012).

2.2 mRNA surveillance and quality control

Formation of an export compatible mRNP requires multiple steps as overviewed above. Exchange and post-translational modifications of associated proteins have to be tightly coordinated for efficient and error-free mRNA production (Kelly and Corbett, 2009; Tutucci and Stutz, 2011). As this many factors and steps are involved, mRNA maturation is prone to errors. To avoid that faulty mRNAs reach the cytoplasm and result in unfunctional proteins, several mechanisms have evolved to survey the mRNAs quality and initiate its nuclear degradation in case it does not match certain criteria.

2.2.1 Degradation of incorrect mRNAs by the quality control machinery in the nucleus

Two major pathways of nuclear mRNA degradation involve the 5' exonuclease Rat1 and the nuclear exosome (Houseley and Tollervey, 2009). Besides its already mentioned role in RNAP II release upon transcription termination (Kim et al., 2004), Rat1 can degrade faulty mRNAs already during transcription from the 5' site (Mosrin-Huaman et al., 2016). Consistently, cells that lack Rat1's binding partner Rai1 accumulate mRNAs with defective 5' cap structures (Jiao et al., 2010).

Another component of the mRNA degradation machinery is the exosome. Its core structure composed of nine subunits is together with the exonuclease Dis3/Rrp44 present in both cellular compartments – the nucleus and the cytoplasm. Additionally, the catalytically active subunit Rrp6 is unique to the nuclear exosome and has been studied in detail. Degradation of mRNAs by the exosome is coupled to recruitment of the co-factor TRAMP, which strongly enhances activity of Rrp6 (Callahan and Butler, 2010). The TRAMP/exosome machinery not only degrades byproducts of mRNA maturation like spliced-out introns, but also is crucial for turnover of mRNAs that were not produced correctly. The TRAMP complex consists of a poly(A) polymerase (Trf4 or Trf5), the Air1/2 proteins and the RNA helicase Mtr4 (Houseley and Tollervey, 2009). While the TRAMP5 (Trf5, Air1, Mtr4) predominantly acts in nucleolar rRNA processing, the TRAMP4 complex (Trf4, Air2, Mtr4) mostly mediates quality control in the nucleoplasm (Callahan and Butler, 2010; San Paolo et al., 2009). Upon detection of defect transcripts, the poly(A) polymerase adds a short oligo(A) tail to the mRNA, which creates a single stranded landing platform for the subsequently recruitment of the Rrp6-containing exosome for degradation (Fasken and Corbett, 2009; Houseley and Tollervey, 2009). Length of the oligo(A) tail is regulated by Mtr4 that as well unwinds secondary structures and dissociates bound proteins on the defective mRNA thus enabling its decay (Houseley and

Tollervey, 2009; Jia et al., 2011). Recruitment of the TRAMP complex to incorrect mRNAs appears to be possible at different steps during mRNA production. Already at the very 5' site of an mRNA, where capping needs to be performed and binding of Npl3 to the correctly processed mRNA could allow Mex67 recruitment (Lei et al., 2001), components of the TRAMP4 complex can be found as revealed by crosslinking and analysis of cDNA (CRAC) experiments (Tuck and Tollervey, 2013). Furthermore, the TRAMP complex is recruited to pre-mRNAs before splicing and supports this process (Kong et al., 2013). Correctness of splicing is monitored by the SR adaptor proteins Gbp2 and Hrb1 that link the TRAMP complex to the mRNA and promote the transcripts' degradation by Rrp6 in case splicing is delayed or defective (Hackmann et al., 2014). Accordingly, in strains deleted for these proteins unspliced mRNAs leak into the cytoplasm (Hackmann et al., 2014). Like capping and splicing, proper 3' end formation and polyadenylation is crucial for formation of a correct mature mRNA. Should any mistakes occur in this step, again degradation of the mRNA is initiated by the TRAMP/exosome pathway (Tutucci and Stutz, 2011).

The same interrelation between mRNP packing and surveillance was observed for mutants of *YRA1* and *SUB2*, components of the TREX complex, which phenotypes were worsened upon combination with *rrp6Δ* (Zenklusen et al., 2002). Especially defects in the 3' processing and the role of Rrp6 in retaining those improperly processed mRNAs has been studied. Thus, Rrp6 can interact with the RNAP II and the adaptor protein Npl3 (Burkard and Butler, 2000) at the 3' end of the mRNA. Here it regulates together with the poly(A) binding proteins Pab1 and Nab2 formation and length of the poly(A) tail by antagonizing the protective function of Nab2 and triggering mRNA decay (Dunn et al., 2005; Schmid et al., 2012, 2015). The role of Nab2 in this maturation step appears to be crucial, as a deletion of the poly(A) binding protein is lethal. Strikingly, when *NAB2* is deleted together with *RRP6* cells are viable (González-Aguilera et al., 2011), presumably because defective mRNAs are exported again and can be translated to partially functional proteins. Synthesizing a poly(A) tail of correct length further depends on mRNA export, as in mutants of this process, transcripts not only accumulate, but rather have a longer hyperadenylated 3' tail (Jensen et al., 2001; Qu et al., 2009). In case the poly(A) tail is not formed or is too short, which can be observed in THO/sub2 mutants or in strains mutated for the poly(A) polymerase Pap1, transcripts can be retained at their site of transcription. This accumulation requires Rrp6, as in mutant strains additionally deleted for this exosome component transcripts are released again (Hilleren et al., 2001; Libri et al., 2002; Rougemaille et al., 2007). Thus, polyadenylation is tightly controlled by quality control

mechanisms that monitor 3’ formation for correctness and efficiency and induce degradation in case of errors (Saguez et al., 2008). This surveillance is important to assure only correct mRNAs are exported and translated. Otherwise defective proteins might be produced that can be toxic to cells (Kallehauge et al., 2012). Therefore, a last checkpoint consisting of Mlp1/2 and Pml39 is established at the nuclear pore complex, directly before export, to retain faulty transcripts (Galy et al., 2004; Palancade et al., 2005).

2.2.2 Quality control resembles fine tuning between export and degradation of mRNAs

Smooth gene expression requires the correct timing of maturation processes and assembly of important factors. Errors during this process might result in mRNAs that cannot be translated into functional proteins, which possibly be harmful to the cell. Malformations in mRNAs can originate from incorrect capping of the 5’ end, no or incomplete splicing or mistakes in 3’ end formation and polyadenylation. Beside these effects any other disruption of the intended mRNP composition or secondary structure could also lead to defects. Therefore, transcription of mRNAs is tightly linked with its packing in export competent particles, which further supports cooperative mRNA export (Meinel and Sträßer, 2015). If errors occur during this procedure, quality control and degradation mechanism prevent accumulation and export of erroneous transcripts (Fasken and Corbett, 2009; Houseley and Tollervey, 2009). Even though several mechanisms are known that allow discrimination between correct and faulty RNAs, over the past years the opinion emerged, quality control is a general mechanism controlling nuclear transcript levels. In fact, it is often not clearly distinguishable, if an mRNA is right or wrong. Rather, its decay relies on kinetic competitions of biogenesis, export and function versus turnover that is enabled if certain checkpoints cannot be reached in time (Doma and Parker, 2007; Mühlemann and Jensen, 2012; Soheilypour and Mofrad, 2016). Consistent with this are findings, where the mRNA binding proteins Gbp2 and Hrb1 control correctness of splicing and recruit either Mex67 to allow export, or the TRAMP complex to promote degradation in case splicing is inefficient or erroneous (Hackmann et al., 2014).

2.3 The impact of stress on cellular functions and mRNA export

2.3.1 The different forms of stresses and their sensing

Cells have to cope with a huge set of different and often changing conditions. Everything that differs from a perfect environment can lead to limitation or impairment of growth. Those changes include restriction of nutrients like glucose or amino acid starvation, exposure to cytotoxic substances, oxidative stress caused by oxide radicals that can be evoked by UV radiation, as well as changes of the outside pH optimum to acidic or alkaline conditions, or high or low salt conditions. Further, decrease or increase of the outer temperature affects fidelity of the cell. While low temperatures mainly only cause slowing down of processes and reduce the speed of growth, increase of temperature over the optimum (25-30 °C for *S. cerevisiae*) leads to perturbations of processes as a result of proteins that became instable or even denatured.

Each of the above-mentioned stresses needs different counteractions to allow the cell to survive and return to normal growth once the stress is over. Such an elaborate stress response is facilitated by a complex network of sensing and signaling pathways.

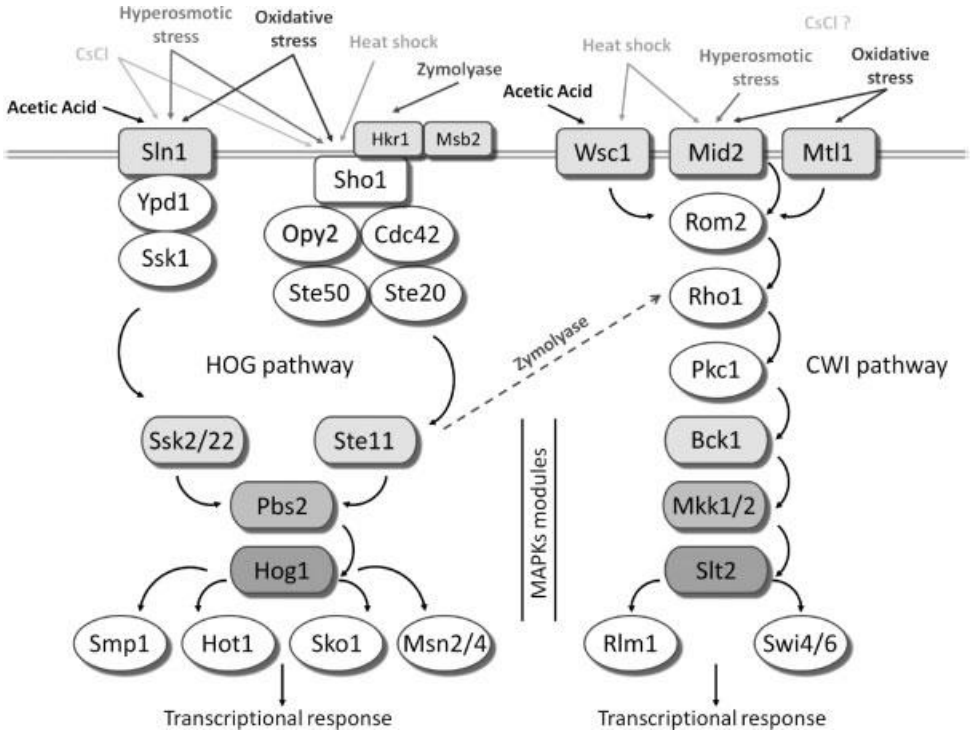

Figure 8: Schematic model for transduction of stress signals in *S. cerevisiae*. Factors involved in the HOG or the CWI pathway are displayed. Sensors on the cell wall receive a stress signal, which they lead on to their specific receptors. These regulate MAPK cascades, which in turn leads to activation of transcription factors (taken from Rodríguez-Pena et al., 2010).

The main pathways acting together in response to environmental changes are the cell wall integrity (CWI) and the high-osmolarity glycerol (HOG) pathway (Figure 8). The CWI pathway is mainly composed of the cell wall associated stress sensors Mid2 and Wsc1. Even though both receptors collaborate in sensing of stresses, Mid2 is more involved in sensing of low pH while Wsc1 plays a more important role in sensing heat stress (Fuchs and Mylonakis, 2009). This signal is transferred to Rom2, a guanyl nucleotide exchange factor (GEF) of the GTPase Roh1. Upon activation Rho1 activates Pkc1, which is the regulating kinase of the downstream mitogen-activated protein kinase (MAPK) cascade (Figure 8). This kinase cascade is composed of Bck1 acting as the MAPK kinase kinase (MAPKKK) that is regulating the MAPK kinases Mkk1 and Mkk2 (MAPKK). These kinases in turn activate the MAPK Slt2, which then leads to phosphorylation of target proteins like the transcription factors Rlm1 or Swi4/Swi6 (Fuchs and Mylonakis, 2009). Slt2 is the key kinase of the CWI pathway and recent studies reveal further targets as the MAPK phosphatase Msg5 or the translational repressor Caf20 (Alonso-Rodríguez et al., 2016).

The HOG pathway has a comparable composition. Sensors like Sln1 or Sho1 receive the different stresses and activate their receptors, which in turn activate a MAPK cascade. In this case, the cascade involves the MAPKKK Ssk2/22 and Ste11, the MAPKK Pbs1 and the MAPK Hog1. The Hog1 kinase regulates among others, Msn2 and Msn4 two of the main stress responsive transcription factors (Figure 8), (Rodríguez-Pena et al., 2010). Recent studies reveal details about the role of Hog1 in response to osmotic stress as the kinase can selectively induce stress responsive genes by re-localizing RNAP II and regulating chromatin remodeling (Nadal-Ribelles et al., 2012) or by phosphorylating nucleoporins, which brings promoters close to nuclear pores thus controlling export of specific mRNAs (Regot et al., 2013).

Despite increasing amount of information is collected for Hog1's role in response to salt stress, exact regulation of the heat stress response and most kinases involved in it remain elusive. However, a third pathway that is involved in heat stress and starvation response is the cyclic AMP protein kinase A (cAMP-PKA) pathway, which is as well involved in activating the transcription factors Msn2 and Msn4 (Fuchs and Mylonakis, 2009; Thevelein and De Winde, 1999). Additionally, the target of rapamycin (TOR) pathway which generally regulates growth and is involved in nutritional sensing, can slow down growth under conditions of stress to support maintenance of cellular processes. (Wei et al., 2013).

While much is known about activation of several pathways under stress, regulation of the heat shock response (HSR) is still broadly unknown. However, the HSR following stress requires

activation of the heat shock transcription factor Hsf1, which regulates reprogramming of transcription to induce HS gene expression (Morano et al., 2012). Details of how Hsf1 itself regulates the heat shock response will be referred later (2.3.6). It was described that the histone deacetylase Sir2 is involved in Hsf1's response to heat stress and the transcription factor Yap1 is required for Hsf1 activation under oxidative stress (Nussbaum et al., 2014). Additionally, the HSR is via Sir2 connected to the unfolded protein response (UPR) and both cross regulate each other under heat stress. The UPR is another stress responsive mechanism that senses unfolded proteins in the endoplasmic reticulum (ER) and regulates activity of the transcription factor Hac1 over the kinase Ire1 (Weindling and Bar-Nun, 2015).

In general, most pathways are often activated together and can act simultaneously and phosphorylation or dephosphorylation of any of these factors can reverse the effect of others or fine tune the response needed. Presumably one kind of stress leads to perturbations at several sites, which are sensed by different, partly antagonistic factors thus enabling a holistic stress response.

2.3.2 The stress response on cellular level

The ways cells can react to a stressful situation are quite diverse and strongly dependent on the kind and especially the intensity of stress the cell needs to cope with. This study focusses on salt and mainly heat stress. Initially, application of heat stress led to the discovery of a certain set of proteins called heat shock proteins (HSPs). Expression of these proteins is strongly enhanced upon heat stress and most of them function as chaperones, which are needed to refold proteins that denatured under high temperatures (Bond, 2006). Even though certain amounts of chaperones are regularly expressed under normal conditions to help newly translated proteins to fold, these would presumably not be sufficient to refold all proteins if the cells face global denaturation. Besides, chaperones mark proteins that are irreversibly damaged for degradation, mostly by ubiquitinylation. In yeast members of the Hsp70- and Hsp90-families together with Hsp60, Hsp104 are among the main HSP proteins that support cellular integrity after stress (Bond, 2006). These proteins can work together and build complexes to disaggregate wrongly folded proteins (Glover and Lindquist, 1998). Additionally, these HSPs can facilitate tolerance to higher temperatures as pre-incubation of cells at 37 °C, which is not enough to initiate a full stress response, but leads to expression of heat shock proteins, allowed cells to survive exposure to 55 °C a lot better (Bond, 2006; Piper, 1996).

2.3.3 Changes in the nucleus upon stress

Clearly, cells need chaperones to overcome threatening conditions. To ensure a fast and preferential expression of HSP genes the cell drastically changes many of its essential processes. When cells are exposed to severe heat stress at 42 °C, normal so-called housekeeping genes are no longer transcribed and only transcription of heat shock (HS) mRNAs is carried out (Huch and Nissan, 2014). Together with that, splicing of pre-mRNAs is blocked and unspliced mRNAs accumulate in the nucleus (Yost and Lindquist, 1986, 1991). How this splicing block is accomplished is still not completely understood, but studies from HeLa cells suggest changes in the composition and amount of the U4/U5/U6 and U2 snRNPs upon heat stress (Bond, 1988; Bracken and Bond, 1999). The intensity of the splicing block is strongly dependent on the intensity of the heat stress. While most other kinds of stresses and mild heat stress conditions at 37 °C seem not to impact splicing, severe heat stress at 42 °C causes such blockage. Again, it was shown that pretreatment with mild heat stress conditions can facilitate to maintain splicing at severe stress (Bond, 1988; Yost and Lindquist, 1986). This thermotolerance and - once the stress is over - the regeneration of the splicing machinery is supported by heat shock proteins like Hsp70 and Hsp104 (Bracken and Bond, 1999). Downregulation of splicing might seem negligible as most yeast mRNAs do not contain introns and especially in HS mRNAs introns are very rare, but even in higher eukaryotes where nearly all mRNAs have to be spliced most HS mRNAs lack introns (Biamonti and Caceres, 2009) arguing for an evolutionary well conserved mechanism that allows HS mRNAs to bypass this maturation step. This explains why splicing is dispensable for expression of HS mRNAs and can be suppressed under heat stress. Not only is the synthesis and maturation of new housekeeping mRNAs inhibited, but also the export of already transcribed mature mRNAs is blocked as indicated by experiments showing a strong nuclear accumulation for poly(A) containing RNAs (Saavedra et al., 1996). Many factors that play a crucial role for export of housekeeping mRNAs under normal conditions seem to be dispensable under stress. Thus, the general mRNA export adaptor Npl3 dissociates from bulk mRNA in the nucleus and is not involved in the export of HS mRNAs (Krebber et al., 1999; Rollenhagen et al., 2007). Comparably, the essential poly(A)-binding protein Nab2 becomes phosphorylated by the MAPK Slt2 under heat stress at 42 °C. Activity of Slt2 appears to be essential for bulk mRNA export block, even though only phosphorylation of Nab2 is not sufficient to establish the retention. Further, Nab2 accumulates together with the mRNA-binding protein Yra1 in nuclear foci. Another protein that can be found in these foci is the quality control factor Mlp1 (Carmody

et al., 2010), indicating these proteins are not necessary for HS mRNA export, but rather need to be sequestered under stress. The same effect can be found for the mRNA-binding adaptor protein Gbp2 that aggregates strongly but reversibly in the nucleus upon heat shock (Wallace et al., 2015).

While the mRNA adaptor Yra1 is dispensable, some argue this is the same for the THO complex in HS mRNA export as in mutants HSPs are still produced (Rollenhagen et al., 2007), while others show nuclear accumulation of HS mRNAs in *THO* mutants (Strässer et al., 2002). Thus, the exact role of THO in expression of HS genes needs to be further studied.

Only few proteins have been described to be essential for the export of mRNAs under stress. Interestingly, these are those factors resembling the 'core' mRNA export pathway under normal conditions. Among them are the export receptor Mex67 (Hurt et al., 2000; Sträßer et al., 2000), and components of the NPC like the DEAD box helicase Dbp5/Rat8, its anchoring nucleoporin Nup159/Rat7 and its cofactor Gle1 (Hodge et al., 1999; Rollenhagen et al., 2004). Another nucleoporin Nup42/Rip1, which is directly interacting with Gle1, is so far the only protein essential for HS mRNA export that is not required under normal conditions (Rollenhagen et al., 2004; Saavedra et al., 1996; Stutz et al., 1997). However, pretreatment of cells lacking Rip1 with a mild heat stress (1 h, 37 °C) can again maintain nuclear export at 42 °C. Together, many nuclear processes like bulk mRNA transcription, splicing and export are interrupted and expression of HS mRNAs is favored while just a small set of proteins is involved in their export, thus presumably enhancing speed and effectiveness of the heat stress response.

2.3.4 Cytoplasmic changes in response to stress

Expression of HS mRNAs is not only enhanced by mechanisms in the nucleus, which generally should result in a fast and unhindered export of stress responsive transcripts, but also supported by changes in the cytoplasm. Upon intense stress the translation machinery undergoes drastic alterations as general protein synthesis is inhibited. Polysomes, several actively translating ribosomes bound to one mRNA, can no longer be detected and only monosomes (single ribosomes) remain (Brengues et al., 2005; Shalgi et al., 2013) . General ribosomal stress is sensed by the ribosome quality control complex (RQC) that signals perturbations to the transcription factor Hsf1 (Brandman et al., 2012).

Globally, stress leads to inhibition of translation initiation, which can to date be narrowed down to two main mechanisms depicted in Figure 9.

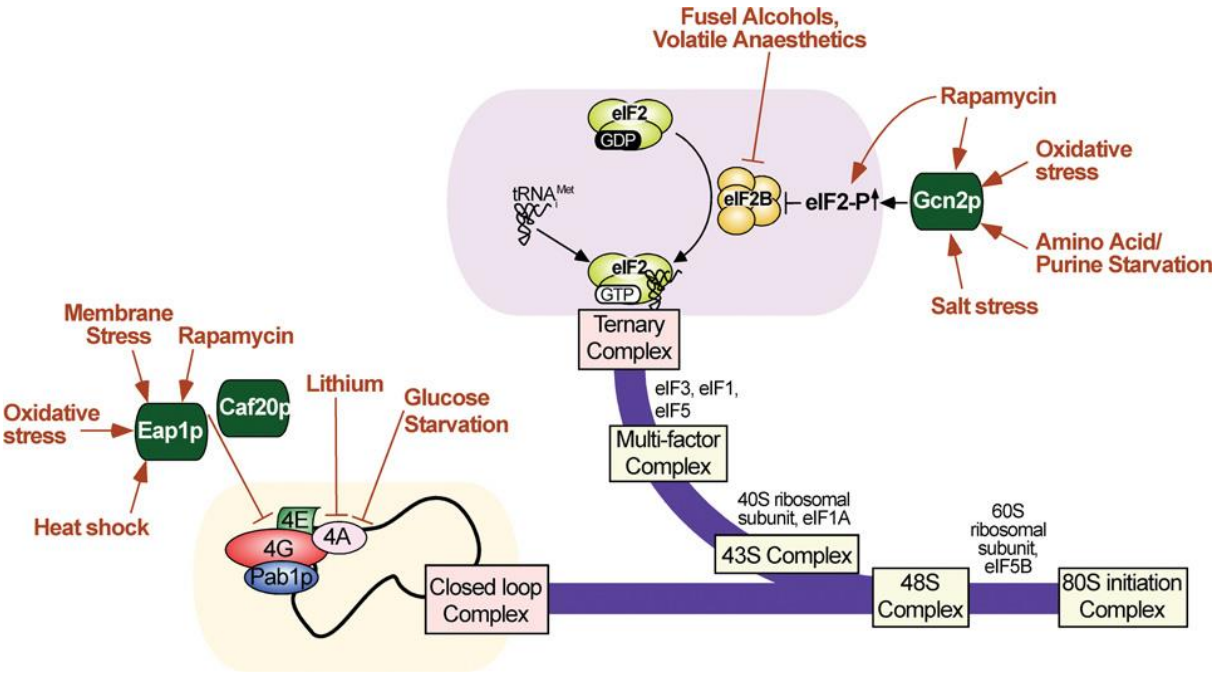

Figure 9: Mechanism of translation initiation control under stress. Different forms of stress lead to inhibition of formation of the closed loop or the ternary complex, which are necessary for translation initiation (taken from Simpson and Ashe, 2012).

The first is the inhibition of the closed loop complex formation. The closed loop complex consists of the mRNA with the poly(A) binding protein Pab1 on its poly(A) tail and the eukaryotic initiation factors eIF4A, eIF4E and eIF4G that bind to the mRNA cap structure. Interaction of this cap binding complex with Pab1 results in the closed loop structure prior to initiation (Hinnebusch, 2011). Under stress, building of this structure is disturbed by the factors Eap1 and Caf20, which both bind to eIF4E and thus competing for its binding to eIF4G (Costello et al., 2015; Simpson and Ashe, 2012). Another mechanism to abolish translation under stress is regulated by the kinase Gcn2. Following activation it phosphorylates eIF2α, a subunit of the initiation factor eIF2 (Dever et al., 1992). In this state eIF2α is a competitive inhibitor for the guanine-nucleotide exchange factor eIF2B. Increasing amounts of phosphorylated eIF2α result in high levels of inactive eIF2-GDP, which in turn reduces the amount of ternary complexes and translation initiation (Krishnamoorthy and Pavitt, 2001; Simpson and Ashe, 2012). Not only the translation machinery itself reacts to harmful conditions, but also the expression pattern of tRNAs undergoes changes in response to stress (Pang et al., 2014).

At the same time the preferentially exported HS mRNAs are translated and chaperones are produced. Studies in higher eukaryotes show that some transcripts can omit eIF2-dependent translation initiation by directly recruiting ribosomes over an internal ribosome-entry site

(IRES) (Yamasaki and Anderson, 2008). Other data suggest a role for the 5' UTR of RNAs were the methylation status of adenosines can initiate translation without the need of a 5'-N^7-methylguanosine cap (Zhou et al., 2015) or by directly acting over the poly(A)-binding protein Pab1 (Wallace et al., 2015). How in detail a privileged translation of HS mRNAs in yeast is ensured, which proteins are involved and which are dispensable needs to be further examined.

2.3.5 Formation of stress granules and P-bodies

How are normal housekeeping mRNAs kept away from translation and do not occupy the translation machinery instead of HS mRNAs? It is known for quite a long time that under stress conditions microscopically visible aggregates form, which contain mRNAs as well as cytoplasmic proteins. The nature of these aggregates is quite diverse and even though more and more of such granules are defined and linked to certain functions (Shah et al., 2014), the two most prominent under stress are so called stress granules and P-bodies (Anderson and Kedersha, 2009). Even though some components are enriched in the one or the other kind of aggregate, many proteins can be found in both (Mitchell et al., 2013). Stress granules are widely referred to as storage compartments that contain intact mRNAs and parts of the translation machinery, thus keeping the "normal" translation apparatus stored until the stress is over. The composition of stress granules is prone to changes and severely relies on the type of stress the cell faces. If glucose is depleted, stress granules build without ribosomal subunits or eIF3 (Pizzinga and Ashe, 2014). On the other hand the stress intensity contributes to stress granule formation as only the combination of mild heat stress (37 °C) and mild ethanol stress (5 %) induces their generation (Yamamoto and Izawa, 2013). In yeast as well as in higher eukaryotes prion-like RNA-binding proteins like Pub1/TIA-1 and Pbp1/Atx2 are known to promote stress granule assembly over self-attracting binding sites (Gilkes et al., 2004; Protter and Parker, 2016). Additionally, the amount of free mRNAs seems to play the role of a scaffold for stress granule formation (Bounedjah et al., 2014). In *S. cerevisiae* a protein called Whi3 is presumably involved in the mRNA composition of these granules (Holmes et al., 2013), while the ubiquitin-specific protease Ubp3 is essential in their general assembly (Nostramo et al., 2015; Nostramo and Herman, 2016). Cells carrying mutants of the THO complex struggle forming stress granules, though the role of THO in this cytoplasmic process remains elusive (Eshleman et al., 2016).

While stress granules represent a repository of translatable mRNAs, processing bodies (P-bodies) are rather the places of mRNA degradation, even though stress in general leads to

stabilization of cytoplasmic mRNAs (Huch and Nissan, 2014). In P-bodies mRNA degradation factors active in decapping (Dcp1/Dcp2, Edc3) or degradation (Xrn1) are enriched together with components of translation initiation (Pizzinga and Ashe, 2014; Sheth and Parker, 2003). Edc3 and Lsm4 are described to aid assembly of these granules by prion-like interactions with themselves (Decker et al., 2007). Recent studies show that in unstressed cells actively transcribed mRNAs form granule-like structures, which transform into P-bodies upon stress (Lui et al., 2014). Even though P-bodies are compartments of degradation and stress granules rather store mRNAs until the stress abates, it was described that some RNAs can leave P-bodies again, as shuttling to polysomes - as far as they are present - has been observed (Brengues et al., 2005) and re-entering of translation after stress can occur like for stress granule bound mRNAs (Pizzinga and Ashe, 2014). Once the stressful situation abates the granules' content is recycled and mRNAs can re-enter the translation machinery, a process which is supported by heat shock proteins like Hsp70 that assist refolding and disassembly (Walters et al., 2015).

2.3.6 Regulation of stress RNA expression by the transcription factor Hsf1

Stress requires the fast expression of stress responsive genes. *S. cerevisiae* has three main transcription factors involved in this response: Msn2 and Msn4 are responsible for expression of stress mRNAs following a broad set of different stresses, while Hsf1 mainly acts in the heat shock response. As described above Msn2/4 are activated by the HOG and the PKA pathway (see 2.3.1) and bind to so called stress responsive elements (STRE) in the promoter regions of their target genes to activate them (Morano et al., 2012). Msn2/4 are dispensable under normal conditions, while contrarily the transcription factor Hsf1 is essential at all conditions. Hsf1 is a trimeric protein binding to a sequence motif that was identified nearly 30 years ago termed the heat-shock element (HSE) (Sorger and Pelham, 1987). This motif consists of three repeats of the nTTCn respective nGAAn sequence to which presumably each subunit of the Hsf1 trimer binds (Sorger, 1991).

HSE type	Consensus sequence	Example genes
perfect	nn**GAA**nn**TTC**nn**GAA**nn	***HSP26, HSP104, SSA1***
gap	nn**GAA**nn**TTC**nnnnnnn**GAA**nn	***HSP82, CPR6, CUP1***
step	nn**GAA**nnnnnnn**GAA**nnnnnnn**GAA**nn	***HSP12, SSA3, YDJ1***

Figure 10: Different HSE elements in promoters of Hsf1 regulated genes. Three different HSEs were described to promote Hsf1 binding. They differ in the number of unconserved nucleotides (n) between the conserved GAA or TCC motifs (Morano et al., 2012). Examples of genes carrying these HSEs are listed (Yamamoto et al., 2005).

To date there are three different forms of HSE elements described, differing mainly in the number of nucleotides between each of the three conserved repeats (Figure 10).

Analysis with a mutant of Hsf1 and chromatin co-immunoprecipitation (ChIP) experiments identifying Hsf1 bound promoters, revealed 60 to over 150 genes, whose heat-induced expression relies on the transcription factor. Many of them are coding for chaperones, others function in protein degradation or cell wall integrity. Though still about a third of the found genes is not classified yet and many promoters contain HSEs that cannot be sorted into one of the above mentioned classes (Hahn et al., 2004; Yamamoto et al., 2005). The sequence of the HSE might be involved in the strength of Hsf1 binding to it, as the factor is nuclear and bound to some transcripts already at normal conditions (Gross et al., 1990). In fact, increased binding of Hsf1 to HSE-containing promoters under stress has been described (Hahn et al., 2004). This contrasts the situation in mammals as four different isoforms (HSF1-4) with specialized functions have evolved and the conserved HSF1 factor only trimerizes and binds to DNA upon stress (Morano et al., 2012; Nakai, 2016). Nevertheless, stress results in strong phosphorylation of Hsf1 in all species and it is likely that this is one mode of Hsf1 regulation to control HS mRNA expression (Morano et al., 2012; Sorger, 1991).

The fast switch from 25 °C to 42 °C and activation of Hsf1 is accompanied by changes in the chromatin structure by the remodeling complex SWI/SNF (Shivaswamy and Iyer, 2008). The correctly timed expression of genes from Hsf1-regulated promoters utilizes or omits a fine-tuned set of general transcription factors. One possibility to explain the finding of Hsf1 being promoter bound already at 25 °C is the identification of partial preinitiation complexes that contain most of the factors required for initiation, but lack RNA polymerase II and the transcription factor TFIIH (Zanton and Pugh, 2006). These partial preinitiation complexes can become functional by an additional signal as a response to stress and recruit the polymerase or

by dissociation of repressing elements like the histone Hho1 (Zanton and Pugh, 2006). Another complex involved in transcription initiation is Mediator that coordinates information from regulatory elements with the transcription machinery (Björklund and Gustafsson, 2005). Its activity is closely connected with Hsf1's function, as on the one hand Hsf1 can directly recruit this complex and thus RNAP II under heat stress and on the other hand Mediator seems to control and reduce expression of HS genes under normal conditions (Kim and Gross, 2013; Singh et al., 2006). Interestingly, expression of Hsf1 target genes under stress can be induced without factors normally essential for initiation like the C-terminal domain (CTD) of RNAP II or Taf9 (Apone et al., 1998; McNeil et al., 1998). Newly emerging data exhibit an even broader role for Hsf1, not only as the essential factor for a fast response to heat stress, but also in an elaborate regulation of its target genes under normal conditions (Pincus, 2016; Solís et al., 2016).

2.4 Scope of the study

The fast switch from normal housekeeping mRNA expression to induction and export of stress responsive transcripts is essential to facilitate cellular survival under existence-threatening conditions. Much information has been gathered over the last decades about how certain areas of the cell react to stress. Thus, upon severe heat stress export of normal bulk mRNA from the nucleus to the cytoplasm is blocked (Saavedra et al., 1996) and the splicing process is stalled (Yost and Lindquist, 1986, 1991), while HS mRNAs are transcribed and exported from the nucleus in an enhanced fashion and subsequently in the cytoplasm are preferentially translated by the ribosomes.

On the other hand mRNA adaptor proteins like Npl3, Nab2 and Yra1, which are important for mRNA maturation and transport are no longer essential under heat stress, as Npl3 dissociates from bulk mRNAs (Krebber et al., 1999). In parallel Nab2 and Yra1, together with the NPC-associated quality control checkpoint factor Mlp1 aggregate in nuclear foci in response to heat stress (Carmody et al., 2010). Overall does expression of HS mRNAs only rely on a reduced set of proteins of which all, except for the NPC component Nup42/Rip1, are all essential under normal conditions (Hodge et al., 1999; Hurt et al., 2000; Rollenhagen et al., 2004; Sträßer et al., 2000).

As both, normal and HS mRNA export, utilize the same proteins for their export, there has to be a mode of differentiation that favors export of HS transcripts upon stress. So far, no general mechanism has been described to allow for this discrimination. Therefore, one aim of this study was to elucidate by what means normal housekeeping mRNAs are retained in the nucleus and how HS mRNA export is different from that.

Furthermore, it was addressed what roles the adaptor proteins take over during normal mRNA export, as this function seems to be dispensable at heat stress, which is already known for Npl3, Nab2 and Yra1.

3 Materials and Methods

3.1 Chemicals and Consumables

Table 1: Chemicals and Materials used in this study

Chemical / Consumable	Supplier / Source
Alpha-factor, custom made peptide	Thermo Fischer Scientific (Schwerte/Germany)
Agarose NEEO Ultra	Carl Roth (Karlsruhe/Germany)
Amersham Hybond N^+ Nylon Membrane	GE Healthcare (Freiburg/Germany)
Amersham Protran 0.45 µm nitrocellulose membrane	GE Healthcare (Freiburg/Germany)
Blocking Reagent	Roche (Mannheim/Germany)
Complete EDTA-free protease inhibitor	Roche (Mannheim/Germany)
CSPD	Roche (Mannheim/Germany)
Deionized Formamide	Applichem (München/Germany)
dNTPs	Thermo Fischer Scientific (Schwerte/Germany)
5-Fluoroortic acid	Apollo Scientific (Derbyshire/UK)
Formaldehyde 37 %	AppliChem (München/Germany)
GFP-Trap beads	ChromoTek (Planegg-Martinsried/Germany)
GoTaq® qPCR Master Mix	Promega (Mannheim/Germany)
IgG Sepharose 6 FastFlow	GE Healthcare (Freiburg/Germany)
Microscope slides, 12 well, 5.2 mm, PTFE-coating	Thermo Fischer Scientific (Schwerte/Germany)
Phenol/chloroform/isoamyl alcohol (25:24:1)	Carl Roth (Karlsruhe/Germany)
Poly-L-lysine hydrobromide	Sigma-Aldrich (München/Germany)
Protino Ni-IDA Resin	Macherey-Nagel (Düren/Germany)
qPCRBIO SyGreen Mix Lo-ROX	Nippon Genetics (Düren/Germany)
RiboLock RNase Inhibitor	Thermo Fischer Scientific (Schwerte/Germany)
Rotiphorese Gel 30 (37.5:1) acrylamide	Carl Roth (Karlsruhe/Germany)
Salmon Sperm DNA	Sigma-Aldrich (München/Germany)
Trizol® Reagent	Life Technologies (Darmstadt/Germany)
Whatman® Blotting Paper 0.8 mm	Hahnemühle (Dassel/Germany)

Table 2: Kits used in this study

Kit	Supplier / Source
Amersham ECL Prime Western Blotting Detection Kit	GE Healthcare (Freiburg/Germany)
DIG RNA labeling mix, 10x	Roche (Mannheim/Germany)
DIG-dUTP oligonucleotide tailing kit	Roche (Mannheim/Germany)
NucleoBond PC100	Macherey-Nagel (Düren/Germany)
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel (Düren/Germany)
NucleoSpin Plasmid	Macherey-Nagel (Düren/Germany)

NucleoSpin RNA	Macherey-Nagel (Düren/Germany)
peqGOLD Gel Extraction Kit	Peqlab (Erlangen/Germany)
WesternBright Chemilumineszenz Substrate Quantum	Biozym (Hess.Ohlendorf/Germany)

Table 3: Marker and standards used in this study

Marker / Standard	Supplier / Source
CozyHiTM Prestained Protein Ladder	HighQu (Kraichtal/Germany)
GeneRuler 100bp DNA Ladder	Thermo Fischer Scientific (Schwerte/Germany)
Lambda DNA/*EcoRI* + *Hind*III Marker	Thermo Fischer Scientific (Schwerte/Germany)
PageRuler Prestained Protein Ladder	Thermo Fischer Scientific (Schwerte/Germany)
PageRuler Unstained Protein Ladder	Thermo Fischer Scientific (Schwerte/Germany)

Chemicals, consumables or other material that are not specifically stated above were purchased from the companies listed below:
AppliChem (München/Germany), BD Biosciences (Heidelberg/Germany), Carl Roth (Karlsruhe/Germany), GE Healthcare (Freiburg/Germany), Life Technologies (Darmstadt/Germany), Merck (Darmstadt/Germany), New England Biolabs (Frankfurt a.M./Germany), OMNILAB GmbH (Bremen/Germany), Peqlab (Erlangen/Germany), Promega (Mannheim/Germany), Roche (Mannheim/Germany), Sarstedt (Nürnbrecht/Germany), Serva (Heidelberg/Germany), Sigma-Aldrich (München/Germany), Thermo Fischer Scientific (Schwerte/Germany), Th.Geyer (Renningen/Germany), VWR (Darmstadt/Germany)

3.2 Equipment and Hardware

Table 4: Equipment and hardware used in this study

Machine	Supplier / Source
Bio Photometer	Eppendorf AG (Hamburg/Germany)
Cell homogenizer FastPrep-24	MP Biomedicals (Illkirch/France)
Centrifuge Heraeus Multifuge X3R with swinging bucket rotor TX-750 or Fiberlite F15-8x50cy fixed-angle rotor	Thermo Fischer Scientific (Schwerte/Germany)
Electro Blotter PerfectBlue Semi-Dry, Sedec M	Peqlab (Erlangen/Germany)
Fluorescence microscope DMI6000B with Leica DFC360 FX camera	Leica (Wetzlar/Germany)
Hemocytometer Neubauer improved	Carl Roth GmbH (Karlsruhe/Germany)
INTAS UV-System for Gel detection	INTAS GmbH (Göttingen/Germany)

Luminescence Detection System Fusion SL 3500.WL	Peqlab (Erlangen/Germany)
NanoDrop 2000 spectrophotometer	Thermo Fischer Scientific (Schwerte/Germany)
Optimax X-ray film processor	PROTEC (Oberstenfeld/Germany)
qPCR Cycler CFX Connect	BioRad (München/Germany)
qPCR Cycler Rotor-Gene Q	Qiagen (Hilden/Germany)
Sonifier Cell Disrupter S-250A	Branson Ultrasonics (Dietzenbach/Germany)
Thermocycler MyCycler	BioRad (München/Germany)
UV-Crosslinker Bio-Link BLX-E365	Vilber Lourmat (Eberhardzell/Germany)
Water Purification Milli-Q	Millipore (Eschborn/Germany)

3.3 Software

Table 5: Software used in this study

Software	Supplier / Source
Adobe Illustrator CS5; Adobe Photoshop CS5	Adobe Systems (San Jose/USA)
ApE Plasmid Editor	M.Wayne Davis (University of Utah/USA)
Bio-1D used for signal quantification	Peqlab (Erlangen/Germany)
Fiji (1.48s) used for signal quantification	W. Rasband (NIH/USA)
Microsoft Office 2010	Microsoft Corporation (Redmond/USA)
Microscopy LAS AF 1.6.2	Leica (Wetzlar/Germany)
SnapGene Viewer	GSL Biotech LLC (Chicago/USA)

3.4 Antibodies and Enzymes

Table 6: Antibodies used in this study

Antibody (organism)	Dilution	Supplier / Source
Anti-Aco1 (rabbit)	1:2,000	courtesy of R. Lill (Marburg/Germany)
Anti-Digoxigenin-AP, Fab fragments (sheep)	1:10,000 (NB)	Roche (Mannheim/Germany)
Anti-Digoxigenin-FITC, Fab fragments (sheep)	1:200 (FISH)	Roche (Mannheim/Germany)
Anti-GFP (mouse)	1:4,000	Thermo Fischer Scientific (Schwerte/Germany)
Anti-GST (B-14) (mouse)	1:2,000	Santa Cruz (Heidelberg/Germany)
Anti-Hem15	1:7,000	courtesy of R. Lill (Marburg/Germany)
Anti-HA (12CA5) (mouse)	1:1,000	Santa Cruz (Heidelberg/Germany)
Anti-mouse IgG-HPR (goat)	1:20,000	Dianova (Hamburg/Germany)
Anti-Mex67 (rabbit)	1:40,000	custom-made by Davids Biotechnologie (Regensburg/Germany)

Anti-myc (9E10) (mouse)	1:1,000	Santa Cruz (Heidelberg/Germany)
Anti-myc (A-14) (rabbit)	1:1,000	Santa Cruz (Heidelberg/Germany)
Anti-Npl3 (rabbit)	1:1,000	custom-made, H. Krebber
Anti-Nop1 (mouse)	1:1,000	Santa Cruz (Heidelberg/Germany)
Anti-rabbit IgG-HPR (goat)	1:20,000	Dianova (Hamburg/Germany)
Anti-Rps3 (rabbit)	1: 500	custom-made by Davids Biotechnologie (Regensburg/Germany)
Anti-Zwf1 (rabbit)	1: 4,000	courtesy of R. Lill (Marburg/Germany)

Dilutions are in general for Western blot (WB) application. Dilutions for Northern blot (NB) or fluorescence in situ hybridization (FISH) are indicated.

Table 7: Enzymes used in this study

Enzyme	Supplier / Source
DreamTaq DNA polymerase	Thermo Fischer Scientific (Schwerte/Germany)
FastAP Alkaline Phosphatase	Thermo Fischer Scientific (Schwerte/Germany)
Maxima Reverse Transcriptase	Thermo Fischer Scientific (Schwerte/Germany)
Q5 DNA polymerase	New England Biolabs (Frankfurt/Germany)
Restriction Enzymes	Thermo Fischer Scientific (Schwerte/Germany)
Restriction Enzymes	New England Biolabs (Frankfurt/Germany)
RNase A	Qiagen (Hilden/Germany)
T4 DNA Ligase	Thermo Fischer Scientific (Schwerte/Germany)
T5 Exonuclease	New England Biolabs (Frankfurt/Germany)
Taq DNA Ligase	New England Biolabs (Frankfurt/Germany)
Velocity DNA polymerase	Bioline (Luckenwalde/Germany)
Zymolyase 20T	Amsbio (Abingdon/UK)

All enzymes were used with the supplied buffer according to manufacturer's protocols.

3.5 Strains

3.5.1 Escherichia coli strains

Table 8: Escherichia coli strains used in this study

Strain	Genotype	Application
DH5α™	F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	plasmid amplification
Rosetta 2 (DE3)	F- ompT gal dcm lon hsdSB (rB-,mB-) λ(DE3) pRARE2(CamR)	protein expression

3.5.2 Saccharomyces cerevisiae strains

Table 9: Saccharomyces cerevisiae strains used in this study

Number	Genotype	Source
HKY36	S288C wild type *MATα ura3-52 leu2Δ1 his3-200*	(Winston et al., 1995)
HKY56	*MATa ura3-52 leu2Δ1 rip1::HIS3*	(Zander et al., 2016)
HKY124	*MATα ura3-52 leu2Δ1 his3Δ200 rat7-1*	(Gorsch et al., 1995)
HKY157	*MATa ura3 leu2 his3 ade npl3::HIS3 + p CEN URA3 NPL3-myc*	(Shen et al., 1998)
HKY168	*MATa ura3 leu2 trp1 his3 lys2 ade2 ade8 gbp2::HIS*	(Hackmann et al., 2014)
HKY194	*MATa ura3 leu2 trp1 his3 lys2 ade2 ade3 hrb1::HIS3*	(Shen et al., 1998)
HKY298	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 hrb1::kanMX4*	Euroscarf
HKY369	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 gbp2::kanMX4*	Euroscarf
HKY380	*MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 npl3::kanMX4*	Euroscarf
HKY381	BY4742 wild type *MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*	Euroscarf
HKY428	*MATα ura3-52 leu2Δ1 his3Δ200 mtr4-G677D*	(Hackmann et al., 2014)
HKY644	*MATα ura3 leu2 trp1 his3 ade2 mex67::HIS3 + p CEN LEU2 mex67-5*	(Segref et al., 1997)
HKY719	*MATα his3Δ1 leu2Δ0 ura3Δ0 npl3::kanMX4 gbp2::kanMX4 hrb1::kanMX4 + p CEN URA3 NPL3*	(Zander et al., 2016)
HKY820	*MATα ura3-52 leu2Δ1 his3Δ200 GBP2-3xmyc:HIS3*	(Hackmann et al., 2014)
HKY821	*MATα ura3-52 leu2Δ1 his3Δ200 HRB1-3xmyc:HIS3*	(Hackmann et al., 2014)
HKY891	*MATa ura3 leu2 his3 lys2 mtr2::kanMX4 + p CEN TRP1 mtr2-21 + p CEN URA3 MTR2*	(Baßler et al., 2001)
HKY892	*MATa ura3 leu2 his3 lys2 mtr2::kanMX4 + p CEN TRP1 mtr2-33 + p CEN URA3 MTR2*	(Baßler et al., 2001)
HKY1028	*MATα leu2Δ0 his3Δ1 lys2Δ0 ura3Δ0 rrp6::kanMX4*	Euroscarf
HKY1115	*MATa ura3 leu2Δ nab2::HIS3 + p CEN URA3 nab2ΔN*	(Marfatia et al., 2003)
HKY1203	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 lys2Δ0 hrb1::HIS3 rrp6::kanMX4*	(Hackmann et al., 2014)
HKY1204	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ade2 ade8 trp1 gbp2::HIS3 rrp6::kanMX4 + p CEN URA3 GBP2-GFP*	(Hackmann et al., 2014)
HKY1263	*MATa leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 ssa4::kanMX4*	Euroscarf
HKY1266	*MATa ura3Δ0 leu2Δ0 his3Δ met15Δ0 MEX67-GFP:HIS3MX6*	Invitrogen
HKY1409	*MATa leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 mlp1::kanMX4*	Euroscarf
HKY1457	*MATa ura3Δ0 leu2Δ0 his3Δ met15Δ0 HPR1-GFP:HIS3MX6*	Invitrogen
HKY1458	*MATa ura3Δ0 leu2Δ0 his3Δ met15Δ0 YRA1-GFP:HIS3MX6*	Invitrogen
HKY1475	*MATa ura3Δ0 leu2Δ0 his3Δ met15Δ0 NAB2-GFP:HIS3MX6*	Invitrogen
HKY1506	*MATa ura3 leu2Δ his3Δ1 nab2::HIS3 rrp6::kanMX4 + p CEN URA3 nab2ΔN*	(Zander et al., 2016)

HKY1507	*MATa ura3Δ0 leu2Δ0 his3Δ met15Δ0 cet1-2::kanR*	Laboratory of Heike Krebber
HKY1509	*MATa ura3Δ0 leu2Δ0 his3Δ met15Δ0 rat1-1::kanR*	Laboratory of Heike Krebber
HKY1569	*MATa ura3 leu2 trp1 his3 ade2 mex67::HIS3 + p CEN LEU2 mex67Δ409-435aa*	(Zander et al., 2016)
HKY1570	*MATa ura3 leu2 trp1 his3 ade2 mex67::HIS3 + p CEN LEU2 mex67KR>AA*	(Zander et al., 2016)
HKY1571	*MATa ura3Δ0 leu2Δ0 rrp6::kanMX4 CYC1-GFP:HIS3MX6*	(Zander et al., 2016)
HKY1572	*MATa ura3Δ0 leu2Δ0 mtr4-G677D CYC1-GFP:HIS3MX6*	(Zander et al., 2016)
HKY1580	*MATa ura3Δ0 leu2Δ0 his3Δ met15Δ0 HSF1-GFP:HIS3MX6*	Invitrogen
HKY1640	*MATa mex67::HIS3 CYC1-GFP:HIS3MX6 ura3Δ0 leu2Δ0 + p CEN LEU2 mex67-5 + p CEN URA3 P_{CYC1}:CYC1-GFP*	(Zander et al., 2016)
HKY1641	*MATa mtr2::kanMX4 CYC1-GFP:HIS3MX6 trp1::kanMX4 ura3Δ0 leu2Δ0 + p CEN TRP1 mtr2-21 + p CEN URA3 P_{CYC1}:CYC1-GFP*	(Zander et al., 2016)
HKY1654	*MATa ura3Δ0 leu2Δ0 his3Δ1 cet1-2::kanR npl3::KANMX6*	Laboratory of Heike Krebber
HKY1655	*MATα ura3Δ0 leu2Δ0 his3Δ1 rat1-1::kanR npl3::KANMX6 + p* CEN LEU2 *+ p* CEN URA3 GFP-NPL3	Laboratory of Heike Krebber

3.6 Plasmids

Table 10: Plasmids used in this study

Number	Features	Source
pHK12	*CEN URA3 P_{ADH} NLS-NES-GFP-GFP*	(Taura et al., 1998)
pHK20	*CEN LEU2 MEX67-GFP*	(Segref et al., 1997)
pHK87	*CEN LEU2*	(Sikorski and Hieter, 1989)
pHK88	*CEN URA3*	(Sikorski and Hieter, 1989)
pHK103	*2µ LEU2*	(Christianson et al., 1992)
pHK104	*2µ URA3*	(Christianson et al., 1992)
pHK144	*2µ URA P_{GAL1} GFP-NPL3*	(Hacker and Krebber, 2004)
pHK154	*CEN LEU2 npl3-17*	(Lee et al., 1996)
pHK358	*CEN LEU2 P_{GAL1} -NAB2-GFP*	(Duncan et al., 2000)
pHK367	*CEN URA3 GBP2-GFP*	(Windgassen and Krebber, 2003)
pHK422	*2µ URA P_{GAL1} GBP2-GFP*	(Windgassen and Krebber, 2003)
pHK418	*CEN LEU2 GFP-NPL3*	(Gilbert et al., 2001)
pHK439	*GST*	(Zenklusen et al., 2001)

pHK765	*CEN URA3 GFP-NPL3*	(Hackmann et al., 2014)
pHK778	*CEN LEU2 9xmyc-NPL3*	(Hackmann et al., 2014)
pHK779	*CEN URA3 9xmyc-NPL3*	(Hackmann et al., 2014)
pHK1276	*GST-NPL3*	(Baierlein et al., 2013)
pHK1279	*PT7:HIS:MTR2*	(Zander et al., 2016)
pHK1372	*PTRC:HIS:TEV:MTR2:MEX67*	(Yao et al., 2007)
pHK1373	*PTRC:HIS:TEV:MTR2:mex67loopKR>AA*	(Yao et al., 2007)
pHK1374	*PTRC:HIS:TEV:MTR2:mex67-409-435aaK343E*	(Yao et al., 2007)
pHK1376	*CEN LEU2 mex67Δ409-435aa*	(Yao et al., 2007)
pHK1377	*CEN LEU2 mex67loopKR>AA (K415A, K416A, K419A, K424A, R426A, R427A)*	(Yao et al., 2007)
pHK1443	*CEN URA3* P_{CYC1}*:CYC1-GFP*	(Zander et al., 2016)
pHK1444	*CEN URA3* P_{HSP12}*:CYC1-GFP*	(Zander et al., 2016)
pHK1445	*CEN URA3* P_{HSP12}*:HSP12-GFP*	(Zander et al., 2016)
pHK1464	*CEN URA3* $P_{CYC1+HSE}$*:CYC1-GFP*	(Zander et al., 2016)
pHK1470	*CEN URA3* P_{GPM1}*:GPM1-GFP*	(Zander et al., 2016)
pHK1472	*CEN URA3* P_{HSP12}*:GPM1-GFP*	(Zander et al., 2016)
pHK1517	*CEN URA3* P_{CYC1}*:HSP12-GFP*	(Zander et al., 2016)
pHK1518	*CEN URA3* P_{GPM1}*:HSP12-GFP*	(Zander et al., 2016)
pHK1547	*CEN URA3* P_{HSP12}*:RPL23B-GFP*	(Zander et al., 2016)
pHK1548	*CEN URA3* P_{RPL23B}*:RPL23B-GFP*	(Zander et al., 2016)
pHK1553	*CEN URA3* $P_{GPM1+HSE}$*:GPM1-GFP*	(Zander et al., 2016)

3.7 Oligonucleotides

Oligonucleotides were purchased at Sigma-Aldrich and stored as 100 µM solutions at -20 °C. Fluorescently labeled oligonucleotides were aliquoted and the current aliquot was kept at 4 °C.

Table 11: Oligonucleotides used in this study

Number	Sequence	Name
HK1002	5'-TGCTAAGGCTGTCGGTAAGG-3'	*TDH1* forward
HK1003	5'-TCAGAGGAGACAACGGCATC-3'	*TDH1* reverse
HK1451	5'-AGGCTCGTAGCGGTTCTGAC-3'	*25S rRNA* forward
HK1452	5'-CGAGCTTCTGCTATCCTGAGGG-3'	*25S rRNA* reverse
HK1511	5'-GTCTTCCTCCGCTCAAACTTCC-3'	*SSA1* forward
HK1512	5'-GAACAGCAGCACCGTAAGCAAC-3'	*SSA1* reverse
HK2022	5'-AAGATGGAAGCGTTCAACTAGC-3'	*GFP* probe forward
HK2023	5'-taatacgactcactatagggATCCATGCCATGTGTAATCC-3'	*GFP* probe reverse with T_7-promoter
HK2040	5'-Cy3-CCATTAACATCACCATCTAATTCAACAAG AATTGGGACAACTCCAGTGAA-Cy3-3'	*GFP* reverse
HK2041	5'-Cy3-CTTGACTTCAGCACGTGTCTTGTAGTTCCC GTCATCTTTGAAAAATATAG-Cy3-3'	*GFP* reverse
HK2055	5'-Cy3-TAAAAGGACAGGGCCATCGCCAATTGGAG TATTTTGTTGATAATGGTCTGCT-Cy3-3'	*GFP* reverse

HK2098	5'-ccgggCAGAACATTCTAGAAAGc-3'	*HSE* forward
HK2099	5'-ccgggCTTTCTAGAATGTTCTGc -3'	*HSE* reverse
HK2128	5'-Cy3-TTCTTTAAAATCAATACCTTTTAAC TCGATTCTATTAACAAGGGTATCAC-Cy3-3'	*GFP* reverse
HK2129	5'-Cy3-TCCGGGTATCTTGAAAAGCACTGAACACC ATAAGTGAAAGTAGTGACAAG-Cy3-3'	*GFP* reverse
HK2134	5'-ATGCCCGAAGGTTATGTACAGG-3'	*GFP* forward
HK2135	5'-CATTCTTTTGTTTGTCTGCCATG-3'	*GFP* reverse
HK2170	5'-Cy3-CTCTTTTCGTTGGGATCTTTCGAAAGGGCA GATTGTGTGGACAGGTAATG-Cy3-3'	*GFP* reverse
HK2171	5'-Cy3-CCTGTACATAACCTTCGGGCATGGCACTCTT GAAAAAGTCATGCCGTTTC-Cy3-3'	*GFP* reverse
HK2226	5'-GGTTGGCAACAGCAGCGGCACCAGCAGCGGC AGCTTCTGGGTCCAAGTAG-Cy3-3'	*GPM1* reverse
HK2227	5'-CCAATGGAATACCAGTTGGGATGTTCAACTTAG CAATGTCAGCATCAGAG-Cy3-3'	*GPM1* reverse

3.8 Cell cultivation

All media were autoclaved before use. Heat-labile compounds like antibiotics or galactose were sterile-filtered and added after autoclaving. For generation of plates 1.5 % (for *E. coli*) or 1.8 % (for *S. cerevisiae*) agar was added to the corresponding liquid medium.

3.8.1 Cultivation of *E. coli* cells

For general cultivation of *E.coli* cells LB medium with the respective antibiotics was used according to (Sambrook et al., 1989). Expression of recombinant proteins (see 3.12.1) was performed using 2xYT medium.

LB medium (low salt) pH 7.5	1.0 % (w/v) Peptone 0.5 % (w/v) Yeast extract 0.5 % (w/v) NaCl	
2xYT medium pH 7.0	1.6 % (w/v) Peptone 1.0 % (w/v) Yeast extract 0.5 % (w/v) NaCl	
Antibiotic concentration	Ampicillin	100 µm/ml
	Kanamycin	20 µg/ml
	Chloramphenicol	34 µg/ml

Single colonies from selective plates were used to inoculate liquid medium cultures and grown with agitation overnight at 37 °C.

3.8.2 Transformation of *E. coli* cells

E.coli transformation was performed as described earlier (Inoue et al., 1990). Chemically competent *E. coli* cells were thawed on ice. A ligated construct or already present plasmid was added up to 1:10 ratio and incubated 30 min on ice. Cells were heat-shocked for 2 min at 42 °C and 1 ml LB medium was added. After incubation for 1 h at 37 °C cells were plated onto selective medium plates.

3.8.3 Cultivation of *S. cerevisiae* cells

For cultivation of *S. cerevisiae* yeast cells, full (YPD) or synthetic selective medium was used and standard protocols were followed (Rose et al., 1990; Sherman, 1991).

YPD medium	2.0 % (w/v) Peptone 1.0 % (w/v) Yeast extract 2.0 % (w/v) Glucose
Selective Medium	0.2 % (w/v) Yeast dropout mix 0.17 % (w/v) Yeast nitrogen base 0.5 % (w/v) Ammonium sulfate 2.0 % (w/v) Glucose

Full medium was used for cultivation of strains with no need for selection for a specific marker gene. Strains with plasmid- or genome-encoded markers that complement an auxotrophy were grown in selective medium with every amino acid and nucleobase in the dropout mix except for the metabolic product resulting from the marker gene.
To select for the loss of an URA3 gene-containing plasmid FOA plates were used.

FOA plates	0.2 % (w/v) Yeast dropout mix 0.17 % (w/v) Yeast nitrogen base 0.5 % (w/v) Ammonium sulfate 0.1 % (w/v) 5-Fluoroortic acid (FOA) 2.0 % (w/v) Glucose 1.8 % (w/v) Agar

The agar was autoclaved and combined with the other sterile filtered compounds. Cells that still carry a URA3 gene produce the toxic 5-Fluorouracil from FOA which is lethal.

Yeast strains were kept on agar plates with the appropriate composition at 4 °C and were unless stated otherwise cultivated at 25 °C. Liquid cultures were inoculated with single colonies or liquid precultures and grown to logarithmic phase with a density of generally 1-3 x 10^7 cells/ml.

Cell density was counted using a hemocytometer and cells were harvested at 4,000 g for 5 min, once washed with water and used directly or frozen in liquid nitrogen and stored at -20 °C for later use. Activation of a temperature sensitive allele or induction of stress response was performed by shifting the cells to the necessary temperature or by adding NaCl in the indicated amounts. For expression of galactose-inducible genes cells were grown in medium containing 2 % raffinose or saccharose and expression induced by addition of 2 % galactose.

Drop dilution test

Growth on certain media or at different temperatures was analyzed by serially diluting the cells to 10^1-10^5 cells/ml, spotting equal amounts (7.5 µl on YPD, 10 µl on selective plates) on plates and incubation for 2-3 days.

New yeast strains were generated by mating two strains of opposite mating types (*MAT* a or *MAT* α). The resulting diploid cells were selected according to their markers and brought into sporulation medium (Sherman, 1991; Sherman and Hicks, 1991).

Super-SPO medium	Solution 1:	0.5 % (w/v) Yeast extract
		306 mM Potassium acetate
	Solution 2:	5 mM Glucose
		5.9 mM Threonine
		1.2 mM Phenylalanine
		0.7 mM Uracil
		0.4 mM Adenine and Tyrosine
		0.3 mM Leucine and Methionine
		0.2 mM Histidine, Lysine, Tryptophan and Arginine

Autoclaved solution 1 was mixed with sterile filtered solution 2. Because of the low amounts of nutrients, diploid yeast cells undergo meiosis and produce four haploid cells called tetrads. These were separated by digestion of the mother's cell wall with Zymolyase followed by dissecting tetrads into single cells on full medium plates using a tetrad microscope. The resulting single colonies were analyzed on different plates to identify their genetic background. In addition, PCR analysis was used to verify certain markers. To identify the mating type of the new strains cells were plated on *MAT* a or *MAT* α tester strains and after 1 day plated on B- plates according to (Sprague, 1991).

B-plates	0.17 % (w/v) Yeast nitrogen base 0.5 % (w/v) Ammonium sulfate 2.0 % (w/v) Glucose 3.0 % (w/v) Agar

After 2 days growth was analyzed. Only strains that have mated with one of the tester strains that have leucine, isoleucine and valine auxotrophies are able to grow on the minimal plates. Therefore, only the opposite mating type of the tester strain should show growth.

3.8.4 Transformation of *S. cerevisiae* cells

Yeast cells were transformed using chemical lithium acetate transformation described in (Gietz et al., 1992).

TE/Lithium acetate pH 7.5	100 mM Lithium acetate 10 mM Tris/HCl 1 mM EDTA
PEG/TE/Lithium acetate pH 7.5	40 % PEG 4000 100 mM Lithium acetate 10 mM Tris/HCl 1 mM EDTA

Log phase cells were harvested, washed twice with TE/Lithium acetate and brought to a density of 10^9 cells/ml. 50 µl cell suspension, 5 µl salmon sperm carrier DNA, 1 µg plasmid and 300 µl PEG/TE/Lithium acetate were incubated at 25 °C for 30 min. Cells were heat-shocked for 15 min at 42 °C and subsequently plated onto selective medium plates. Single colonies became visible after 2-4 days and were streaked on selective plates.

3.9 Cloning, DNA purification and DNA analysis

For most cloning approaches standard methods were used as described (Sambrook et al., 1989).

3.9.1 Isolation of chromosomal DNA from yeast

Genomic chromosomal DNA (gDNA) from yeast cells was prepared using phenol/chloroform extraction (Rose et al., 1990).

Detergent Lysis Buffer pH 8.0	100 mM NaCl 10 mM Tris/HCl 1 mM EDTA 2 % (v/v) Triton X-100 1 % (w/v) SDS
TE-Buffer pH 8.0	10 mM Tris/HCl 1 mM EDTA

A saturated 10 ml liquid culture was harvested, washed once with water and the cell pellet was disrupted with 200 µl detergent lysis buffer, 200 µl phenol/chloroform/isoamylalcohol (25:24:1) (P/C/I) and 200 µl glass beads by using the FastPrep-24 machine at 4.5 m/s, 20 s for 3 times. After addition of 200 µl TE-buffer and intense mixing the aqueous phase was separated by centrifugation (21,000 g, 5 min). The upper, aqueous phase was washed twice with 200 µl P/C/I and once with C/I before the DNA was precipitated by addition of 1 ml 100 % ethanol and 6 µl 7.5 M ammonium acetate. To pellet the DNA, the solution was centrifuged 30 min at 21,000 g, 4 °C. Afterwards, the pellet was washed with 70 % ethanol, air dried and resuspended in 100 µl deionized water. DNA was stored at -20 °C for further use.

3.9.2 Isolation of plasmid DNA from *E. coli*

Plasmid DNA from *E. coli* was extracted using the commercially available Macherey-Nagel kits. Small amounts from 5 ml cultures were purified using the NucleoSpin Plasmid kit. Isolation of larger amounts from 100-200 ml cultures was performed using the NucleoBond PC 100 kit. Plasmids were eluted in deionized water, brought to a concentration of 1 µg/µl and stored at -20 °C.

3.9.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify DNA fragments with gene specific oligonucleotides (primers) from plasmids or gDNA. Analytical PCRs were run to verify a certain genotype or cloning step and performed using the DreamTaq polymerase. For products

that were subsequently used for cloning approaches proofreading polymerases like Velocity (Bioline) or Q5 (NEB) were chosen. Each of the polymerases, templates and primers required slightly different reaction conditions and in general manufacturer's instructions were followed. Still each PCR has in principle the same setup, which is shown for a DreamTaq PCR below.

25 µl DreamTaq PCR reaction		PCR program			
1 x	DreamTaq buffer	95 °C	3 min	Initial denaturation	
0.2 mM	dNTPs	95 °C	30 s	Denaturation	x cycles
1 µM	Forward primer	x °C	30 s	Annealing	
1 µM	Reverse primer	72 °C	1 min/kb	Extension	
50 ng	Template DNA	72 °C	10 min	Final extension	
1.25 U	DreamTaq polymerase				

Annealing temperature depended on the melting temperature of the primers. The length of the extension time was selected according to the product size.

3.9.4 Digestion with restriction enzymes

Analytical restriction digest of plasmids was performed to verify cloning steps. Plasmids or PCR products were digested to produce compatible ends to use them in cloning approaches. Both were realized following manufacturer's instructions and in general prepared as described below.

20 µl Restriction digest reaction	
1x	Enzyme specific buffer
1 µl	Restriction enzyme
1-5 µg	DNA

3.9.5 Agarose gel electrophoresis and DNA extraction

Agarose gels were used to analyze analytical PCRs or to purify DNA fragments from PCRs or restriction digests.

TEA-Buffer pH 8.5	40 mM Tris acetate 1 mM EDTA
6x DNA loading dye	10 mM Tris pH 7.5 60 % (v/v) Glycerol 0.03 % (w/v) Bromophenol blue 0.03 % (w/v) Xylene cyanol

For standard applications gels with 1 % (w/v) agarose in 1x TAE buffer were used. Agarose solution was microwaved until completely dissolved and the chilled solution was supplemented with 0.5 µg/ml ethidium bromide to stain the DNA. Gels were cast in trays with combs fitting in size to the application (analysis or purification). Samples were mixed with 6x loading dye, loaded onto the gel and let run in 1x TAE at 120 V for 30-60 min. A DNA marker served as a size control. The separated DNA fragments were visualized using UV-light and pictures taken with the INTAS system. Fragments that were run to purify were cut out of the gel with a scalpel and extracted with the peqGOLD Gel extraction kit (Peqlab) according to manufacturer's instructions. Concentration of the eluted DNA was measured with the NanoDrop spectrophotometer.

3.9.6 Ligation of DNA fragments

Ligation of compatible ends of plasmid fragments and/or PCR products was used to create new plasmids. The purified DNA fragments were mixed in relation to their size. In general, 100 ng vector backbone was combined with 2-fold excess of insert, 1x Ligase buffer and 1 µl T4 DNA Ligase in a total volume of 10 µl. The mixture was incubated at 16 °C overnight. During this time, the ligase connects the 5'- phosphate of the vector with the 3'- hydroxyl group of the insert. The ligated plasmid was used to transform *E. coli* cells and subsequently selected for positive clones.

3.9.7 Gibson Assembly (GA)

Beside standard ligation Gibson Assembly was used to generate new plasmids. This method (described in (Gibson, 2011; Gibson et al., 2009)) allows joining of multiple DNA fragments with compatible overhangs in one reaction by utilizing three enzymatic activities.

2x GA master mix	5 % (v/v) PEG 8000
	100 mM Tris/HCl pH 7.5
	10 mM $MgCl_2$
	10 mM DTT
	200 µM dNTPs
	1 mM NAD
	0.004 U/µl T5 exonuclease
	0.025 U/µl Phusion DNA polymerase
	4 U/µl *Taq* DNA ligase

5'- ends of double stranded fragments were digested by the T5 exonuclease leaving 3'- overhangs. These overhangs allow complementary annealing of the fragments. Subsequently, 3' gaps in the sequence are filled up by the Phusion DNA polymerase. The resulting free ends of each strand are ligated by the *Taq* DNA ligase. A 20 µl reaction contained 1x GA master mix, 100 ng of vector and 2-3-fold excess of the insert. After 1-3 h incubation at 50 °C the newly ligated plasmid was used to transform *E. coli* cells.

3.9.8 Sequencing

New plasmids were sequenced to verify the correct position and DNA sequence of the insert. 200-500 ng of the plasmid together with 20 pmol of a gene specific primer were mixed in 14 µl and send to LGC Genomics (Berlin/Germany) for sequencing.

3.10 Molecular biological methods with yeast

3.10.1 Extraction of RNA from yeast and reaction mixtures

Cells or mixtures were treated as indicated before RNA preparation. Extraction of total RNA from yeast pellets or liquid mixtures (see nucleo-cytoplasmic fractionation) as well as cytoplasmic RNA was performed using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions. RNA that was obtained by RNA- Co-IP experiments (see 3.13.2) was purified using Trizol®. 1 ml Trizol® was added to the beads with the bound protein-RNA complexes and incubated with agitation at 65 °C for 10 min. Addition of 200 µl chloroform lead to formation of two phases – one aqueous, one organic, which were separated by centrifugation at 20,000 g for 10 min. The resulting upper phase was mixed with 1 µl glycogen and 500 µl isopropanol to precipitate the RNA. After centrifugation for 30 min at 20,000 g, 4 °C the pellet was washed once with 70 % ice-cold ethanol and let dry. RNA was resuspended in DEPC-treated deionized water, concentration measured and kept at –20 °C for short and at -80 °C for long term storage.

3.10.2 Quantitative RT-PCR (qRT-PCR)

Quantitative RT-PCR was used to examine relative induction of certain genes or the differences in protein-bound RNA quantities. This method is based on molecules like SYBR® Green that become fluorescent upon binding to double stranded DNA. After each PCR- cycle the

fluorescence is measured and thus the DNA product increase over time can be correlated with the starting amount of DNA. Equal amounts of RNA were transcribed into coding DNA (cDNA) using the Maxima reverse transcriptase and following manufacturer's instructions. In general, random hexamer primer were used. As negative controls (RT^-) samples without reverse transcriptase were treated equally. The resulting cDNA was diluted 1:20-1:50 depending on the amount of transcribed RNA. For analysis of ribosomal RNAs dilutions again were further diluted 1:1,000 because of the high abundance of these RNAs. 5 µl of diluted cDNA was mixed with 2x qRT-PCR master mix and gene specific primers (final concentration 0,08 µM) in 15 µl. Reactions and analysis were carried out using mainly the qPCR Cycler CFX Connect (BioRad) and the following program:

PCR program

95 °C 5 min	Initial denaturation	
95 °C 5 s	Denaturation	45 cycles
60 °C 10 s	Annealing	
60 °C 10 s	Extension	
65 °C–95 °C	Melt Curve	

At the end of the PCR a melt curve was recorded to verify that only one specific product was produced. Resulting Ct - values were normalized where applicable and/or fold changes were set in relation to total RNA, a wildtype isolate or a specific gene according to requirements of the experiment and basically applying the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.10.3 Synthesis of digoxigenin labeled RNA probes and oligonucleotide tailing

Digoxigenin (DIG)-labeled probes of 200-300 nt were used to visualize RNA in Northern Blot approaches or *in situ* hybridization microscopy studies. Templates for RNA specific probes were prepared by PCR using primers pairs with one carrying a *T7*-promoter (HK 2022 and HK 2023 for the GFP probe). These purified templates were used for *in vitro* run off-transcription reactions with DIG-UTP labeling mix (Roche). This mix contains all four NTPs that naturally occur in RNAs together with DIG-11-UTP in smaller amounts, which leads to incorporation of DIG-UTP every 20^{th}-25^{th} nucleotide depending on RNA composition. Reactions were prepared as suggested by the manufacturer.

DIG-labeling reaction 20 µl	200-250 ng DNA template 1x Transcription buffer 1x DIG RNA labeling mix 20 U RiboLock RNase Inhibitor 40 U T7 RNA polymerase

After incubation at 37 °C for 2 h the DNA template was digested with 2 µl DNase I for 15 min at 37 °C. RNA probes were precipitated with 0.25 volumes 4 M LiCl, 1 µl glycogen and 3 volumes 100 % ethanol overnight at -20 °C. The following centrifugation at 20,000 g for 30 min pelleted the RNA, which was washed with 70 % ethanol and air-dried. The probe was resuspended in 20 µl formamide, 20 µl 0.5x TE pH 7.5 and 60 µl HybMix before stored at -20 °C.

Hybridization mix (HybMix)	50 % (v/v) deionized formamide 5x SSC 1x Denharts solution 0.1 mg/ml Heparin
50x Denharts solution	1 % (w/v) Ficoll 1 % (w/v) Polyvinylpyrrolidone 1 % (w/v) BSA

For identification of poly(A)-containing RNAs on Northern dot blots, a DIG-labeled oligo-dT probe was prepared. Since in vitro transcription of poly(A) stretches is not very effective a different method was chosen. A 50 nt long dT oligo was 3' end tailed with approximately 100 nt DIG-dUTP and dATP using an DIG-dUTP tailing kit (Roche) and following the enclosed protocol. The probe was precipitated and restored as described above.

3.10.4 Nucleo-cytoplasmic fractionation

Extraction of cytoplasm was, with modifications, performed as described (Sklenar and Parthun, 2004). For nucleo-cytoplasmic fractionation cells were grown to log-phase in selective medium. After harvest cells were washed once with 1ml YPD/ 1 M Sorbitol/ 2 mM DTT and resuspended in YPD/ 1 M Sorbitol/ 1 mM DTT. Cells were spheroblasted using Zymolyase and diluted in 50 ml YPD/ 1 M Sorbitol to recover for 30 min at 25 °C and shifted to indicated temperature if applicable. Cells were put on ice, centrifuged at 900 g for 5 min and resuspended in 500 µl Ficoll buffer.

Ficoll buffer pH 6.0	18 % (w/v) Ficoll 400 10 mM HEPES
Buffer A pH6.0	50 mM NaCl 1 mM $MgCl_2$ 10 mM HEPES

Total RNA sample was taken and subsequently cells were broken by addition of 1 ml buffer A. The suspension was mixed and centrifuged at 1,500 g for 15 min. The supernatant was used for cytoplasmic analyses. To verify correct fractionation of the cytoplasmic lysates, samples were analyzed in western blot for the presence of the cytoplasmic protein Zwf1 and nucleolar protein Nop1. RNA was purified as described above

3.10.5 RNA agarose gels, Northern blot and Dot blot experiments

Total or cytoplasmic RNA was analyzed in Northern blots and analysis was performed as described earlier (Sambrook et al., 1989; Wu et al., 2014). For gel-electrophoresis all solid material was cleaned with 0.1 M NaOH and all aqueous solutions treated with 0.1 % DEPC to avoid RNA degradation by contaminating RNases.

10x MOPS pH 7.0	200 mM MOPS 50 mM Sodium acetate 10 mM EDTA
RNA loading dye	50 % (v/v) deionized formamide 6 % (v/v) formaldehyde 1x MOPS 25 ng/ml ethidium bromide 10 % Glycerol Bromophenol blue and Xylene cyanol
20x SSC	3 M NaCl 300 mM Sodium Citrate

Gels containing 1x MOPS, 1 % agarose and 2 % formaldehyde were cast under the hood. Equal amounts of RNA were mixed 1:2 with RNA loading dye, denatured for 10 min at 65 °C and chilled on ice. Samples were loaded onto the gel and let run in 1x MOPS at 120 V for 1-2 h. Integrity of the RNA and equal loading was analyzed under UV light before the gel was washed once with DEPC-treated water and twice with 20x SSC to remove the formaldehyde before blotting. RNA was transferred to a nylon membrane with dry capillary blot overnight. For this the gel was set on a glass plate followed by the membrane, 3 layers dry Whatman paper and a

stack of paper towels, 500 g weight compressed the setup. The next day, the transferred RNA was cross-linked to the membrane using 5,000 J/cm^2 UV light for 7 min and baking at 80 °C for 2 h. For dot blot experiments the eluted RNA was spotted on the membrane, let dry, cross-linked as above and treated the same.

Hybridization buffer pH 7.2	500 mM Sodium phosphate pH 7.2 7 % (w/v) SDS 1 mM EDTA
1M Sodium phosphate pH 7.2	68.4 ml 1M Na_2HPO_4 31.6 ml 1M NaH_2PO_4
5x Maleic acid buffer pH 7.5	0.5 M Maleic acid 750 mM NaCl
1x Blocking solution	1 % Blocking Reagent (Roche) 1x Maleic acid
Washing buffer	1x Maleic acid 0.3 % (v/v) Tween 20
Detection buffer pH 9.5	100 mM Tris pH 9.5 100 mM NaCl

The membrane was prehybridized with hybridization buffer for 1 hour at hybridization temperature (42 °C for oligo dT probes, 68 °C for gene specific probes) with agitation. 1 µl of the respective probe was added to the membrane and hybridized overnight. Next day the membrane was washed once in 2x SSC, 0.1 % SDS and 1x SSC, 0.1 % SDS at room temperature und twice with 0.5x SSC, 0.1 % at hybridization temperature for 15 min. After shortly washing with washing buffer, the membrane was blocked for 30 min in 1x blocking solution and incubated with anti-DIG antibody (1:10,000 in blocking buffer) for 60 min. Two 15 min washing steps with washing buffer were performed before the membrane was equilibrated in detection buffer and CSPD (diluted 1:100 in detection buffer) was applied. To reduce background, the membrane was incubated at 37 °C for 10 min before detection of the signal with either the Fusion camera or X-ray sensitive films (Fuji) depending on signal intensity.

3.11 Microscopic studies / Cell biological methods with yeast

All microscopic studies were performed at the Leica DMI6000B fluorescence microscope and pictures obtained with the Leica DFC360 FX camera using the LAS AF 1.6.2 software.

3.11.1 Fluorescence *in situ* hybridization (FISH)

FISH experiments were performed to visualize nucleic acids by using sequence-specific fluorescently labeled probes and essentially performed as described earlier (Hackmann et al., 2014). Yeast cells were grown to log-phase in appropriate medium and shifted as indicated if applicable. For visualization of *GPM1* mRNA cells were grown in 1 % glucose to provoke a slight limitation and expression of the gene was induced by addition of 4 % glucose 15 min prior to heat stress application. Cells were fixed with 4 % formaldehyde for 45-60 min. After centrifugation at 2,000 g, 4 °C for 5 min cells were washed three times with P-solution before resuspended in 100 µl P-solution and treated with 10 mM DTT for 10 min.

0.1 M Phosphate buffer pH 6.5	33 mM K_2HPO_4 67 mM KH_2PO_4
P-solution pH 6.5	0.1 M Phosphate buffer 1.2 M Sorbitol
1x PBS pH 7.4	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
Mounting medium pH 8.0	2 % (w/v) n-Propyl-gallate 80 % (v/v) Glycerol 1x PBS pH 8.0

Cells were spheroblasted by addition of 0.5 mg Zymolyase and incubated until approximately 70 % of the cells appeared dark. Centrifugation at 400 g for 5 min was perform to pellet the cells, washed once with P-solution and 25 µl suspension were brought on a poly-lysine coated slide. Incubation at 4 °C for 30 min was followed by treatment with 0.5 % Triton X-100 in P-solution for 10 min. To block polar groups cells were equilibrated with 0.1 M triethanolamine (TEA), pH 8.0 before incubation with 0.25 % (v/v) acetic anhydride in 0.1 M TEA for 5 min. Prehybridization with HybMix (see 3.10.3) to which 0.5 mg/ml tRNA and 0.5 mg/ml denatured salmon sperm DNA was added was performed at 37 °C for 1 h. Subsequently, HybMix with 0.5 mg/ml tRNA and 0.5 mg/ml salmon sperm DNA and a specific probe was brought onto the

slide and incubated at 37 °C overnight. For detection of poly(A)$^+$ containing RNAs an Atto488- or Cy3-labeled oligo dT_{50} probe was used in a 1:100-1:500 dilution. For detection of specific RNAs Cy3-labeled oligos (see Table 11) were used 1:50-1:500, *in vitro* transcribed DIG-RNA probes were used 1:10-1:100. After incubation slides were washed with 2x SSC and 1x SSC for 60 min each and twice with 0.5x SSC for 30 min at 37 °C and 25 °C. If fluorescently labeled probes were used, slides were washed with 0.5 % Tween 20 in PBS for 30 min and DNA was stained with Hoechst 33342 (1:10,000 in PBS) for 5 min. After washing twice with PBS for 5 min slides were let dry and sealed with mounting medium, a cover slip and nail polish. If DIG-labeled probes were used slides were blocked with antibody blocking buffer (ABB) for 1 h at room temperature.

Antibody blocking buffer (ABB)	1x PBS 5 % (w/v) heat-inactivated FCS 0.3 % (v/v) Triton-X-100

Anti-DIG antibody was diluted 1:200 in ABB and applied overnight at 4 °C. Washing was performed twice with ABB for 15 min and once for 30 min before 30 min washing with 0.5 % Tween 20 in PBS and staining DNA as described above.

3.12 In vitro binding studies

3.12.1 Expression of recombinant proteins

For interaction analysis of Mex67 and its mutants with Npl3 *in vitro*, recombinant proteins were expressed in *E. coli* Rosetta 2 (DE3) cells (Novagen). These cells carry a chromosomal T7-RNA polymerase under the control of a *lacUV5*-promoter, which allows controlled induction of protein expression by addition of galactose, in general the galactose mimicking compound isopropyl-β-D-thiogalactopyranosid (IPTG) is used. Additionally, these cells contain a plasmid with genes encoding for a chloramphenicol resistance and tRNAs that are rare in *E. coli*, but frequently used in eukaryotes, which might help to improve expression. Untagged Mex67 was expressed together with His_6-Mtr2 and was already present and purified by affinity chromatography using Ni-NTA agarose (Macherey & Nagel) by Dr. Lysann Bender and Michael Mitter (according to (Yao et al., 2007)). Wild type Mex67 (pHK1372), mex67KR>AA (pHK1373) and mex67Δloop (pHK1374) were diluted in elution buffer to a final concentration of 0.45 mg/ml and stored at -80 °C.

Elution buffer pH 7.5	20 mM HEPES pH 7.5 100 mM KCl 10 mM NaCl 4 mM $MgCl_2$ 20 % (v/v) Glycerol 0.5 % (v/v) NP-40 Protease inhibitor (Roche)

For GST-Npl3 bacterial lysate *E. coli* Rosetta 2 cells were transformed with pHK1276 (pGEX4T-1-GST-*NPL3*) and grown at 37 °C in 2xYT medium, supplemented with ampicillin and chloramphenicol, to an OD_{600} of 1.2 before addition of pre-cooled medium and induction with 0.5 mM IPTG at 16 °C overnight. Induction was controlled by taking samples before and after induction and performing SDS-PAGE and Coomassie staining (see 3.13.3 and 3.13.4). Afterwards, cells were harvested and pellets stored at -20 °C or directly resuspended in lysis buffer (4 ml lysis buffer for a pellet of 50 ml culture).

Lysis Buffer pH 7.5	20 mM HEPES pH 7.5 100 mM NaCl 4 mM $MgCl_2$ 10 % (v/v) Glycerol 1 mM DTT 0.1 % (v/v) NP-40 Protease inhibitor (Roche)

The suspension was sonified 2-3 times for 5 min (30 % duty cycle, output 3) until lysate became visibly clearer. Insoluble parts were separated by centrifugation at 20,000 g for 20 min at 4 °C and the amount of GST-Npl3 in the lysate was adjusted to approximately 1 µg/µl. This was achieved by comparing the signal intensities of the lysate with different amounts of bovine serum albumin (BSA) after running an SDS-gel and staining the proteins with Coomassie. Aliquots of the lysate were stored at -20 °C until use.

3.12.2 *In vitro* co-immunoprecipitations

In vitro co-immunoprecipitations with Mex67, mex67KR>AA or mex67Δloop with Npl3 were performed by combining 50 µg of the respective Mex67 protein, 250 µl GST-Npl3 bacterial lysate, 100 µg RNase A and 550 µl lysis buffer (see 3.12.1) with Protein G sepharose beads (Amersham Biosciences) loaded with Mex67 antibody (rabbit). Samples were incubated at 4 °C on an overhead-rotator for 1.5 h before beads were washed six times with lysis buffer. Beads and input samples were mixed with 2x SDS-loading buffer and subjected to SDS-PAGE

followed by Western blot to detect proteins with the indicated antibodies (Mex67 (rabbit, serum) 1:50,000); GST (mouse, Santa Cruz) 1:2,000).

3.12.3 *In vitro* competition assay

The influence of nucleic acids on Mex67-Npl3 binding was analyzed with a competition assay using RNA and DNA. A complex of *in vitro* purified Mex67 and GST-Npl3 was allowed to form as described in 3.12.2. The beads were washed 3 times and resuspended in 1 ml lysis buffer with 1 µl RiboLock to which the indicated amount of total yeast RNA (prepared from cells that were shifted to 42 °C for 30 min) or DNA (a 7.3 kb plasmid was digested with *Ban*I resulting in 6 fragments ranging from 94 bp to 3.1 kb) were added. After further incubation for 1 h at 4 °C on an overhead-rotator beads were washed four times with lysis buffer and proteins were detected as described above. For quantification of pull-down intensities, the Bio1D software was used.

3.13 Protein and RNA biochemical methods

3.13.1 Preparation of yeast cell lysate

After yeast cells were grown to log phase and treated as indicated for each experiment they were transferred in a 2 ml screw-cap tube or a 15 ml falcon tube. Cells were lysed with the same amount of glass beads and lysis buffer, which was chosen according to assay requirements and supplemented with protease inhibitor (complete EDTA-free, Roche), phosphatase inhibitor and if applicable RiboLock (Thermo) to avoid RNA degradation. Homogenization and rupture of cells was performed three times using the FastPrep-24 machine at 4.5 m/s for 2 ml tubes and at 5.5 m/s for 15 ml tubes for 20 seconds. This was followed by centrifugation at 4,000 g for 5 min and at 21,000 g for 10 min resulting in the cleared lysate directly used for experiments.

3.13.2 Protein-protein and protein-RNA co-immunoprecipitation

Co-immunoprecipitations (IPs) were utilized to analyze interactions between proteins or proteins and their bound RNAs and performed as described earlier (Gross et al., 2007; Hackmann et al., 2014). For most analysis 10 µl slurry of GFP-Trap beads (Chromotek) with covalently bound anti-GFP antibodies were used to purify GFP-tagged proteins. Precipitation of other proteins was performed by coupling 20 µg of the specific antibody (anti-myc,

anti-Mex67) with 20 µl slurry of IgG-sepharose beads. Beads were washed 4 times with the corresponding buffer before use.

PBSKMT buffer pH 7.5	1x PBS pH 7.5 3 mM KCl 2.5 mM $MgCl_2$ 0.5 % (v/v) Triton-X-100
RNA-IP buffer pH 7.5	25 mM Tris/HCl pH 7.5 100 mM KCl 0.2 % (v/v) Triton-X-100 0.2 mM PMSF 5 mM DTT RiboLock, Protease Inhibitor

Commonly 400 ml cell culture was used, treated as indicated and pelleted. Cells were lysed in PBSMT buffer for protein-protein IPs and in RNA-IP buffer for protein-RNA IPs as described above. To control and compare protein contents of each sample 50 µl lysate were mixed with the same amount 2x SDS loading buffer, boiled 5 min at 95 °C and stored at -20 °C to be used as input control. For RNA Co-IPs 100 µl were taken and immediately frozen in liquid nitrogen as total RNA sample. Lysates and antibody-coupled beads were combined and if applicable additional substances (RiboLock, phosphatase inhibitor) added. Where indicated, lysates were treated with 0.2 mg/ml RNase A to degrade single-stranded RNAs. For most precipitations samples were incubated 3-4 h at 4 °C with overhead rotation. Immunoprecipitation of Hsf1-GFP was performed for 20 min at 25 °C for the untreated cells and at 37 °C for cells that were prior heat stressed at 42 °C. Incubation of the stressed lysate was not performed at 42 °C because after 20 min lysates started to get cloudy - potentially because proteins started to denature and precipitate. After incubation beads were washed a minimum of six times with 1 ml of the corresponding buffer with centrifugation between each washing at 400 g for 2 min at 4 °C. For reduction of unspecific signals beads were transferred in a new Eppendorf tube before the last washing step followed by elution in 35 µl 2x SDS loading dye, boiling at 95 °C for 5 min and storage at –20 °C until use. In RNA Co-IP applications beads were split in two portions – one for control of protein precipitation in western blot, one for isolation of co-precipitated RNA as described in 3.10.1.

3.13.3 SDS-acrylamide gel-electrophoresis (SDS-PAGE)

Total cell lysates or proteins that were purified via co-immunoprecipitation were separated by size and analyzed by denaturing SDS polyacrylamide gel-electrophoresis (SDS-PAGE) essentially described in (Garfin, 2009). A Tris-glycine buffer and denaturing system (Laemmli, 1970) were used together with an SDS-gel composed of 5 % stacking gel and 10 % resolving gel.

Staking gel (5 %)	16.7 % (v/v) Rotiphorese Gel 30 acrylamide mix 125 mM Tris/HCl pH 6.8 0.1 % (w/v) SDS 0.1 % (w/v) Ammoniumpersulfate (APS) 0.1 % (v/v) Tetramethylethylendiamin (TEMED)
Resolving gel (10 %)	33.3 % (v/v) Rotiphorese Gel 30 375 mM Tris/HCl pH 8.0 0.1 % (w/v) SDS 0.1 % (w/v) APS 0.1 % (v/v) TEMED
Running buffer	25 mM Tris 192 mM Glycine 0.1 % (w/v) SDS
2x SDS-loading buffer	125 mM Tris/HCl pH 6.8 25 %(v/v) Glycerol 2 % (w/v) SDS 5 % (v/v) β-Mercaptoethanol Bromophenol blue

Gel components were mixed and polymerization initiated by addition of APS and TEMED. Samples were mixed with SDS-loading buffer, boiled at 95 °C for 5 min and centrifuged at 22,000 g for 1 min. A size standard (10 µl unstained or 3 µl prestained marker) was loaded together with the samples and electrophoresis performed at 25 mA to let samples run into the staking gel, followed by 40 mA for resolving gel or at 5-7 mA if the gel was run overnight.

3.13.4 Coomassie staining of SDS-gels

Coomassie Brilliant blue was used to stain proteins in SDS-polyacrylamide gels as in general described earlier (Neuhoff et al., 1985). This method was mainly used to control expression of recombinant proteins.

Staining solution	50 % (v/v) Methanol 10 % (v/v) Acetic acid 0.25 % (w/v) Coomassie Brilliant Blue R250
Destaining solution	5 % (v/v) Methanol 10 % (v/v) Acetic acid

Gels were incubated in staining solution for 15 min, followed by destaining with destaining solution until bands became visible. Heating in a microwave and tissues that bound color particles accelerated destaining.

3.13.5 Western blot analysis

To detect proteins with specific antibodies, samples separated by SDS-PAGE were transferred on a nitrocellulose membrane using Western blot. The method described in (Towbin et al., 1979) was modified as follows.

Blotting buffer	25 mM Tris base 192 mM Glycine 20 % (v/v) Methanol (freshly added)
Ponceau S solution	5 % (v/v) Acetic acid 0.2 % (w/v) Ponceau S
TBST pH 7.4	50 mM Tris pH 7.4 150 mM NaCl 0.1 % (v/v) Tween 20

A semi-dry blotting system was used to transfer the proteins on the membrane. The Anode was wetted with blotting buffer, then one layer Whatman paper in size of the gel was put on the anode followed by the membrane (Protran 0.45 µm), then the gel and finally another layer of Whatman paper. All components were equilibrated in blotting buffer and air bubbles removed before blotting for 1.5-2 h depending on the size of the proteins of interest. The size of the membrane defined the amperage (1.5 mA/cm^2). After transfer proteins on the membrane were stained with Ponceau S solution. Washing with water made separated proteins as well as marker protein bands visible, which allowed marking of the size standard and cutting the membrane. Unspecific protein binding sites were blocked with 5 % milk powder in TBST for 1 h. Afterwards, primary antibodies (see 3.4) in 2 % milk powder/TBST were applied for 2 h at room temperature or overnight at 4 °C. Membranes were washed three times with TBST before incubation with secondary HRP-coupled antibody in 2 % milk powder/TBST for 2 h at room

temperature. This was followed by two washing steps in TBST and one in TBS (TBST lacking Tween 20) before the 1:1 mixed detection substrate was added and signals were detected using the Fusion-SL chemoluminescence detection system.

3.14 Microarray analysis

Preparation of mRNA samples was performed by Dr. Alexandra Hackmann and Daniel Becker as published earlier (Zander et al., 2016). In general, cells were grown to log phase and kept at 25 °C or heat stressed for 30 min at 42 °C before cultures were UV-crosslinked. Cells were harvested and lysates prepared in RIP-buffer, which was used for co-immunoprecipitation of Npl3-myc (HKY 157) or Mex67-GFP (HKY 1266) with GFP-Trap beads or protein G sepharose beads coupled with c-myc antibody at 4 °C for 4 h. Co-precipitated RNA was purified using Trizol® and possibly contaminating DNA degraded with the TURBO DNA-free kit (Ambion). The following microarray analysis was carried out by the Transkriptomanalyselabor (TAL) Göttingen. 200 µg RNA were used to prepare cDNA and further processed using Affimetrix products. A detailed description can be found in Zander et al. 2016. The resulting data was deposited with the accession number GSE83267 in the NCBI GEO archive.

3.15 Quantification and statistical analysis

Quantification of dot blot and northern blot signals was performed using the Fiji software by measuring signal intensities, subtracting background and calculating relative intensities. Western blots were quantified using the Bio1D software. For quantification of e.g. nuclear accumulation microscopy pictures were analyzed by counting a minimum of 50 cells for each strain and calculating the percentage of a displayed phenotype. For statistical analyses an unpaired, two-sided students t-test was performed. Depending on the type of the experiment equal or unequal variance was applied to calculate p-values. These are indicated as follows: ***: $p<0.001$, **: $p<0.01$, *: $p<0.5$.

4 Results

4.1 The role of Mex67 in mRNA export under stress

4.1.1 Mex67 dissociates from mRNAs but not its adaptor proteins under stress

Mex67 is one of the few factors that is essential for nuclear export of mRNAs under normal and stress conditions (Hurt et al., 2000; Sträßer et al., 2000). It was published earlier that adaptor proteins like Nab2 and Npl3 are not required for export of HS mRNAs (Carmody et al., 2010; Krebber et al., 1999) and that normal mRNAs are no longer exported under stress (Saavedra et al., 1996). This raised the question how cells discriminate between normal mRNAs that are meant to be blocked in the nucleus and HS mRNAs that are predestined for export under stress conditions. As already presented for most factors (Dr. Lysann Bender, PhD thesis), not only Npl3 and Nab2 but all mRNA adaptor proteins including Gbp2, Hrb1 or Yra1 that are loaded by the THO complex, as well as Mex67 showed dissociation of bulk mRNAs under heat stress (15 min, 42 °C) and salt stress (1 M NaCl, 1 h). Further experiments in this study verified the same effect for Npl3 and found the THO complex component Hpr1 as an additional factor dissociating from mRNAs under stress. Figure 11a shows the average amount of poly(A)$^+$-containing RNAs that were bound to the indicated proteins and the reduction under both stress conditions.

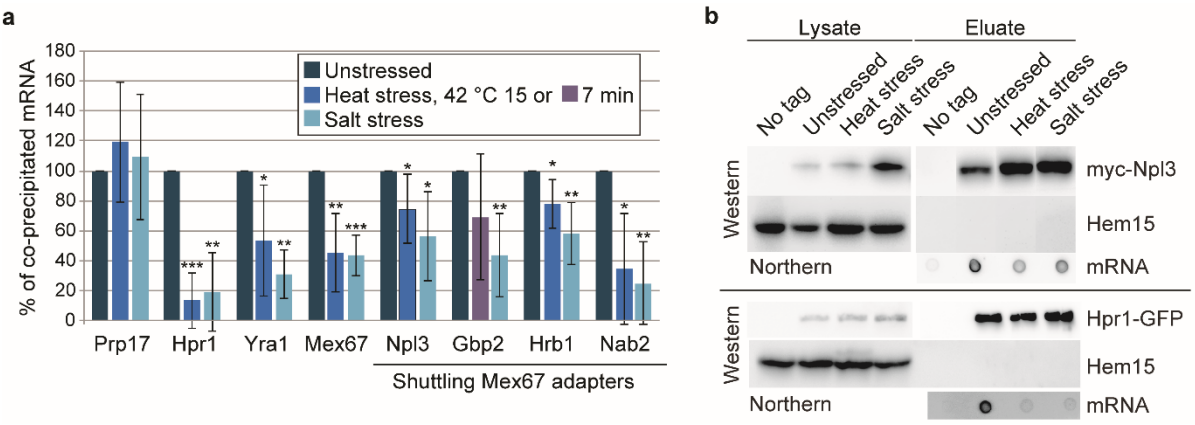

Figure 11: Mex67 and its adaptor proteins dissociate from mRNAs under stress. a, Quantification of a minimum of three RNA-Co-IPs with GFP-or myc-tagged proteins as depicted in (b)**.** Co-purifying RNA was isolated and analyzed with dot blot experiments against poly(A) RNA, normalized with the amount of precipitated protein and set into relation to unstressed conditions. Average and standard deviation was calculated and a two-tailed, two-sample, unequal variance t-test was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (Most analyses were performed by Dr. Lysann Bender). **b**, Western blot analysis show the purified proteins detected by antibodies against their tags and Hem15 specific antibody as a negative control. The co-purified mRNA was analyzed via northern dot blot and detected with a DIG-labeled oligo dT_{50} probe. Published in (Zander et al., 2016).

Examples of some co-precipitations are shown in Figure 11b. Contrastingly, the splicing factor Prp17 stayed bound with comparable amounts of mRNA at all conditions, which resembles the stalled splicing reaction induced by stress (Biamonti and Caceres, 2009).

Notably, the adaptor protein Gbp2 aggregates reversibly under these heat stress conditions (Wallace et al., 2015) for which reason a shorter heat stress was performed. As shown above the interaction of general shuttling export factors like Npl3, Gbp2, Hrb1 and Nab2 with bulk poly(A)$^+$ RNAs was reduced under stress. This finding at least partially explains how the general mRNA export block is accomplished. If now the adaptor proteins dissociated from the mRNA under stress what happens to their binding to the export receptor Mex67? This was addressed by further experiments analyzing this interaction under the same unstressed, heat stress and salt stress conditions as above using co-immunoprecipitations and western blot analyses. Surprisingly, the interaction of Mex67 with its adaptors stayed the same irrespective of the treatment (Figure 12). Again, just a small amount of Gbp2 could be precipitated at heat stress conditions due to its aggregation, but still the ratio of Gbp2 to bound Mex67 was comparable to no stress or salt stress.

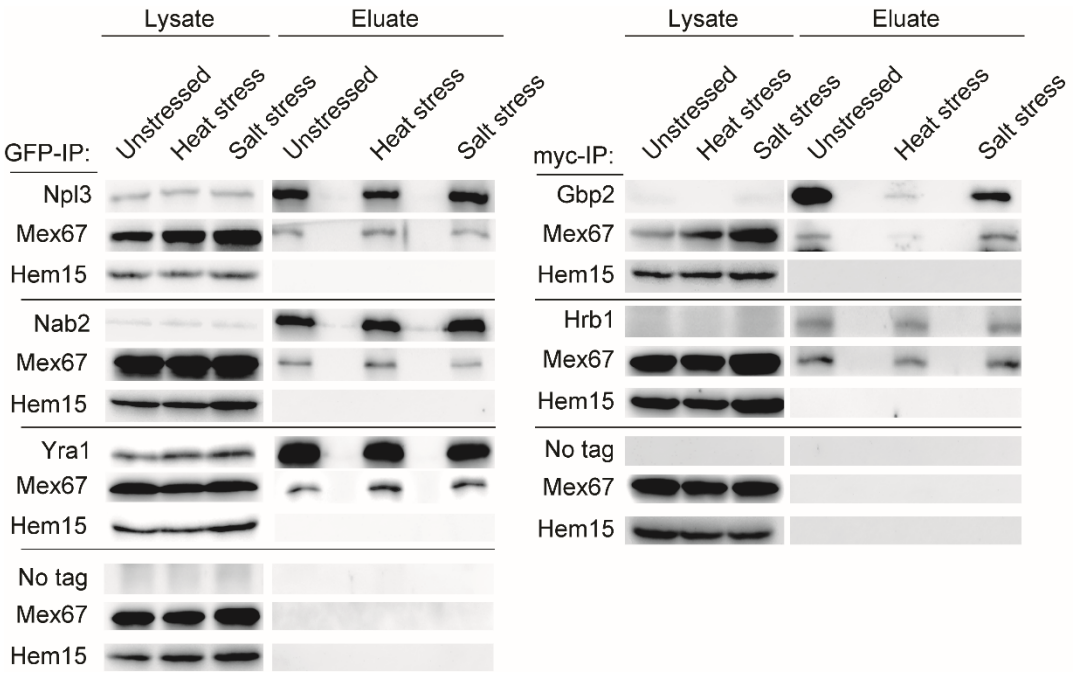

Figure 12: Mex67 does not dissociate from its adaptor proteins during stress. Co-immunoprecipitations with Mex67 adaptor proteins show no change in Mex67 binding under unstressed (25 °C), heat stress (15 min, 42 °C) or salt stress (1 h, 1 M NaCl) conditions. GFP- or myc-tagged adaptor proteins were precipitated and detected in Western blot analyses with GFP- or myc-specific antibodies. Mex67 was detected with a direct antibody. A direct antibody against Hem15 and precipitation from a lysate without any tagged protein served as negative controls. Published in (Zander et al., 2016).

To assure that this interaction is direct and not mediated by mRNAs, to which both analyzed proteins could be bound independently, all lysates were treated with RNase A during the entire incubation period. Completeness of RNA degradation was controlled by isolating total RNA from the lysate after incubation, which was subsequently transcribed into cDNA and analyzed with a semi-quantitative PCR (Figure 13).

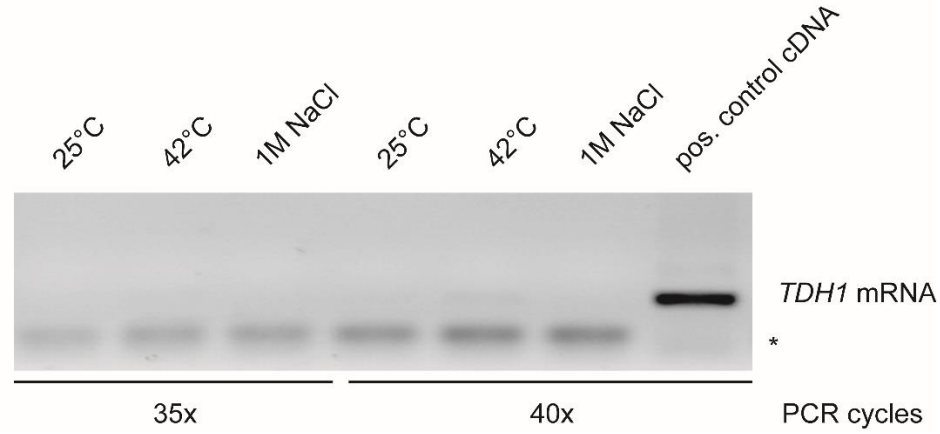

Figure 13: Semi-quantitative PCR verifies complete RNA digestion. Total RNA was isolated from lysates analyzed in Figure 12, cDNA prepared and PCR amplifying the *TDH1*-mRNA was performed. A lysate that was treated equally except for addition of RNase A was used as a positive control. Samples were taken after 35 and 40 cycles and analyzed on an agarose gel. A lower migrating band originates from unconsumed primers (*).

The PCR verified complete mRNA digestion as even after 40 PCR cycles no product was detectable, indicating the interaction between Mex67 and its adaptor proteins shown in Figure 12 is and stays direct at normal and stress conditions.

As the adaptor proteins lose interaction with poly(A)$^+$-RNA but not Mex67 this could be a mechanism to dissociate both the adaptor and the exporter from bulk RNAs to block export. This would be in agreement with the finding in Figure 11 as Mex67 itself was less bound to mRNAs under stress. It is possible that the pool of Mex67 being adaptor protein bound is different from the pool that dissociates from mRNAs and differs due to different experimental setups. Therefore, we visualized these two effects in one experiment. An RNA co-immunoprecipitation with Mex67-GFP was performed, the beads were split and the co-purified myc-Npl3 was detected using western blot analysis. The bound mRNA was visualized using northern dot blot analysis.

Figure 14 shows that myc-Npl3 is equally bound to Mex67 at all conditions (unstressed, heat stress, salt stress) as also displayed in Figure 12. Furthermore, northern dot blots show that for the same precipitation Mex67 bound less mRNA at stress conditions, which is consistent with the findings presented above (Figure 11).

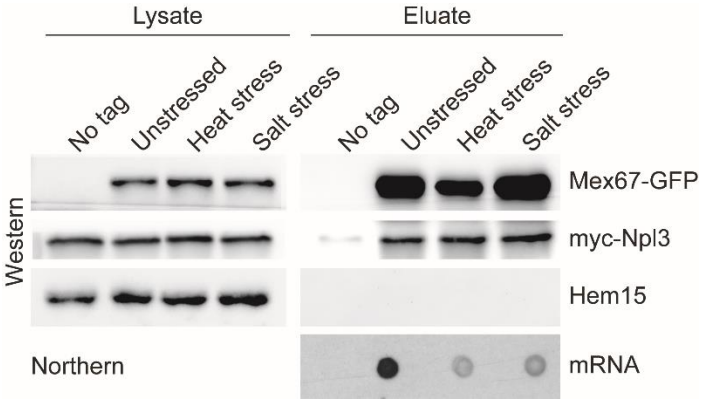

Figure 14: Mex67 dissociates from mRNAs while it stays bound to its adaptor Npl3. Purification of Mex67-GFP from unstressed (25 °C), heat (15 min, 42 °C) or salt stressed (1 h, 1 M NaCl) cells and analysis of bound Npl3 via Western blot together with analysis of bound poly(A)$^+$-RNAs via dot blot is shown. Mex67 and Npl3 were detected with GFP- or myc-specific antibodies. A direct antibody against Hem15 served as a negative control. A DIG-labeled oligo dT_{50} probe detected mRNAs. Published in (Zander et al., 2016).

Together all these data indicate that Mex67 as well as the adaptor proteins dissociate from bulk mRNAs under stress. Since the interaction of Mex67 and the adaptors was the same at all conditions it is quite likely that Mex67 dissociates together with the adaptors and this in turn might lead to retention of bulk mRNAs in the nucleus.

4.1.2 Genome wide analysis shows global dissociation of Npl3 and Mex67 from bulk mRNAs and enrichment of Mex67 on stress responsive transcripts

To gather further insight in the mRNA binding status of Npl3 and Mex67 under normal and stress conditions, genome-wide RNA-binding profile analysis was performed in the department of Prof. Heike Krebber. Transcripts that were Npl3 or Mex67 bound at 25 °C or upon 30 min 42 °C heat shock were purified. These mRNAs were examined via microarray analysis and the precipitated amount that was bound to either protein at the specific temperature (25 °C or 30 min, 42 °C) was determined.

Detailed analysis of the data confirmed that both Npl3 and Mex67 showed dissociation from bulk mRNAs under heat stress even on a global level. When compared to normal conditions the amount of bound mRNA was reduced to 80 % for Npl3 and to 70 % for Mex67 (Figure 15).

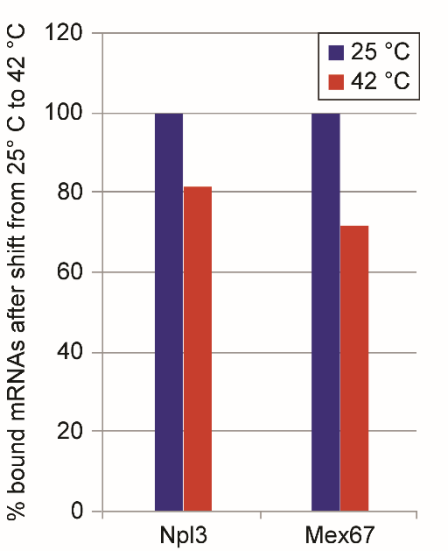

Figure 15: Microarray analysis confirm dissociation of Npl3 and Mex67 from bulk mRNAs. RNA co-immunoprecipitations with Npl3 and Mex67 from either untreated or heat stressed cells were performed. Co-precipitated mRNAs were purified and analyzed via microarray. The intensity for each transcript was normalized to the no tag IP before the number of bound mRNAs at 25 °C ($log_2 > 0.5$) was set to 100 % and the reduction upon heat stress calculated. Published in (Zander et al., 2016).

These numbers only present the mRNAs completely dissociated (log_2 fold change < 0,5) upon stress, but not those with only a slighter reduction in binding. Additionally, one has to consider that only mRNAs that were not exported yet and are still in the nucleus would show a reduced interaction. This pool might be quite small compared to cytoplasmic mRNAs, which likely aggregate in P-bodies or stress granules with several proteins that might include some adaptors (Mitchell et al., 2013). Nevertheless, the reduced mRNA binding of Npl3 and Mex67 observed in dot blot analyses is reinforced by these microarray data.

Beside the information about the total amount of bound RNA, a benefit of microarray analysis is that it identifies the mRNA itself, which allowed to further specify the binding profiles of Npl3 and Mex67 at normal and stress conditions. When each RNA was sorted into its functional group, clear differences between the two proteins and the different conditions could be observed (Figure 16). Functional groups were chosen according to the transcript's main function in the cell as far as this was described in the *Saccharomyces* genome database SGD (www.yeastgenome.org; August 2016).

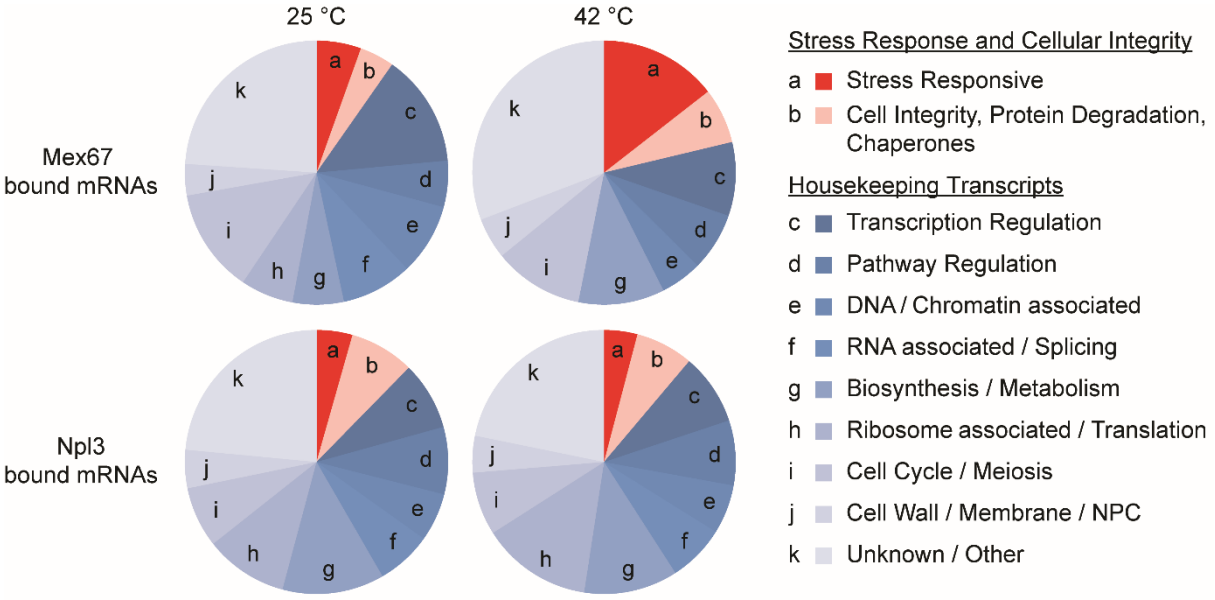

Figure 16: Mex67 binds specifically to HS mRNAs under stress. Microarray analysis shows increased binding of Mex67 to stress mRNAs at 42 °C. Transcripts co-precipitated with Npl3 or Mex67 after treatment of the cells with normal (25 °C) or heat stress (42 °C) conditions were analyzed. Bound mRNAs ($log_2 > 0.5$) at 25 °C or 42 °C were sorted into functional groups (a-k) according to the function annotated in the SGD database. Published in (Zander et al., 2016).

For this analysis Npl3 showed no striking difference in its binding profile when shifted from 25 °C to 42 °C and mRNAs of all functional groups were bound at heat stress. However, one has to keep in mind that at 42 °C only 80 % of the RNA was associated with Npl3 (Figure 15). This would indicate that Npl3 dissociates equally from all RNAs under stress and not staying bound to a certain population.

In contrast, Mex67 showed a drastic change following stress. About a threefold increase for stress responsive mRNAs (group a) and a twofold increase for transcripts encoding for chaperones or components involved in protein degradation (group b) could be found. The latter group is not directly described to be stress responsive, but one can easily assume their function in coping with heat stress. Interestingly, the third group of Mex67-bound mRNAs that became noticeably larger under stress was the one containing yet unknown factors (group k) (Figure 16). It is quite likely that new components involved in stress response will be found among them. Together, this genome-wide analysis supports the dissociation of export factors from mRNAs under stress and identified Mex67 to be enriched on stress responsive transcripts.

4.1.3 Mex67-Mtr2 but no known adaptor protein is involved in export of HS mRNAs

As shown above Mex67 and all adaptors dissociate from bulk mRNA under stress and it is known that poly(A)$^+$ RNA accumulates in the nucleus. To analyze if this block is also detectable

for a specific mRNA and is detectable in mutants of all adaptor proteins, fluorescent *in situ* hybridization (FISH) experiment were performed. Cells were shifted to 42 °C for 30 min and the specific housekeeping *GPM1*-mRNA encoding for phosphoglycerate mutase, an enzyme that acts in glycolysis and is constantly expressed, was detected. This was accompanied by detection of poly(A)$^+$-containing RNAs.

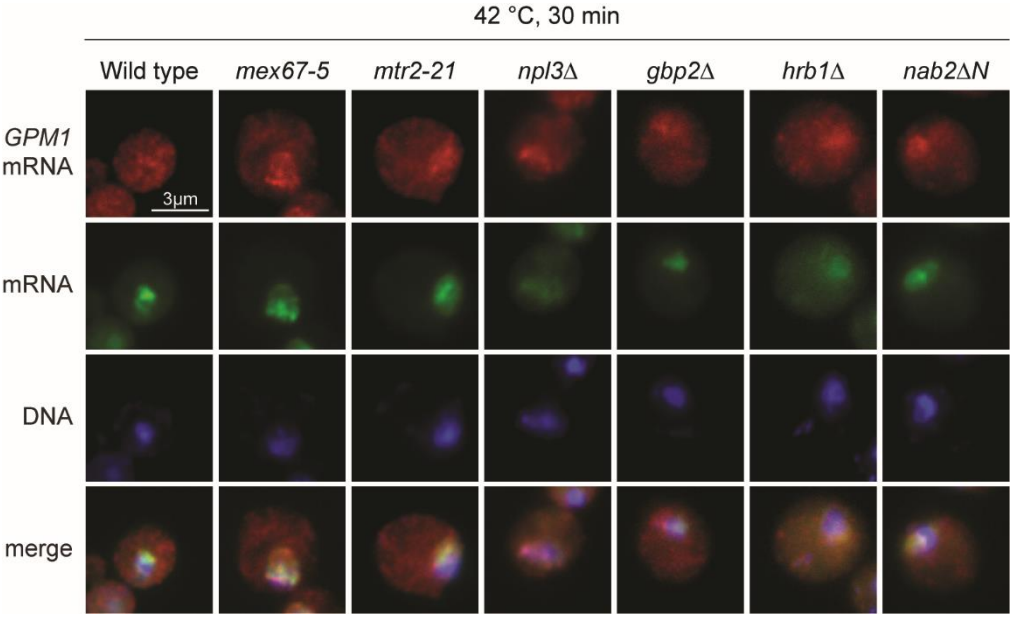

Figure 17: Export of the housekeeping *GPM1*-mRNA is blocked under heat stress conditions. FISH analysis of the indicated strains after a 30 min shift to 42 °C was performed. A sequence-specific Cy3-labelled probe detected the *GPM1*-mRNA (red). Bulk mRNA was detected with an Atto488-labelled oligo d(T)$_{50}$ probe (green). DNA was stained with Hoechst (blue). Published in (Zander et al., 2016).

In order to increase the *GPM1*-specific signal, cells were grown on half the amount of glucose (1 %) that is normally added to the medium and RNA expression increased by addition of 4 % glucose 15 min before application of heat stress. Figure 17 shows that the *GPM1*-mRNA accumulated in all strains including the wild type in the same manner bulk mRNA accumulated in the nucleus. Staining of *GPM1*-mRNA expectedly did not show as a strong signal as for total RNA, because in this case only a single mRNA was detected. Nevertheless, in all strains at least some dots in the nucleus -indicated by total RNA staining and DNA staining- could be observed. This indicates that in fact normal and frequently expressed housekeeping mRNAs like *GPM1* are blocked in their export under stress. Furthermore, this holds true for transcripts just induced shortly before heat application as performed here.

The specificity of the obtained signal is underlined when compared with Figure 18, where the exact same setup was used except for omission of RNA-specific probes.

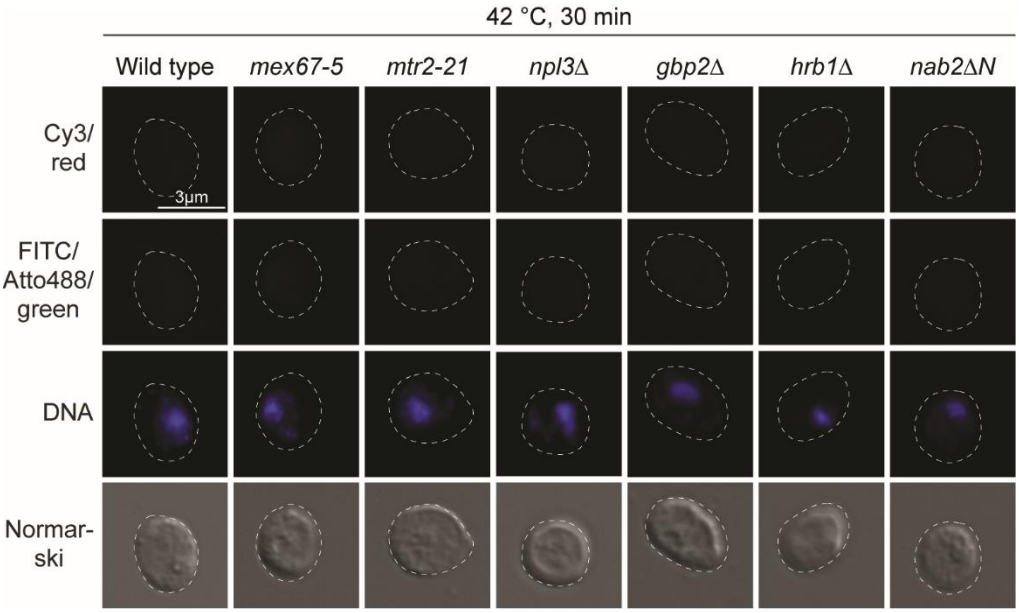

Figure 18: No staining of cellular compartments can be observed when a specific probe is missing. The same experiment as in Figure 17 was performed except no RNA-specific probes were added. DNA was stained with Hoechst (blue). Published in (Zander et al., 2016).

Here no signal was detected for *GPM1*- or poly(A)$^+$-mRNAs indicating a specific staining and not detection of background signal. Signals in all following FISH analysis were analogous. Together this shows that heat stress induces a general export block of housekeeping RNAs, not only in mutants of the general export receptor Mex67 and its cofactor Mtr2 and in the wildtype as described earlier (Rollenhagen et al., 2007; Saavedra et al., 1996), but in all adaptor protein mutants. Even the just induced *GPM1*-mRNA is blocked in all strains. As this block is universal for all housekeeping mRNAs it remains to analyze the fate of a HS mRNA in these mutants. It is known that Mex67 acts together with Mtr2 as a heterodimer in nuclear export at both normal and stress conditions and Nab2 is most likely excluded from HS mRNA export because of its aggregation in nuclear granules. Nevertheless, the other adaptor proteins could still have a function in export of stress mRNAs. To investigate this aspect, FISH analysis (most data by Dr. Lysann Bender, see Zander et al., 2016) of the HS mRNA *SSA4* were performed. As expected and in accordance with published data, in a mutant of *MEX67* (*mex67-5*) this HS mRNA is no longer exported and trapped in the nucleus (Figure 19).

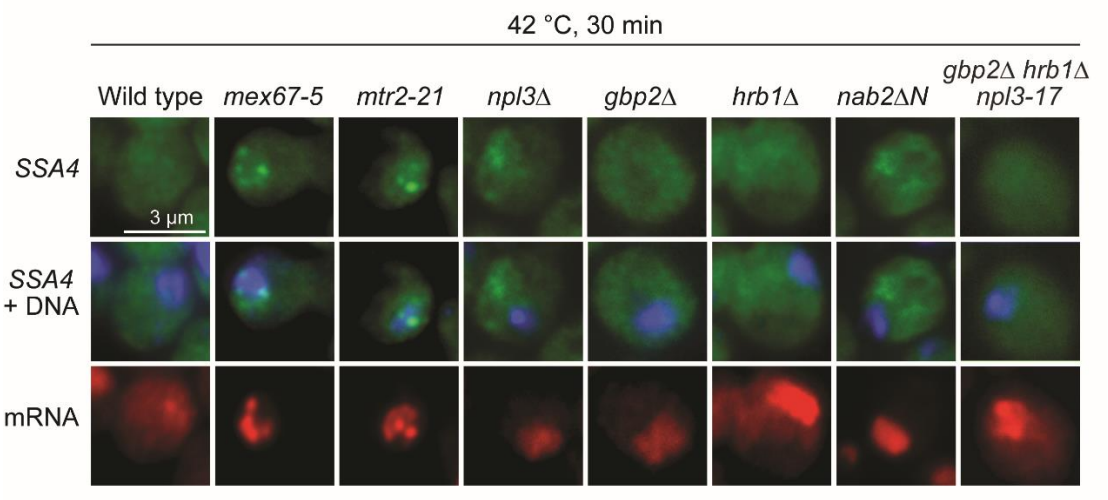

Figure 19: Mex67 and Mtr2 but no export adaptor acts in HS mRNA export. Fluorescence *in situ* hybridization experiments localize the *SSA4* mRNA using a DIG-labelled probe (green). The indicated strains were grown to log phase before shifted to 42 °C for 30 min. DNA was stained with Hoechst (blue) and bulk poly(A)$^+$ RNA visualized with a Cy3-labelled probe (red) (most analyses were performed by Dr. Lysann Bender). Published in (Zander et al., 2016).

The same dot like structures that co-localize with the DNA could be observed for a *MTR2* mutant (*mtr2-21*), which is not surprising as Mex67 and Mtr2 act as a heterodimeric complex in RNA export. Strikingly, in all mutants of the adaptor proteins *npl3Δ, gbp2Δ, hrb1Δ* and *nab2ΔN* no export defect could be found as is the case in wild type cells where these mRNAs are normally exported. Even in the triple mutant *gbp2Δ hrb1Δ npl3-17* no accumulation could be detected, which argues even stronger against a participation of the adaptor proteins in HS mRNAs export since here no adaptor could compensate for the loss of the other.

Even though one cannot exclude that there might be other, yet unknown, factors that support Mex67-Mtr2 in binding to HS mRNAs, these and the following data allow the conclusion that Mex67-Mtr2 acts presumably alone in HS mRNA export. At least an involvement of the known shuttling adaptor proteins Npl3, Gbp2, Hrb1 and Nab2 in expression and export of stress responsive transcripts seems unlikely.

4.2 Mex67 binds Npl3 and RNA via the same region

4.2.1 Mutations of *MEX67* disturb HS mRNA export

Previous publications show that Mex67 is capable of direct binding to the 5S rRNA (Yao et al., 2007) and that a domain that builds a loop on the surface of Mex67 plays a crucial role in this process. As Dr. Lysann Bender could show (PhD thesis), RNA-binding of Mex67 is not exclusive to the 5S rRNA. In fact, Mex67 binds all kinds of RNAs including housekeeping mRNAs as well as HS mRNAs. This information was obtained with two Mex67 versions with mutations in the loop domain created by the lab of Ed Hurt. The mex67KR>AA mutant carries mutations of six lysines and arginines in the loop region (aa 415-427) to alanines which reduces the proteins ability to bind RNA, while the mex67Δloop mutant is deleted for the loop domain from amino acids 409-435 (Yao et al., 2007). Both loop domain mutants fail to bind to HS mRNAs as previous *in vitro* experiments indicate (PhD thesis Dr. Lysann Bender and Zander et al. 2016). To analyze these mutants in more detail for their behavior under heat stress, FISH experiments with staining of bulk mRNAs together with the gene specific *GPM1*-mRNA were performed (Figure 20).

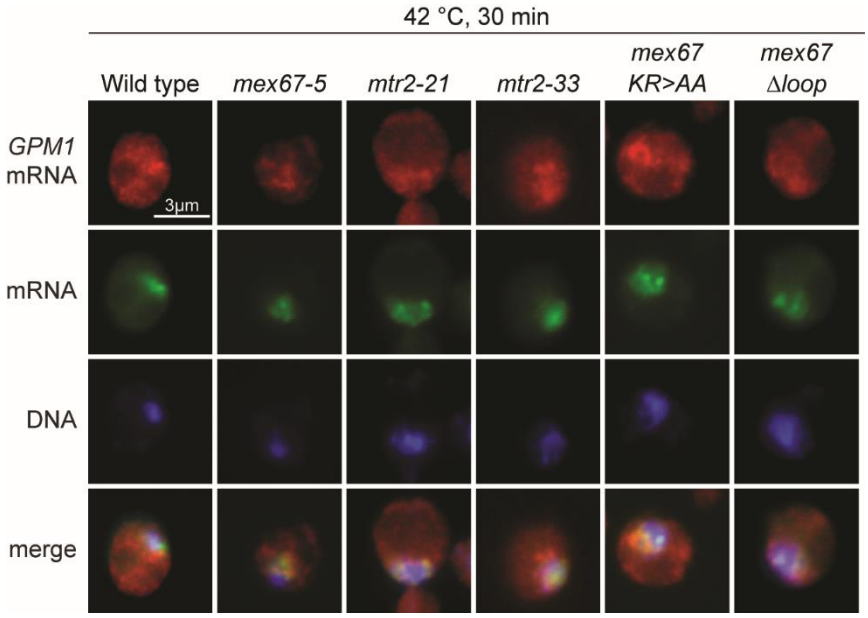

Figure 20: All mutants of *MEX67* and *MTR2* show bulk mRNA export defects. Indicated strains were grown to log phase and shifted to 42 °C for 30 min. FISH experiments with probes against the specific *GPM1*-mRNA (red) and against poly(A)$^+$-RNA (green) were performed. DNA was stained with Hoechst (blue). Accumulation of *GPM1*. and bulk-mRNA was detected in all mutants. Published in (Zander et al., 2016).

The two loop domain mutants of *MEX67* (*mex67KR>AA* and *mex67Δloop*) as well as another *mtr2* mutant (*mtr2-33*) showed the same export defect for bulk poly(A)$^+$-RNA and the *GPM1*-

mRNA that could be detected for wild type cells and the other mutants of *MEX67* and *MTR2* (*mex67-5, mtr2-21*). As all strains accumulated the specific *GPM1*-mRNA and bulk mRNA, the loop mutants of *MEX67* showed no difference to wild type in response to heat stress. Since these mutant strains expectedly accumulated bulk mRNAs under stress, the same strains were analyzed in terms of HS mRNA export. As already published, these *mex67* and *mtr2* mutants failed to export the HS mRNAs *SSA4* and *HSP12* and accumulated them in the nucleus (PhD thesis Dr. Lysann Bender and Zander et al. 2016). One can conclude that especially for Mex67 already single amino acid mutations or the deletion of the loop domain plays a crucial role in the export of HS mRNAs.

Interestingly, strains carrying these permanent (not temperature sensitive) mutant versions of Mex67 in the loop domain are viable even at elevated temperatures (Yao et al., 2007). Therefore, the defect in HS mRNA export cannot be explained by excessive degradation as all proteins were stable in the cell even after 42 °C heat stress for 30 min (Figure 21). The different size of the mex67Δloop mutant protein is caused by deletion of the loop domain which reduces the size of the protein by approximately 3-5 kDa.

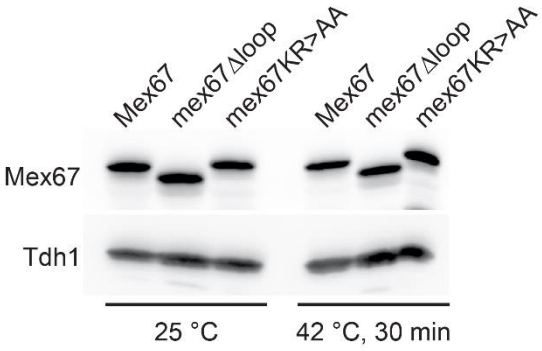

Figure 21: Mex67 and its loop domain mutants are stable at 42 °C. Western blot analysis of Mex67 and two loop domain mutants. Cells carrying the indicated Mex67 constructs were grown to log phase before one half was heat stressed at 42 °C for 30 min. Mex67 is detected with a specific antibody. Detection of Tdh1 with a specific antibody served as a loading control. Published in (Zander et al., 2016).

As these mex67 mutant proteins were stably expressed at normal conditions and stayed stable after stress, degradation cannot be the cause of nuclear HS mRNA retention.

4.2.2 The loop domain of Mex67 is needed for RNA and Npl3 binding

To gather more insight in the mechanism of Mex67 in binding to adaptor proteins and RNA in general, the interaction of Mex67 and its mutants with the adaptor protein Npl3 were analyzed *in vivo*. Cells carrying the respective version of Mex67 and a myc-tagged version of Npl3 were used for co-immunoprecipitations (Figure 22). The myc-tagged and not the wild type version of Npl3 was used to enlarge the protein, as otherwise Npl3 would be the same size as Mex67.

Western blot analyses of the precipitations showed that the interaction between mex67Δloop and Npl3 is significantly reduced in comparison to wild type Mex67. Also for mex67KR>AA a strong reduction in Npl3 binding can be observed.

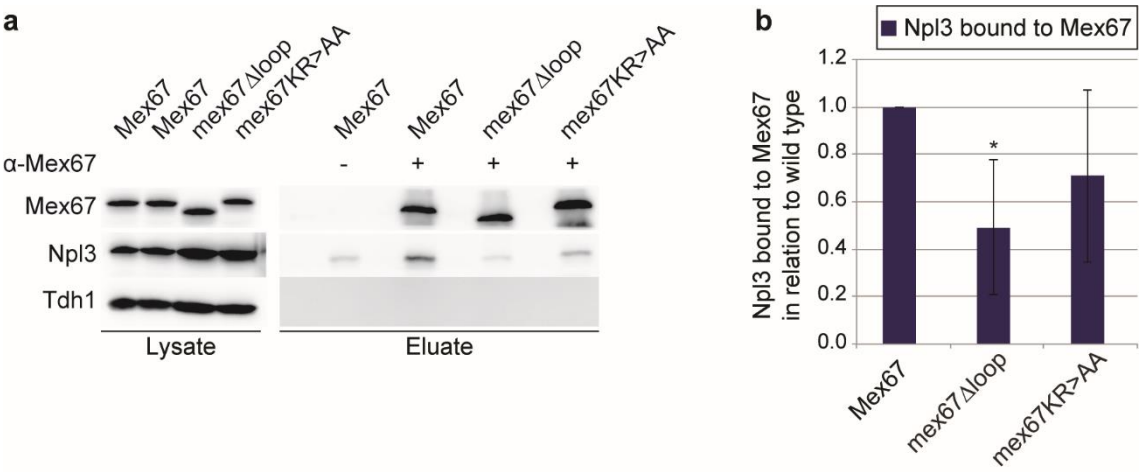

Figure 22: Mex67 binds to Npl3 via its loop domain *in vivo*. a, Western blot analysis of co-immunoprecipitation with the indicated versions of Mex67 with Mex67-specific antibodies is shown. Co-purified Npl3 was detected with a myc-specific antibody. A Tdh1-specific antibody served as a negative control. **b,** Quantification of 4 different experiments shown in (a). Signal intensities were measured, corrected by background and set in relation to Mex67. Average and standard deviation was calculated and a two-tailed, two-sample, unequal variance t-test was performed (*p < 0.05). Published in (Zander et al., 2016).

Even in the negative control, where unspecific precipitation of Mex67 was not possible since no antibody was added, trace amounts of Npl3 could be precipitated, potentially due to unspecific binding to the beads. It is likely that some of the precipitated Npl3 in the other lanes originated from unspecific binding rather from real interaction. Additionally, there might be other proteins binding to Mex67 or its mutants and by this possibly bridging the interaction with Npl3. Nevertheless, the strongest binding of Npl3 was clearly detectable to the unmutated Mex67. In order to analyze only the direct interaction of these two proteins the same experiment was carried out *in vitro*. All three versions of Mex67 were purified recombinantly and were already present (see 3.12.1). GST-Npl3 was expressed recombinantly and used with a defined concentration (1 µg/µl) in bacterial lysate. Bacterial lysate was used to compete for unspecific binding partners while at the same time reducing the amount of interactions with unknown proteins that proteins from yeast lysate might exhibit. As shown in Figure 23 the interaction of Mex67 with Npl3 *in vitro* was drastically and significantly reduced for mex67 mutants.

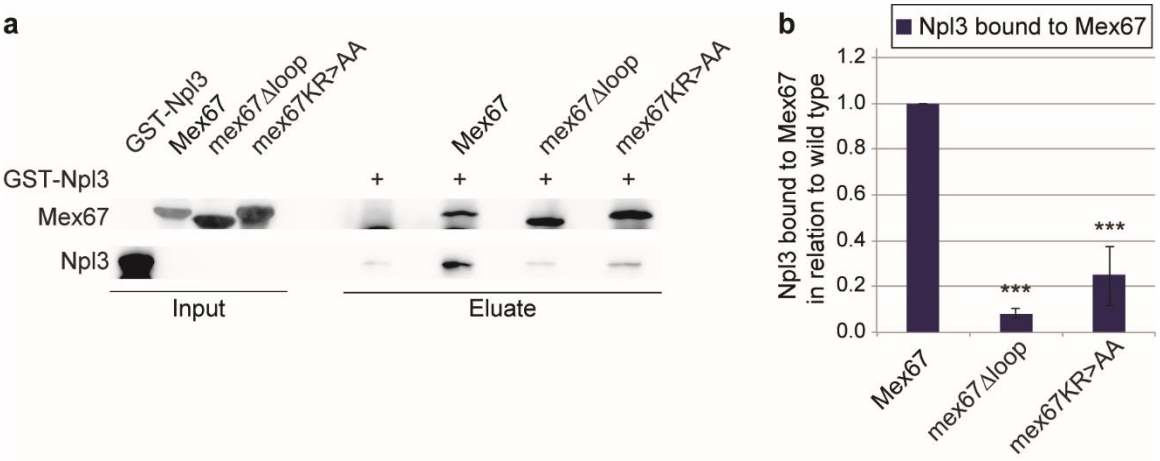

Figure 23: Mex67 binds to Npl3 via its loop domain *in vitro*. **a**, Western Blot analysis of recombinantly expressed Mex67 proteins that were immunopurified with Mex67-speciifc antibody in the presence of recombinantly expressed GST-Npl3 in bacterial lysate. Mex67 was detected by a protein-specific antibody. GST-specific antibody was used to detect Npl3. One reaction without recombinant Mex67 was used as negative control. **b,** Quantification of 5 different experiments shown in (a). Signal intensities were measured, corrected by background and set in relation to Mex67. Average and standard deviation was calculated and a two-tailed, two-sample, unequal variance t-test was performed (***p < 0.001). Published in (Zander et al., 2016).

The effect of binding reduction *in vitro* was even stronger than *in vivo* (Figure 22) which might in fact be due to other factors in the yeast lysate that supported an interaction of the mex67 mutants with Npl3. These data together identify a role of the loop domain of Mex67 not only in RNA binding as already published, but also in binding to the shuttling adaptor protein Npl3.

4.2.3 Binding of Mex67 to Npl3 or RNA is mutually exclusive

If Mex67 binds to RNA and Npl3 over the same or at least a neighboring region, it seems likely that only one partner can bind at a time. To analyze this, *in vitro* competition assays were performed. Here a preformed Mex67-Npl3 as shown in Figure 23 was challenged with increasing amounts of RNA or DNA. The RNA was prepared from yeast cells that were shifted to 42 °C for 30 min prior isolation. In this way, bulk housekeeping as well as HS mRNAs should be present in the total RNA extraction. DNA was prepared from a plasmid that was cut into six pieces ranging from 94 bp to 3.1 kb, sizes that cover most of the sizes of RNAs that can be found in the cell. DNA was chosen as a negative control since it is like RNA, a nucleic acid but contains a different sugar backbone and is, in this case, double stranded. Therefore, this experiment should show the different affinities for the protein binding partner and for a single stranded RNA.

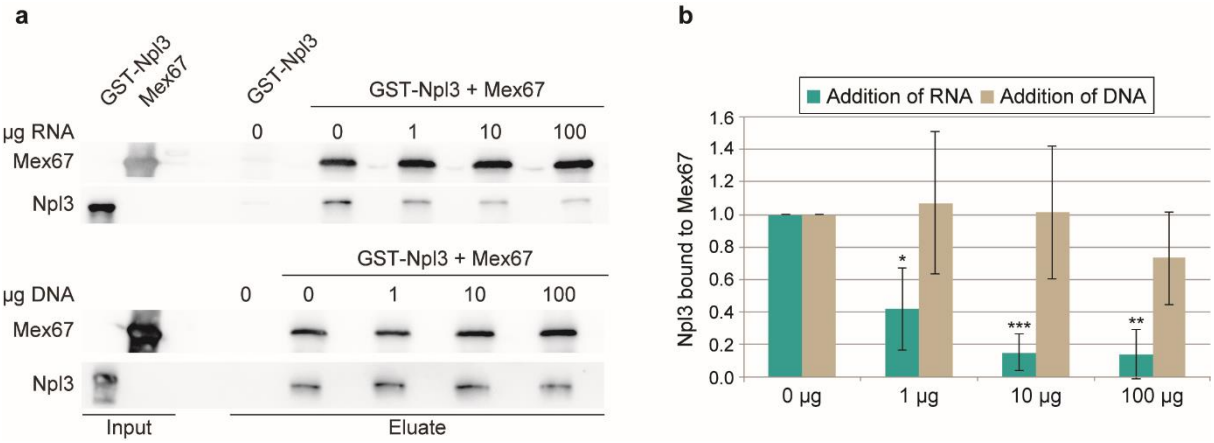

Figure 24: RNA but not DNA disrupts a preformed Mex67-Npl3 complex. a, An *in vitro* preformed complex of recombinantly expressed Mex67 and GST-Npl3 was treated with increasing amounts of RNA or DNA (0 µg-100 µg). Western blot analyses detect precipitated Mex67 with a protein-specific antibody and co-purified GST-Npl3 with a GST antibody. **b,** Quantification of 3 different experiments shown in (a). Signal intensities were measured, corrected by background and set in relation to no RNA/DNA addition. Average and standard deviation was calculated and a two-tailed, two-sample, unequal variance t-test was performed (*p < 0.05, **p < 0.01, ***p < 0.001). Published in (Zander et al., 2016).

Indeed, when incubating a complex of recombinantly expressed Mex67-Npl3 with increasing amounts of RNA the interaction between these proteins was destroyed as depicted in Figure 24. Already the smallest quantity of RNA (1 µg) significantly reduced binding of Mex67 to Npl3 by 60 % and 10 µg of RNA resulted in more than 80 % loss of interaction (Figure 24 b). Contrastingly, addition of DNA had no significant effect when 1 or 10 µg were added to the complex. Only when 100 µg of digested DNA was added to the sample less than 30 % of the complexes were destroyed. Potentially, Npl3 can bind to both nucleic acids. However, the affinity of Npl3 for RNA is a lot higher as this experiment suggested. The mutant versions of Mex67 are not degraded, and cells carrying them are viable. This and the strong effect that RNA had on the Mex67-Npl3 complex argues for at least an overlap of the Mex67 regions involved in binding to RNA and that Mex67's binding to either RNA or Npl3 seems to be mutually exclusive.

Together, under normal conditions Mex67 binds RNA-bound Npl3 and exports this particle. If the cells must cope with stress it appears that an Npl3-Mex67 complex is dissociated from the RNA, which thus blocks export of bulk mRNAs. The data further suggest that newly transcribed HS mRNAs are directly bound by free Mex67 and exported without the help of adaptor proteins.

4.3 Mex67 is directly recruited to the transcription site of HS mRNAs

4.3.1 Mex67 interacts with Hsf1 under heat stress conditions

Under normal conditions newly transcribed mRNAs are co-transcriptionally loaded with adaptor proteins like Npl3, Gbp2, Hrb1 and Nab2, which in turn are bound by the export receptor heterodimer Mex67-Mtr2. However, all these proteins dissociate from mRNAs under stress as shown in 4.1.1 and are not involved in the export of HS mRNAs. This lead to the question how Mex67 is linked with the stress responsive transcripts that ought to be exported. Either HS mRNAs are transcribed and move freely in the nucleoplasm until Mex67 randomly binds to them and promotes their export or there is a more organized and thus potentially more efficient way that allows the binding of Mex67 to the emerging HS mRNAs. To ensure a fast loading of Mex67 this process is presumably co-transcriptional.

One factor that is involved in the strong expression of heat stress responsive genes is the trimeric transcription factor Hsf1. It becomes highly phosphorylated under heat stress and binds to the promoters of HS genes via the specific heat shock element (HSE) (Sorger, 1991). This factor is in close proximity to newly transcribed HS mRNAs as it interacts with the transcription machinery (see 2.3.6). Potentially, Mex67 is directly recruited to the place of transcription to ensure a fast export. As Hsf1 is essential for heat stress responsive gene expression, it was used to analyze this model in more detail. Therefore, co-immunoprecipitation experiments with an endogenously GFP-tagged version of Hsf1 with and without the application of heat stress were performed and analyzed if Mex67 co-purified with this transcription factor.

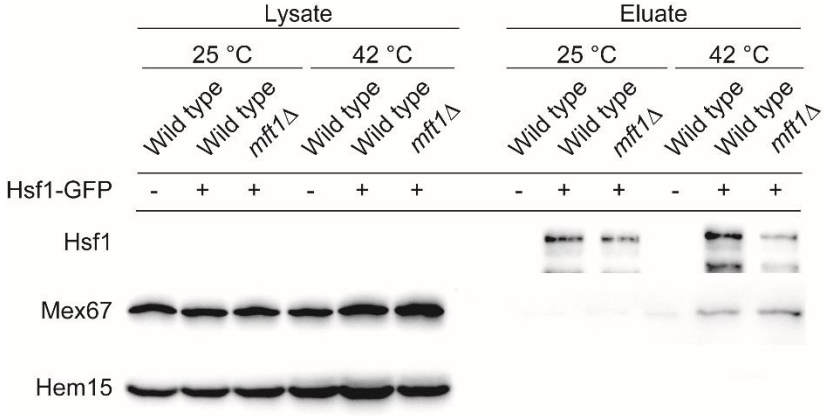

Figure 25: Mex67 binds to the transcription factor Hsf1 upon heat stress. Cells with the indicated genetic background were either kept at 25 °C or shifted to 42 °C for 15 min. GFP-trap beads were used to precipitate Hsf1-GFP from the lysates. Western blot analysis with GFP-specific antibody confirmed the pull-down. Protein-specific antibodies were used to detect the co-purifying Mex67 and the negative control Hem15. Published in (Zander et al., 2016).

The results depicted in Figure 25 show an exemplary western blot with the detectable pull-down of Hsf1-GFP from cells treated with or without heat stress (constant 25 °C or 15 min at 42 °C). Due to a low number of the protein in the cell (approximately 300-400 molecules/cell (Kulak et al., 2014)) and its large size (120 kDa) the amount of precipitated Hsf1-GFP was comparably small. Still, a slight upshift of Hsf1-GFP was visible at 42 °C indicating its phosphorylation and by this the cellular response to the applied stress. Strikingly, the export receptor Mex67, which could not be detected to interact with Hsf1-GFP at 25 °C showed interaction at 42 °C. This was the case not only in wild type cells, but also for a strain deleted for *MFT1*, a subunit of the THO complex. The THO/TREX complex is important for expression, transcription elongation and export factor loading of mRNAs under normal conditions as exemplarily Mex67 is recruited to Nab2 by the help of Yra1 (Iglesias et al., 2010). While the THO complex is crucial for normal mRNA expression, its importance in transcriptional stress response is not clear. However, even though THO might still be involved in transcription elongation and factor loading under stress, deletion of one of its components, *MFT1*, did not influence interaction of Hsf1 with Mex67 arguing for a direct recruitment of the export receptor to the newly transcribed HS mRNA. Together, under heat stress Mex67 can bind the transcription factor Hsf1, most likely without the need of the loading complex THO. This in turn would allow direct and early binding of Mex67 to the HS mRNA and promote its export.

4.3.2 Mex67 is enriched on transcripts controlled by Hsf1

Co-immunoprecipitations above show that Mex67 interacts with the transcription factor Hsf1. If this is a mechanism to ensure direct recruitment of Mex67 to HS mRNAs during transcription the exporter might be bound to all mRNAs whose transcription is under the control of Hsf1. To investigate this on a genome-wide scale the microarray data already analyzed for global binding profiles of Mex67 and Npl3 (see 4.1.2) were used. Several genes controlled by Hsf1 were already identified (Yamamoto et al., 2005) though still the number is growing. Binding of Mex67 at 42 °C and 25 °C to the transcripts deriving from these genes was analyzed. The ratio resulting from comparing both temperatures was set into relation with the same ratio that was calculated for binding of Npl3 to these mRNAs at both temperatures. This allowed to visualize the changes in binding not only between the two temperatures, but as well between a protein that dissociates from bulk mRNA under stress (Npl3) and one that is crucial for HS mRNA export (Mex67). Since the microarray was performed only once the results might not display

the average status in a cell in response to heat stress, but gives broad overview of the general tendencies. In fact, analysis of the microarray data revealed that Mex67 was enriched on nearly all Hsf1-controlled transcripts under stress when compared to Npl3 as depicted in Figure 26.

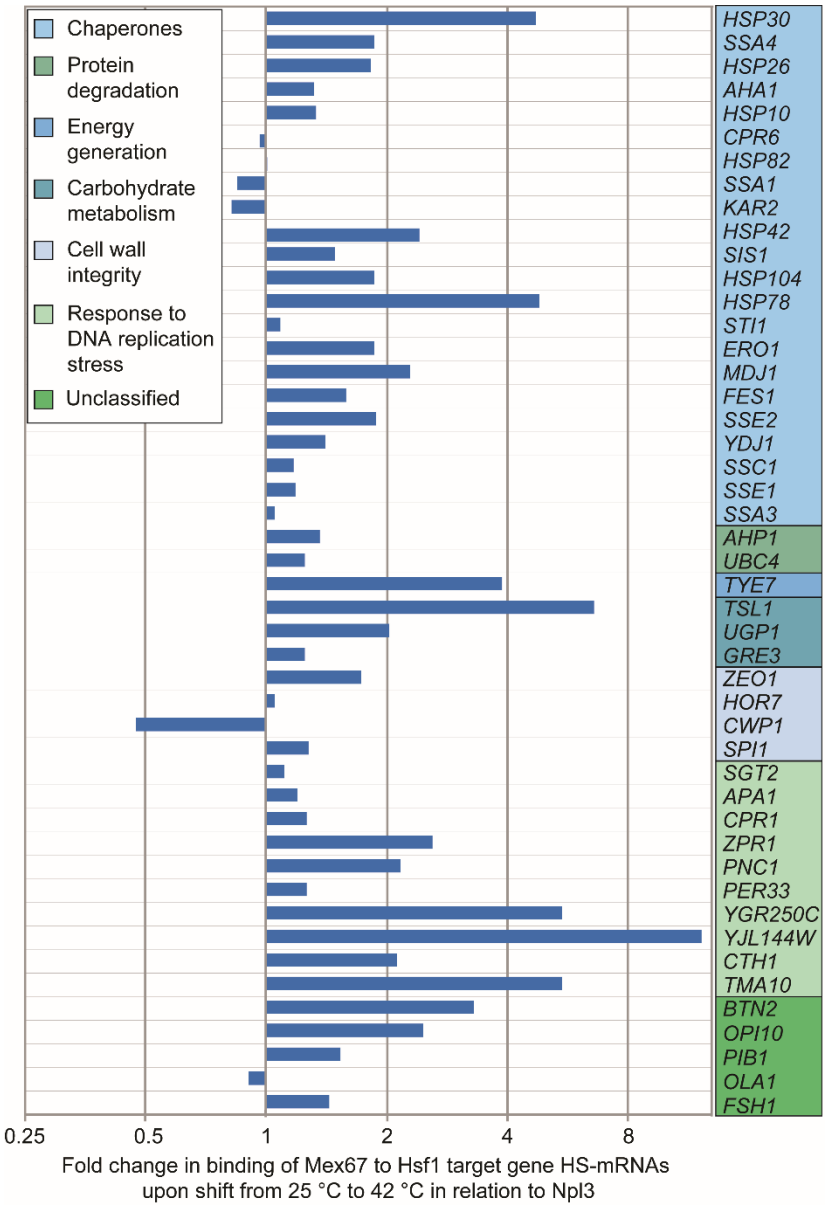

Figure 26: Mex67 is enriched on mRNAs produced under the control of Hsf1. Microarray analysis of mRNAs bound to Npl3 and Mex67 at 25 °C and 42 °C was used to compare binding of both proteins to Hsf1-dependent mRNAs. Ratio of Mex67 compared with Npl3 was calculated and fold changes are depicted. Genes under the control of Hsf1 were grouped according to their function in the cell. Published in (Zander et al., 2016).

This enrichment could not only be found for chaperones like Hsp30 or Hsp70 (encoded by *YRO1* and *SSA4*, respectively), but also for mRNAs encoding proteins that act in energy generation or respond to DNA replication stress. The grouping of genes and their transcripts was performed according to their described functions in SGD (www.yeastgenome.org; August

2016), though there might be other, maybe yet unknown functions that require their expression upon stress. As already described (see 2.3.6) Hsf1 is also involved in expression of some of its target genes under normal conditions. This might be the reason that a few transcripts were about equally or less bound to Mex67 compared to Npl3 as both proteins should collaborate in their export at 25 °C and loose binding to them at 42 °C if they are treated like normal "housekeeping" mRNAs. Another explanation for a negative ratio of Mex67 to Npl3 binding is that some mRNAs were not expressed under normal conditions and showed negative binding values for both proteins at 25 °C when set in relation to unspecific binding (no tag). This is the reason that even though under heat stress Mex67 bound stronger to this transcript than Npl3, calculation using all values resulted in reduced binding. However, overall Mex67 bound stronger to Hsf1-controlled HS mRNAs under stress than Npl3. This again argues for a direct role of Mex67 in export of stress responsive transcripts and underlines the connection Hsf1 creates between the export receptor and the newly emerging HS mRNA as suggested by Hsf1 co-precipitation experiments with Mex67 (Figure 25).

4.4 The fate of an mRNA is controlled by its promoter

4.4.1 Change of the promoter can turn a housekeeping gene into a stress responsive gene

As Hsf1 interacts with Mex67 under stress and potentially thus allows direct recruitment of the export factor to HS mRNAs to ensure their preferential export, it seems reasonable that the heat shock element (HSE) in the HS gene promoter essential for Hsf1 binding is crucial for this process. Therefore, the promoter and especially the HSE therein should direct expression and favored mRNA export following stress. Indeed it was shown that under glucose starvation the promoter and the HSE or stress responsive (STRE) elements therein influence the cytoplasmic localization of mRNAs (Zid and O'Shea, 2014).

To analyze this in more detail and especially for heat stress conditions, several plasmids were created. These constructs carried the housekeeping gene *CYC1* encoding for a cytochrome C involved in mitochondrial respiration that is low expressed under normal fermentative conditions and the gene *HSP12* encoding for heat shock induced Hsp12. The promoter of *HSP12* contains a HSE, which sequence is classified as step HSE (listed in Figure 10) that is bound by Hsf1 and this way expression is promoted. Constructs with *CYC1* and *HSP12* under the control of the *CYC1*-promoter as well as both genes under control of the *HSP12*-promoter

were assembled and a fifth construct with *CYC1* under its own promoter was created, that additionally contained a 17 bp fragment resembling the perfect HSE from *SSA4* (Figure 10). This artificial HSE was inserted in the *CYC1* promoter at approximately the same distance from the potential transcription start site as in *SSA4*. To investigate only the changes of theses specific transcripts upon applying heat stress to the cell, the open reading frame (ORF) encoding for the GFP protein was inserted behind the ORF encoding for *CYC1* or *HSP12*. As the *GFP*-sequence is unique to the analyzed constructs no background effects of mRNAs deriving from the endogenous yeast genes should disturb the examination of the effect the promoter has on construct expression upon heat stress.

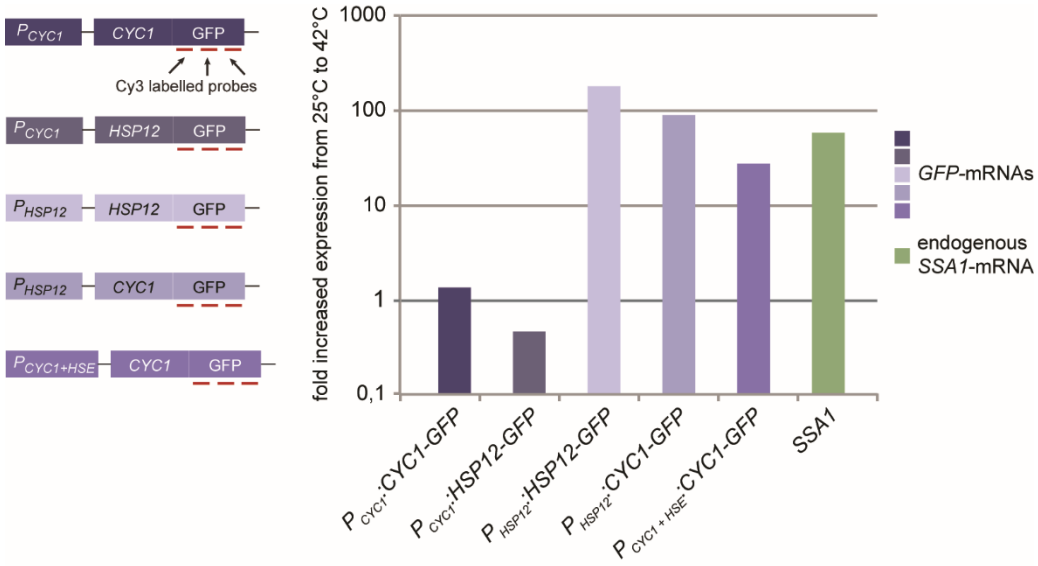

Figure 27: *CYC1*-mRNAs from constructs carrying the *HSP12*-promoter or the *SSA4* HSE are enriched under heat stress. Wild type cells were transformed with the constructs indicated at the left side and were grown to log phase. One portion was kept at 25 °C while the other half was shifted to 42 °C for 30 min. Total RNA was isolated and equal amounts transcribed into cDNA. RT-qPCR analysis was performed detecting the respective GFP-containing mRNA, the endogenous *SSA1* HS mRNA and 25S rRNA at 25 °C and after heat stress. Values were normalized to the 25S rRNA, ratios between normal and stress conditions calculated for each transcript and displayed above. Published in (Zander et al., 2016).

Figure 27 shows an exemplary RT-qPCR analysis detecting the *GFP*-containing mRNAs and their change in expression from 25 °C to 42 °C after 30 min heat stress. As expected expression of *CYC1* under its own promoter did not change substantially between 25 °C and 42 °C as its transcription and degradation are shut down under stress (Bond, 2006). The same effect was detectable for *HSP12* expressed from the *CYC1*-promoter, which argues for a role of the promoter but not the ORF in transcription induction under heat stress. This finding was supported by the observation that both *CYC1* and *HSP12* under control of the *HSP12*-promoter

were equally induced under heat stress like the endogenous *SSA1*-mRNA. Even the *CYC1*-promoter to which an HSE was added was sufficient to induce the *CYC1*-mRNA when heat was applied (Figure 27). Thus, change of the promoter or insertion of the small HSE could turn a housekeeping mRNA into a stress responsive transcript. As the HSE is the binding site for Hsf1 and only addition of this element leads to induction under heat stress, the transcription factor is presumably the essential factor needed to induce a gene's expression in response to stress.

4.4.2 The time point of expression, defined by the promoter, regulates if mRNAs are quality controlled or not

Under normal conditions mRNAs are constantly controlled during their maturation and those identified as faulty are degraded. As earlier studies show, the factors essential in this degradation process are the RNA helicase Mtr4 and the nuclear exosome component Rrp6 (Callahan and Butler, 2010; Fasken and Corbett, 2009; Jia et al., 2011). Cells lacking one of these proteins accumulate faulty transcribed, spliced or otherwise processed mRNAs in their nuclei as they cannot be degraded properly. Adaptor proteins like Npl3, Gbp2, Hrb1 and Nab2 presumably recruit the degradation machinery and prevent export as in cells lacking both, an adaptor protein and Rrp6, faulty mRNAs are exported again and leak into the cytoplasm (Hackmann et al., 2014; Zander et al., 2016).

Under stress these adaptor proteins are not involved in export of HS mRNAs but rather dissociate from bulk mRNA and thus block their export (4.1.1). If adaptor proteins are not needed upon stress as they are for normal mRNA export and HS mRNAs are exported only by Mex67 and Mtr2, the question arises how these stress responsive transcripts act in terms of quality control. Dr. Lysann Bender could show that only in mutants of *MEX67* and *MTR2* export of the HS mRNAs *SSA4* and *HSP12* is blocked. Even application of 42 °C heat stress for one hour did not result in nuclear accumulation of stress mRNAs in the *MTR4* mutant *mtr4-G677D* or the exosome deletion strain *rrp6Δ* while normal bulk poly(A)$^+$ mRNAs were blocked in the nucleus (PhD thesis Dr. Lysann Bender and Zander et al. 2016). The observation that these two stress responsive transcripts did not accumulate in the nuclei of mutants of the degradation machinery could be due to the small number of mistakes that occur during the rather short period of transcription (30 min or 1 h (Zander et al., 2016)), and it takes longer to accumulate a visible amount of faulty export-blocked transcripts. However, expression of HS mRNAs in response to stress is fast and the quantity that is produced outnumbers nearly all single

housekeeping transcripts. Therefore, it seems more likely that controlling and degrading proteins like Mtr4 and Rrp6 do not act in the assurance of HS mRNA quality. If this is indeed the case was analyzed using the set of constructs examined above (Figure 27) in fluorescence *in situ* hybridization experiments to localize these mRNAs in the different cell backgrounds. This approach allowed to visualize the same mRNA expressed at normal or heat stress conditions as only the promoter and not the ORF sequence changes and differences in localization cannot be credited to the diversity of the analyzed transcripts. As shown in Figure 28 the *CYC1* transcript under control of its own promoter (I.) accumulated in the nuclei of wild type cells as well as in the export mutants *mex67-5* and *mtr2-21* and strikingly in mutants of the degradation machinery (*mtr4-G677D* and *rrp6Δ*).

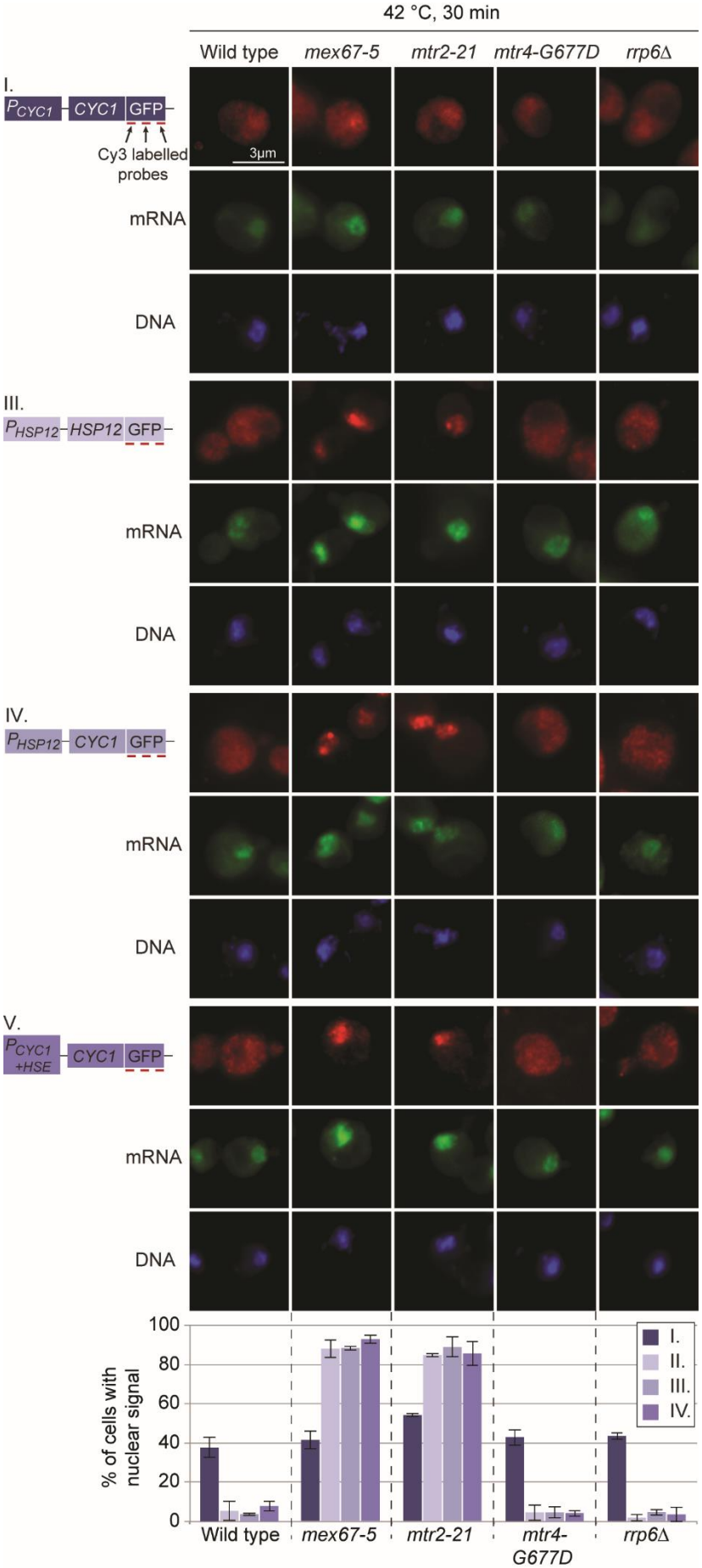

Figure 28: Heat stress induced mRNAs are not quality controlled. Indicated strains were transformed with constructs I.-V. as shown in Figure 27 and grown to log phase before shifting to 42 °C for 30 min. Cells were fixed with formaldehyde and FISH analyses performed detecting the GFP-containing mRNA with probes against the GFP sequence (red). Bulk poly(A)$^+$ RNA was stained with an oligo d(T) probe (green) and DNA detected with Hoechst (blue). While nuclear accumulation of poly(A)$^+$ RNA can be detected in all strains, the analyzed constructs only accumulate when expressed from the housekeeping *CYC1*-promoter but not from heat stress inducible promoters (top). For each construct and each strain the percentage of cells showing nuclear accumulation was counted and the average of three FISH experiments is displayed (bottom). Published in (Zander et al., 2016).

The construct was specifically detected by using probes only against the *GFP*-mRNA part, thus not staining the endogenous *CYC1* mRNA. When compared to total mRNA the accumulation is relatively weak and quantification revealed that only 40 % of cells showed a nuclear signal. This can be explained as Cyc1 is as a Cytochrome C protein part of the respiratory chain. Under laboratory conditions mostly glucose is the main nutrient and *S. cerevisiae* produces energy by fermentation rather than respiration. This is the reason that *CYC1* mRNA is very poorly expressed from its own promoter under this condition. Possibly the 60 % of cells showing no nuclear signal did not measurably express the gene. Another influence that has to be taken into account is that those mRNAs that were not allowed to be exported can be degraded by other mechanisms, even though degradation by the Rrp6-containing exosome is the most prominent pathway. Nevertheless, nuclear accumulation of *CYC1* under its own promoter could be observed in all strains. The *HSP12* transcript derived from the *HSP12*-promoter (III.) on the other hand only accumulates in the export mutants *mex67-5* and *mtr2-21* but not in the degradation mutants *mtr4-G677D* and *rrp6Δ*. Strikingly, the same pattern could be observed for a *CYC1* transcript when expressed from the *HSP12*-promoter (IV.) or after insertion of an HSE in its own promoter (V.), as no accumulation in mutants of the degradation machinery could be detected. Due to the strong export defects in *mex67-5* and *mtr2-21* and the high induction (see Figure 27) a nuclear signal for these transcripts was found for almost every cell and transcript (Figure 28 bottom). Together, all analyzed strains show the expected general bulk mRNA export block under heat stress. In contrast and unlike mRNAs expressed under normal conditions, transcripts induced upon heat possibly omit quality control and are directly exported. As described the *CYC1* gene is poorly expressed under normal fermentative conditions. In order to proof that not only mRNAs with few copy numbers, but also highly transcribed genes underlie quality control under normal conditions and evade it under stress, another mRNA was analyzed. For this approach, the *GPM1* gene was selected, which encodes for a phosphoglycerate mutase. This enzyme catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate in glycolysis and is present in the cell in high numbers. As the protein is needed for efficient growth under normal conditions the corresponding mRNA should be permanently present and highly expressed. The ORF of this gene was used to create constructs with different constitutions as in Figure 27 with *GPM1* under its own and the *HSP12*-promoter. Additionally, *HSP12* under its own and the *GPM1*-promoter and *GPM1* with an HSE inserted in its own promoter were analyzed for their expression in response to stress (Figure 29).

Comparable to experiments with *CYC1*-containing constructs (Figure 27), expression from the *GPM1*-promoter, regardless if the *GPM1* or *HSP12* ORF follows, was not induced upon stress.

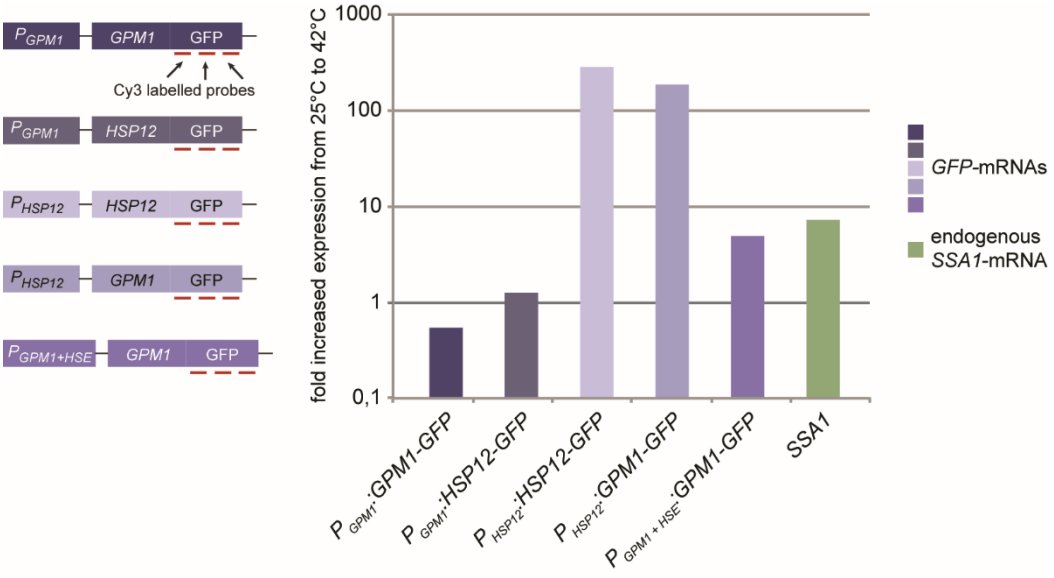

Figure 29: *GPM1*-mRNAs from constructs carrying the *HSP12*-promoter or the *SSA4* HSE are enriched under heat stress. Wild type cells were transformed with the constructs indicated at the left side and were grown to log phase. One portion was kept at 25 °C while the other half was shifted to 42 °C for 30 min. Total RNA was isolated and equal amounts transcribed into cDNA. RT-qPCR analysis was performed detecting the respective GFP-containing mRNA, the endogenous *SSA1* HS mRNA and 25S rRNA at 25 °C and after heat stress. Values were normalized to the 25S rRNA, ratios between normal and stress conditions calculated for each transcript and displayed above. Published in (Zander et al., 2016).

Contrary, expression was induced when any construct was under control of the *HSP12*-promoter or an HSE was inserted. These findings support a general mechanism in gene expression under stress that is controlled by the genes' promoter rather than the transcribed sequence of the gene.

Like the *CYC1* constructs, the *GPM1* constructs are specifically inducible under heat stress. As *GPM1* expression is much stronger under normal conditions these constructs were analyzed in FISH experiments as well (Figure 30). Expectedly, mRNAs derived from ORFs under control of the *GPM1*-promoter (I. + II.) clearly accumulate with visible dot like structures in the nuclei of wild type cells as well as export or degradation mutants. This can be found in approximately 60 % of the cells indicating the general mRNA export block together with retention of faulty transcripts that are predestined for degradation. Constructs under control of the *HSP12*-promoter (III. + IV.) or with a HSE in the *GPM1*-promoter (V.) only show nuclear accumulation in mutants of the export receptor heterodimer (*mex67-5* and *mtr2-21*).

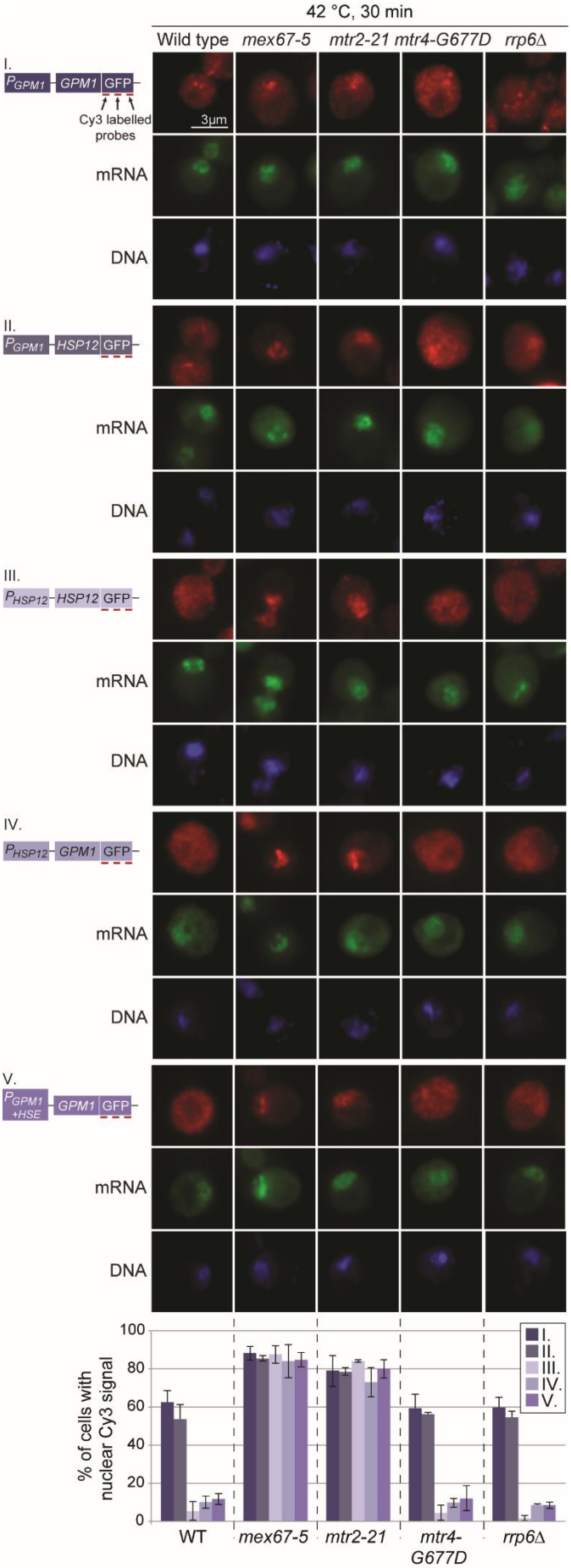

Figure 30: The *GPM1*-mRNA is no longer quality controlled under stress as it does not accumulate in mutants of *MTR4* or *RRP6* when expressed from stress-responsive promoters. Indicated strains were transformed with constructs I.-V. as shown in Figure 29 and grown to log phase before shifting to 42 °C for 30 min. Cells were fixed with formaldehyde and FISH analyses performed detecting the GFP-containing mRNA with probes against the GFP sequence (red). Bulk poly(A)$^+$ RNA was stained with an oligo d(T) probe (green) and DNA detected with Hoechst (blue). While nuclear accumulation of poly(A)$^+$ RNA can be detected in all strains, the analyzed constructs only accumulate when expressed from the housekeeping *GPM1*-promoter but not from heat stress inducible promoters (top). For each construct and each strain the percentage of cells showing nuclear accumulation was counted and the average of three FISH experiments is displayed (bottom). Published in (Zander et al., 2016).

In wild type cells and remarkably in mutants of the nuclear quality control and degradation machinery (*mtr4-G677D* and *rrp6Δ*) these synthetic stress responsive mRNAs are normally exported as only very few cells (~ 10 %) show accumulation in the nucleus (Figure 30 bottom). All together these experiments indicate that normal housekeeping genes can be turned into stress responsive genes only by exchanging their own promoters with a promoter from an HS gene. Even addition of a single heat shock element in the genes original promoter is sufficient to induce this effect. Following heat stress, they are strongly induced, leading to expression of large amounts of transcripts that are treated like all HS mRNAs.

HS mRNAs are not retained in the degradation defective *mtr4-G677D* and *rrp6Δ* strains even though in these mutants nuclear accumulation of poly(A)-containing RNAs can be detected under normal conditions (Zander et al., 2016). Here the same observation can be made for the analyzed synthetic constructs as they seem to omit quality control, when expressed in response to heat shock.

4.5 Adaptor proteins supposably present quality control checkpoint marks, dispensable for stress response

Control of correct pre-mRNA processing and packing into mRNPs is comprehensibly important to assure functionality of cellular processes. The results presented above lead to the assumption that transcripts expressed in response to heat stress are not susceptible to general quality control mechanism and degraded by Mtr4 and Rrp6, but rather directly exported by the export receptor Mex67-Mtr2. While Mtr4 and Rrp6 act in degradation of false mRNAs several other factors were described in supporting this mechanism (see 2.2). Among them Mlp1, which is localized at the nuclear pore complex that senses defective mRNPs and prevents their export (Galy et al., 2004). Furthermore, all shuttling adaptor proteins were described to fulfil roles in controlling correct maturation of mRNAs. Gbp2 and Hrb1 monitor the splicing reaction as they retain incorrectly spliced mRNAs in the nucleus (Hackmann et al., 2014) and Nab2 is crucial for correct length and formation of the 3’ poly(A) tail (Schmid et al., 2015). In addition, Npl3 was shown to interact with the capping complex and to be potentially involved in control of correct capping (Shen et al., 2000).

4.5.1 Combined defects in 5' mRNA maturation-control show synthetic lethality

Npl3 is a general mRNA binding protein and shuttles with the readily packed mRNP from the nucleus to the cytoplasm. As shown before the adaptor protein accompanies the mRNA during its entire maturation and binds as one of the first proteins as soon as the 5' end emerges from the polymerase and the cap binding complex assembles (Lei et al., 2001). There is some evidence that Npl3 only binds the new mRNA if the m^7-G cap is properly processed thus creating a checkpoint for correct processing of one essential mRNA feature (Shen et al., 2000). Cet1 and Ceg1 are the two enzymes carrying out the important reactions for conversion of the free 5' end into a capped mRNA where Cet1 acts as the 5'-triphosphatase. If Npl3 is indeed involved in control of correct capping, disruption of this surveillance mechanism would overflow the cell with incorrect transcripts. Presumably, most of the mRNAs are not exported properly and some become degraded in the nucleus. However, if their amount is too large, export might occur. Without a cap those transcripts cannot be translated in the cytoplasm and are not protected against degradation. This will intervene with growth and proliferation of the cell. To test this hypothesis a strain carrying a deletion of *NPL3* was crossed with a strain containing a temperature sensitive allele of *CET1* (*cet1-2*) and a drop dilution test was performed (Figure 31).

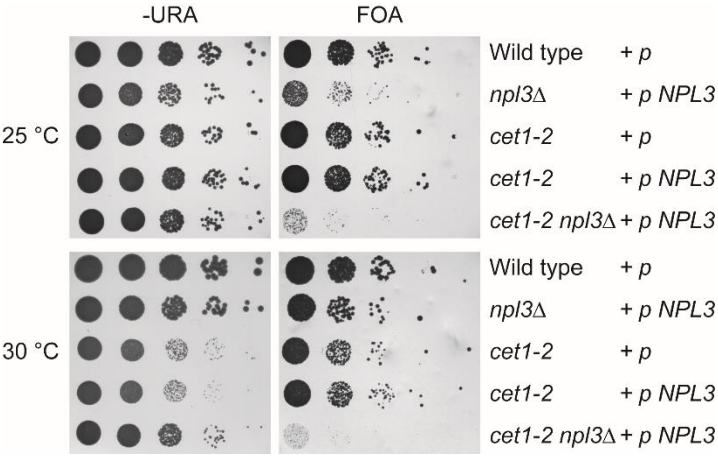

Figure 31: Combined defects in capping and capping control are lethal. Serial dilutions of the indicated strains were spotted on plates selecting for the presence of an empty or *NPL3* containing plasmid (-URA). The same strains were spotted on plates selecting for the loss of the plasmid (FOA) and incubated at 25 °C or 30 °C and incubated for 3 days.

Mutant strains were transformed with a covering plasmid containing the *NPL3* gene together with the *URA3* marker. Propagation or loss of this plasmid was enforced by spotting the cell dilutions on -URA or FOA-plates respectively. The strain deleted for *NPL3* alone is viable without the plasmid at 25 °C as well as 30 °C. The same could be observed for the *CET1* mutated strain, indicating the capping defect induced at these temperatures alone is not toxic to

the cell and can be compensated. Contrastingly, when both mutations were combined and no covering plasmid was present, cells grew very poorly at 25 °C and are dead at 30 °C. This indicates that combined defects in capping itself and control of this process resulted in a perturbation the cell is unable to overcome.

In order to further address this theory, another factor, Rat1, involved in control of the 5' end of a transcribing mRNA was examined. Rat1 is a nuclear 5'-3' exoribonuclease that degrades mRNAs from its 5' end and is involved in release of the RNA polymerase II during transcription termination (Kim et al., 2004). A temperature sensitive allele of *RAT1* (*rat1-1*) was combined with a *NPL3* deletion and used for drop dilution analyses.

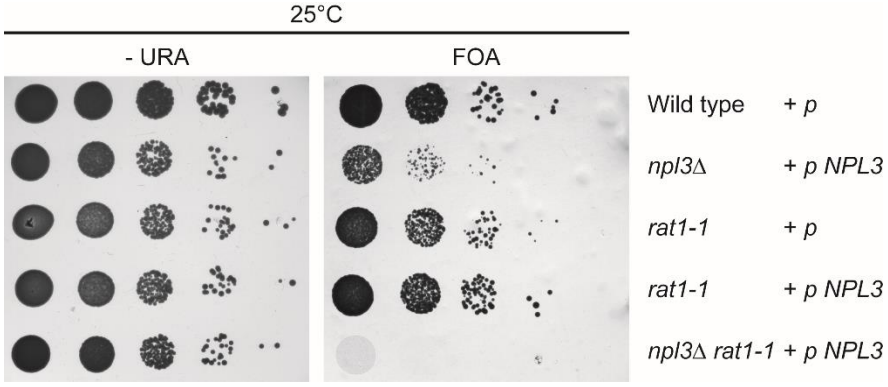

Figure 32: Deletion of *NPL3* is lethal when combined with a mutant of the nuclear 5' exonuclease Rat1. Serial dilutions of the indicated strains with an empty or *NPL3* containing plasmid were spotted on plates selecting for the presence (-URA) or loss of the plasmid (FOA) and incubated at 25 °C for 3 days

As depicted in Figure 32 a combination of these two mutations without a covering plasmid (FOA) was already lethal at 25 °C. Possibly, the cell could not compensate for the simultaneous loss of a control and degradation mechanism in 5'-RNA capping and together with the findings for the mutant of *CET1* these data argue for a role of Npl3 in control of correct processing of the cap. These genetic interactions point to Npl3 as part of the nuclear quality control mechanism in pre-mRNA maturation.

4.5.2 Splicing defects are not sensed under heat stress

Npl3 most likely controls correct capping, Nab2 assures quality during polyadenylation and the two other adaptor proteins Gbp2 and Hrb1 act in surveillance of splicing of intron containing mRNAs. As all these proteins are neglectable for HS mRNA export under heat and salt stress the question arises if execution of quality control is coupled to adaptor protein binding to the mRNA. In other words, if dissociation and not binding of these proteins might allow HS mRNAs to evade this control mechanism. To address this theory, cytoplasmic fractionation with strains deleted for *RRP6* alone or in combination with *HRB1* or *GBP2* was performed. It is known that faulty RNAs accumulate in *rrp6Δ* which leak in the cytoplasm in *hrb1Δ rrp6Δ* and *gbp2Δ rrp6Δ* (Hackmann et al., 2014). As Hrb1 and Gbp2 play a role in control of splicing the presence of the intron-containing *RPL23B*-mRNA in the cytoplasm was detected (Figure 33).

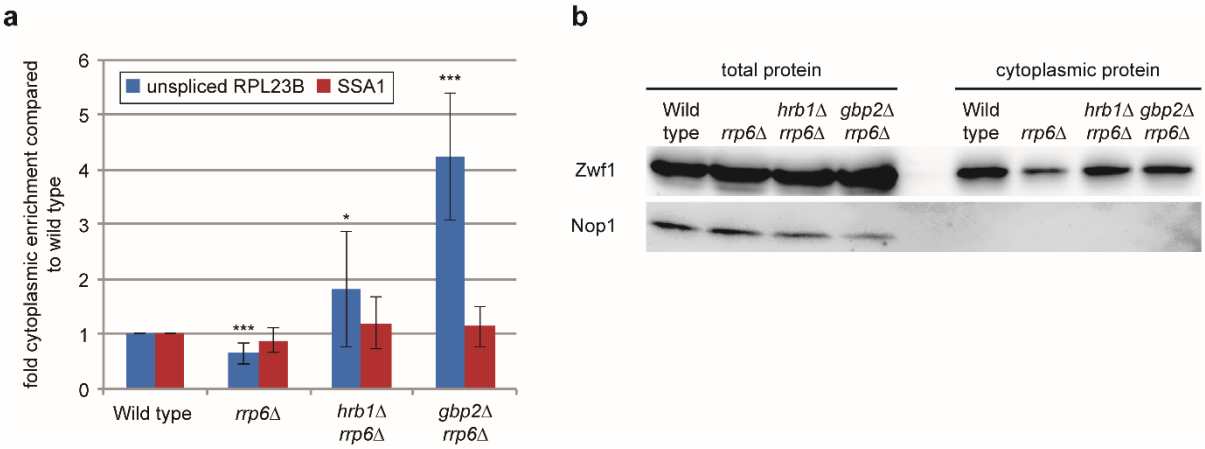

Figure 33: Combined deletions of the adaptor proteins *HRB1* and *GBP2* with the exosome mutant *rrp6Δ* show leakage of unprocessed mRNAs into the cytoplasm. a, Cytoplasmic fractionation and subsequent RNA isolation was performed in the indicated strains. *SSA1*-mRNA expression was induced at 42 °C for 30 min and levels of total and cytoplasmic RNA were determined using RT-qPCR. Average and standard deviation was calculated and a two-tailed, two-sample, unequal variance t-test was performed (*$p < 0.05$, ***$p < 0.001$). **b**, Cytoplasmic fractionation was controlled by detection of the cytoplasmic protein Zwf1 and nucleolar protein Nop1 with protein-specific antibodies using western blot.

RPL23B encodes for the L23B protein of the large ribosomal subunit and is highly expressed. RT-qPCR analysis of total and cytoplasmic mRNAs showed that more intron-containing *RPL23B*-mRNAs accumulate in the nucleus of *rrp6Δ* as the ratio is reduced in comparison to wild type. This resembles the retention of incorrectly processed mRNAs that cannot be degraded by the exosome lacking Rrp6. Contrastingly, in the double deletion strains *hrb1Δ rrp6Δ* and *gbp2Δ rrp6Δ* these faulty mRNAs are significantly enriched in the cytoplasm

as they are neither degraded by Rrp6 nor retained by Hrb1 or Gbp2. When analyzing the HS mRNA *SSA1*, no change in cytoplasmic levels was detected for all strains. Most stress responsive genes do not contain an intron, which is also the case for *SSA1*. For this reason, mistakes in splicing cannot be detected, however other defects like in capping, polyadenylation or faulty mRNP packaging could occur. Correct execution and quality of these steps could still be sensed by the adaptor proteins and degradation assured by the exosome. Since this seems not to be the case, it is either due to the experimental setup, as Hrb1 and Gbp2 only or mostly control intron-containing transcripts and deletion of the degrading *RRP6* was not sufficient to enrich faulty *SSA1*-mRNAs in the nucleus. Or another possibility is that the entire mechanism of mRNA maturation control that most likely is mediated by the adaptor proteins is not active during expression of heat shock genes.

Mistakes that would result in retention of an mRNA by the quality control machinery are diverse and hard to grasp as they can occur from the start of transcription to the release of the correctly packed mRNPs in the cytoplasm. Therefore, a direct and unambiguous experimental readout was required. Because HS mRNAs lack introns, a synthetic construct was designed consisting of the intron-containing *RPL23B* ORF fused with the *GFP*-sequence under control of its own or the *HSP12*-promoter.

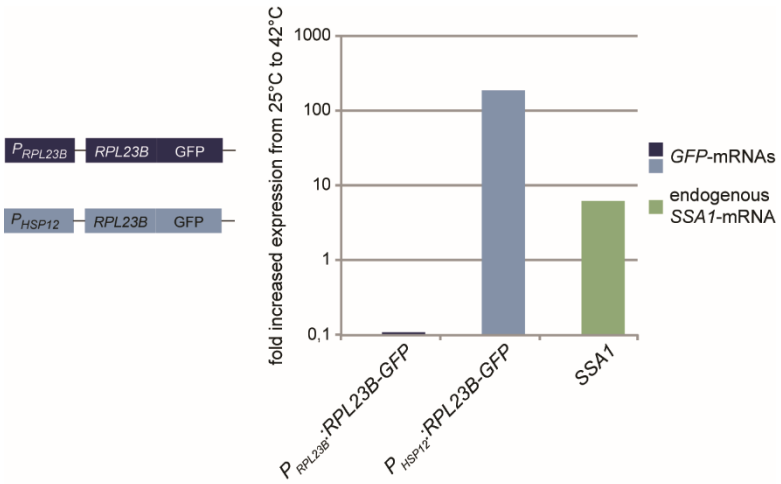

Figure 34: *RPL23B* becomes heat stress inducible under control of the *HSP12*-promoter. The ORF of *RPL23B* was fused with the *GFP*-sequence under the control of its own or the *HSP12*-promoter. Wild type cells were transformed with plasmids carrying these constructs and one part kept at normal conditions (25 °C) while the other was incubated at 42 °C for 30 min to apply heat stress. Total RNA was isolated, cDNA prepared and RT-qPCR performed to detect the *GFP*-sequence part of the mRNAs of interest as well as the endogenous *SSA1*-mRNA. Published in (Zander et al., 2016).

Expression of the *HSP12*-controlled construct is strongly induced in response to 42 °C heat stress, even stronger than the endogenous *SSA1* (Figure 34). In contrast, expression of the *RPL23B* transcript under its own promoter is tenfold reduced under stress compared to normal conditions. These two constructs provided the possibility to analyze the impact of quality control on the same mRNA under both conditions of interest. Due to the added *GFP*-sequence this further allowed to only analyze mRNAs derived from the housekeeping or stress promoter respectively and omits detection of the endogenous transcripts.

Both constructs were analyzed in a wild type strain that was kept at 25 °C or treated with 42 °C heat stress for 30 min before total and cytoplasmic RNA was isolated. Northern blot analysis was performed with equal amounts of RNA and the *RPL23B*-*GFP*-construct derived from the *RPL23B*- or the *HSP12*-promoter was detected using a *GFP*-specific DIG-labelled RNA probe.

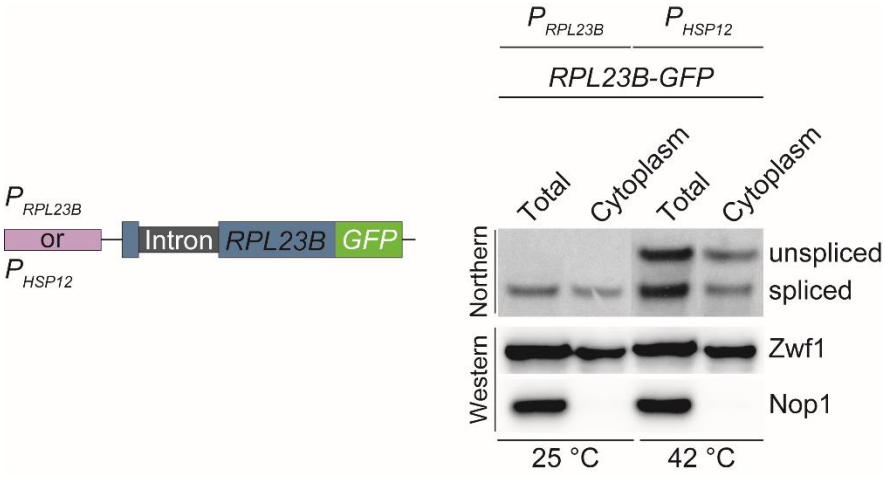

Figure 35: Unspliced *RPL23B*-mRNA is exported to the cytoplasm when expressed from the *HSP12*-promoter in the presence of Gbp2 and Hrb1. Wild type cells carrying the constructs analyzed in Figure 34 were kept at 25 °C or incubated at 42 °C for 30 min before cytoplasmic isolation. Total and cytoplasmic RNA was isolated and analyzed via northern blot using a *GFP*-specific DIG-labelled probe. Cytoplasmic fractionation was controlled by detection of the cytoplasmic protein Zwf1 and nucleolar protein Nop1 with protein-specific antibodies using western blot. Published in (Zander et al., 2016).

The *GFP*-probe detected mRNAs derived from both constructs as depicted in Figure 35. The *RPL23B*-mRNA made from its own promoter at 25 °C was only detected as the spliced version not only in the cytoplasm, but also in the total fraction that also contains the nucleus. This indicates this type of mRNA is directly spliced and exported or presumably degraded when errors in splicing occur. Strikingly this was different for the *HSP12*-promoted *RPL23B*-mRNA. Not only the relative amount compared to total RNA was higher, most likely due to its strong induction upon heat stress, but a clear and strong second band running higher as the first was detected. The increase in size equaled the size of the intron for which reason this band

supposedly resembled the unspliced transcript. Intensity of this longer unspliced band was as strong as the spliced form in the total RNA fraction but even slightly stronger in the cytoplasm arguing for the export of a large amount of unspliced *RPL23B*-mRNAs into the cytoplasm when expressed under stress. This effect was detected even though both adaptor proteins, Gbp2 and Hrb1, that control splicing were present in the cell.

The amount of spliced construct might result from expression at 25 °C as HS mRNAs are strongly induced upon stress but are also expressed to some extend under normal conditions. Another explanation could be that there are still some active spliceosomes present regardless of the stress, indicating that splicing but not control of its correct execution is maintained. Anyway, detection of this faulty construct in the cytoplasmic fraction that is only visible when expressed from a stress responsive promoter and that cannot be translated into a functional protein supports the idea that quality control mechanisms are circumvented under conditions of severe stresses like a 42 °C heat shock. Rather, export of endogenous or synthetic HS mRNAs under stress is a fast and apparently not discriminating process.

4.5.3 Fast and preferential export of HS mRNAs is essential to survive severe heat stress

Stress responsive transcripts are exported directly by Mex67-Mtr2 without the help of adaptor proteins like Npl3, Gbp2, Hrb1 or Nab2 and control of their quality is circumvented. These findings directly point to the question, why cells omit mechanisms that assure a trouble-free and organized gene expression. This might be answered by the nature of heat stress, which is the central stress analyzed in this study. While limitation of nutrients or sensing of a potential mating partner leads to changes in gene expression as well, survival of heat stress requires an immediate response. Therefore, it was examined, if all the differences between normal and heat stress conditions in fact result in a faster export and translation of HS mRNAs. Not only genes under control of HSE-containing promoters are strongly inducible, but also the *GAL1*-promoter exhibits this effect. These different expression pathways were compared to find out when the first proteins appear after induction.

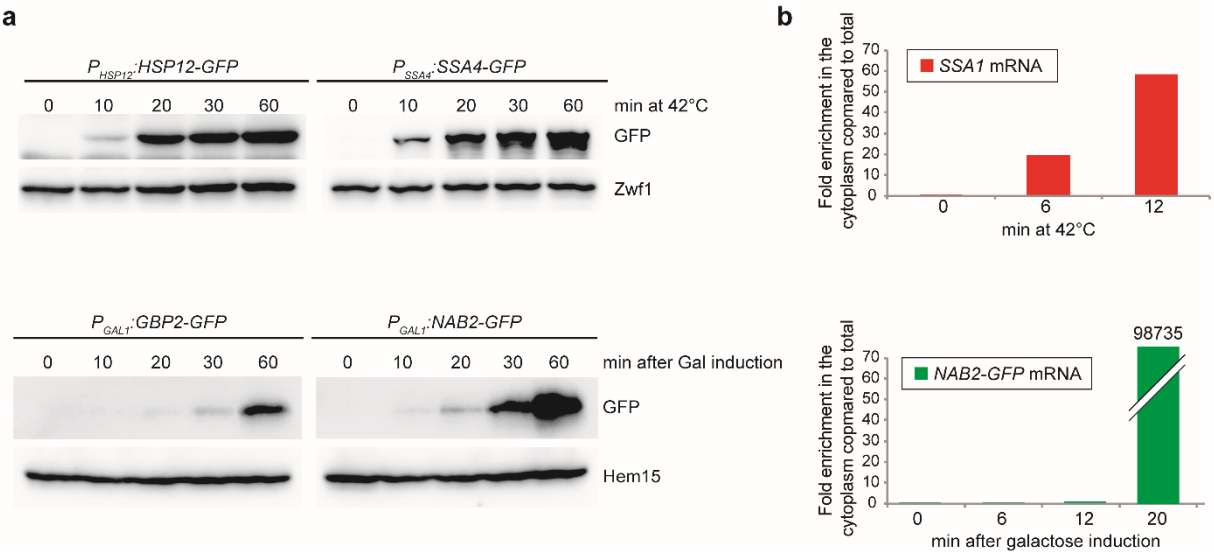

Figure 36: HS mRNAs are exported and translated faster than *GAL1*-induced mRNAs. a, Strains carrying GFP-tagged versions of two heat shock proteins (*HSP12, SSA4*) under their own promoters and two adaptor proteins (*GBP2, NAB2*) under control of the *GAL1*-promoter were induced with 42 °C heat stress or 2 % galactose for 1 h. Samples were taken at time points indicated and western blot analysis with a GFP-specific antibody was performed. **b,** RT-qPCR analysis of the indicated mRNAs in the cytoplasm compared to total mRNA levels at the indicated times after induction (RT-qPCR analysis was performed by Daniel Becker).

Genes encoding two heat shock proteins (*HSP12*, *SSA4*) and two adaptor proteins (*GBP2*, *HRB1*) were analyzed (Figure 36). For both heat inducible transcripts the first GFP-tagged version of the proteins was visible after 10 min incubation at 42 °C in western blot analysis. Contrary, the GFP-tagged adaptor proteins under control of the *GAL1*-promoter were detectable only 20 min, rather 30 min after galactose induction (a). This correlates with the *SSA1*-mRNA that reached the cytoplasm already 6 min following induction, whereas the *GAL1*-induced *NAB2-GFP*-mRNA was not detected in reasonable amounts until 20 min (b). These data visualize the fast export and translation in response to heat stress that is established in half of the time needed normally for a response on expression level as exemplified by galactose induction.

All together a lot of mechanisms required for the general error-free gene expression are bypassed or shut down upon stress and the cell focusses on fast production of heat shock proteins. To examine how crucial this immediate change is for survival, several strains defective for proteins involved in general mRNA expression were analyzed in their response to severe heat stress.

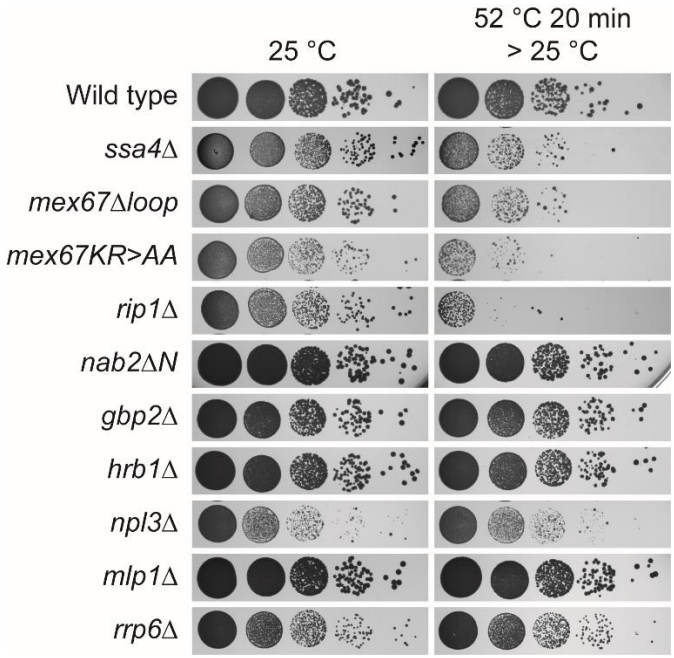

Figure 37: Proteins involved in HS mRNA export but no adaptor proteins or quality control factors are crucial to survive severe heat stress. Serial dilutions of the indicated strains were prepared and spotted on full medium plates. Afterwards the dilutions were subjected to severe heat stress at 52 °C for 20 min and again spotted on full medium plates before both plates were incubated at 25 °C for 3 days. Published in (Zander et al., 2016).

Dilutions of these strains were either spotted directly on full medium plates or incubated at 52 °C for 20 min before spotted (Figure 37). Following stress, cells lacking genes encoding for chaperones (*SSA4*) or the, under heat stress, essential NPC component *RIP1* showed clear growth defects. The same effect was found for the two loop domain mutants of *MEX67*. In contrast, strains lacking one of the adaptor proteins (*NAB2, GBP2, HRB1, NPL3*), the nuclear exosome component *RRP6* or *MLP1*, which acts as the final quality control factor at the NPC were not impaired in growth after heat stress. Hence, proteins involved in HS mRNA export are essential for the immediate export and translation of chaperone encoding mRNAs, while quality control components can be deleted without impact on cellular survival under conditions of severe heat stress.

5 Discussion

5.1 Export of mRNAs in response to heat stress relies on Mex67-Mtr2 but not the adaptor proteins

Response to heat shock needs to be fast and requires the immediate expression of protective chaperones that help the cell to maintain essential functions. For this switch between normal and stress-responsive gene expression it is not sufficient to change transcription, but rather to suppress ongoing mRNA maturation processes including splicing (Bond, 2006). Together with this, normal mRNA export is inhibited (Saavedra et al., 1996), potentially to leave resources of the translation machinery that are not sequestered in cytoplasmic granules, free to the HS mRNA. The present study gives an explanation by what means the fast switch between normal and HS mRNA export, at least in part, is facilitated. Further, the results enlisted above support the idea that not only some adaptor proteins, as published before, but all of the so far known mRNA adaptor proteins in *S. cerevisiae* are dispensable for export under heat stress. In contrast, Mex67 is essential for HS mRNA export (Hurt et al., 2000). How the exporter can discriminate between these two different RNA species could be explained by the data gathered in this work.

5.1.1 Export block of bulk mRNAs is facilitated by a global dissociation of adaptors and Mex67

The block of normal housekeeping mRNAs in the nuclei of cells that experience severe heat stress at 42 °C is a long known phenomenon (Saavedra et al., 1996). A general export block at these conditions cannot explain this phenotype, as stress responsive transcripts are exported and translated. Further, as bulk mRNAs are highly diverse, sequence specific retention mechanisms can also not account for this nuclear block. The export block is rather accomplished by a general dissociation of mRNA adaptor proteins and the export receptor Mex67. Both, analysis of poly(A)-containing RNA bound to adaptor proteins and the genome wide mRNA microarray data show that less transcripts can be co-precipitated with Mex67 and the adaptor proteins under stress (Figure 11 and Figure 15). This is consistent with earlier findings that show Npl3 dissociating from mRNAs under stress conditions and being able to leave the nucleus without cargo (Krebber et al., 1999). On the other hand, the direct, RNA-independent, interaction of the adaptor proteins with the export receptor Mex67 does not change under either salt or heat stress conditions (Figure 12). That the protein-protein interaction does not change, but less mRNA is

bound, argues for the dissociation of Mex67 together with its adaptor as a complex. Thus, Mex67 dissociation seems rather to be promoted by adaptor proteins dissociating from the mRNA. As binding of Mex67 to the mRNP is a crucial prerequisite for general mRNA export, this gives an explanation for the block of bulk mRNA export upon stress. Most likely, this will block mature or almost mature mRNAs that have already the necessary export factors loaded and are not retained at earlier maturation steps like splicing (Yost and Lindquist, 1986, 1991). By what means adaptor proteins lose their contact to the mRNA is not exactly known. Presumably, post-translational modifications like phosphorylations, methylations or ubiquitinylations of the adaptor proteins trigger the dissociation. In fact, Nab2 is phosphorylated under severe heat stress (Carmody et al., 2010). Further, Nab2 forms nuclear foci together with another mRNA-binding protein, Yra1, in response to stress, even though this aggregation seems not to rely on phosphorylation of Nab2 (Carmody et al., 2010). Formation of reversible nuclear aggregates in response to heat stress was also observed for Gbp2 (Wallace et al., 2015). This aggregation might be caused by a post-translational modification of Gbp2, or the high temperature results in conformational changes of the protein, which then can no longer bind to the mRNA and is prone to aggregation. However, it remains to be clarified if aggregation of the adaptors leads to dissociation from the mRNA, or if excess of free adaptors results in this focus formation.

5.1.2 Adaptor proteins are dispensable for HS mRNA export and Mex67 shows mutually exclusive binding to either RNA or the adaptor Npl3

As adaptor proteins dissociate from the mRNA and thus facilitate bulk mRNA retention, they might not bind to stress responsive transcripts as well. This theory is supported by analysis of the global mRNA binding profiles of Npl3 and Mex67. While overall mRNA association of both proteins is reduced, Npl3 shows no changes in the binding profile under heat stress (Figure 15 and Figure 16). For Mex67 in contrast, approximately the double amount of stress mRNAs are bound at 42 °C underlining the necessity of Mex67 in HS mRNA export (Hurt et al., 2000). Dissociation of the adaptor proteins from bulk mRNA and no enrichment on stress mRNAs indicates that these proteins are not needed for export under stress. In fact, export of the HS mRNA *SSA4* is not disturb in mutants of the shuttling adaptor proteins *NPL3, GBP2, HRB1*, and *NAB2* (Figure 19). A compensatory effect of the adaptor proteins is ruled out as even in a triple mutant (*gbp2Δ hrb1Δ npl3-17*) no nuclear accumulation is detectable. Hence, the only known proteins that shuttle with the stress mRNA to the cytoplasm and enable export

are Mex67 and its co-factor Mtr2. Involvement of other, yet unknown, factors in HS mRNA export can certainly not be excluded, but seems rather unlikely. Especially, because Mex67 is able to bind to the 5S rRNA during ribosomal export (Yao et al., 2007) and directly to normal and stress mRNAs in *in vitro* experiments (Zander et al., 2016), this likely is also the case for HS mRNA export *in vivo*. Indeed, for higher eukaryotes constitutive transport elements (CTEs) exist, which enable a direct recruitment of the Mex67-Mtr2 homolog Tap-p15 (Braun et al., 1999; Kang and Cullen, 1999). This special way of export is often exploited by retroviruses (Bray et al., 1994), but as well Tap can bind over a CTE to an alternative splice variant of its own mRNA that still contains an intron (Li et al., 2006). Interestingly, the CTEs contain a conserved AAGACA motif important for Tap-p15 binding (Katahira et al., 2015; Li et al., 2006), which can also be found as a forward and reverse sequence in the ORFs of the yeast stress responsive genes *HSP12* and *SSA4*. If this motif is the actual HS mRNA binding site for Mex67-Mtr2 needs to be further studied. Nevertheless, direct binding of the exporter Mex67/Tap likely is a mechanism that allows viruses or the cell itself to circumvent the general export pathway and enhance nucleo-cytoplasmic transport of a special set of RNAs, which might not have passed quality control.

For the export of normal housekeeping mRNAs Mex67 binds to the mRNP over interaction with the shuttling adaptor proteins, while binding of HS mRNAs most probably is direct. This direct binding to RNAs relies substantially on the loop domain of Mex67, which upon mutation or deletion results in loss of RNA binding (Yao et al., 2007; Zander et al., 2016). The here presented *in vitro* and *in vivo* data identify this loop domain additionally to be important for binding to the adaptor protein Npl3, as in loop-domain mutants of Mex67 interaction between these proteins is reduced (Figure 22 and Figure 23). However, even though export of the 60S-ribosomal subunit by binding of the Mex67 loop to the 5S rRNA is blocked in loop-domain mutants, normal mRNA export is still carried out (Yao et al., 2007). This suggests that the reduced interaction of the loop-domain mutants with Npl3 is sufficient for adaptor protein mediated mRNA export under normal conditions, but these mutants are not capable of direct HS mRNA export under stress (Zander et al., 2016). This bifunctional character of the loop region is supported by the finding that excess of RNA can disrupt the interaction of Npl3 with Mex67 (Figure 24) indicating a mutually exclusive binding of Mex67 to either RNA or Npl3.

Under normal conditions Npl3 is recruited very early to the transcribing mRNA, which might result in covering of the transcript and prevention of direct Mex67 binding. Interestingly, and even though the data differ, the amount of Npl3 molecules outnumbers Mex67 by a factor of

4-27 fold (Ghaemmaghami et al., 2003; Kulak et al., 2014), which further could argue for recruitment of Mex67 only to the mature mRNP a later time point and only prior to export.

5.1.3 Mex67 is directly recruited to Hsf1 controlled genes

In the case of heat stress, Mex67 is found in direct proximity of heat shock genes and to be interacting with the RNAP II subunit Rpb1 (Zander et al., 2016). This could be a mechanism how the export receptor is directly recruited to HS mRNAs without the need for adaptor proteins. As the RNA polymerase II is the central component of every mRNA transcription, discrimination between normal and stress RNA production presumably relies on another factor. Comparable to Rpb1, Mex67 specifically interacts with the transcription factor Hsf1 under severe heat stress conditions (Figure 25). Hsf1 is the essential factor for induction of a strong heat shock response and its interaction with Mex67 would couple this transcription induction with the export machinery. Indeed, when compared to Npl3, Mex67 is under stress enriched on nearly all transcripts that are controlled by Hsf1 in their expression (Figure 26), indicating a general recruitment of Mex67.

Placing Hsf1 as a regulatory switch between normal mRNP assembly and direct Mex67-mediated HS mRNA export seems to be a useful way of regulation. However, Hsf1 is bound to promoters already at normal conditions and can promote a basal expression level of these genes (Pincus, 2016; Solís et al., 2016). If the strong phosphorylation of Hsf1 under stress is a prerequisite for Mex67 binding or if other factors are involved in its loading needs to be further examined. Interestingly, an observation in mammalian cells shows a different connection of the transcription factor with the subsequent fate of an mRNA. Here the translation elongation factor eEF1A1 interacts with HSF1 and activates HSP70 transcription before it accompanies the resulting mRNA during export and to the translating ribosomes (Vera et al., 2014). This likely is another aspect of how a favored expression of heat shock genes is achieved.

5.2 Quality control mechanism are omitted under stress to assure fast HS mRNA export

Several factors that accompany an mRNA during maturation and export under normal conditions are dispensable for the expression of stress responsive genes. The shuttling mRNA adaptor proteins Npl3, Gbp2, Hrb1 and Nab2 dissociate and some of them are sequestered in nuclear foci. The nuclear mRNA-binding protein and component of the TREX complex, Yra1,

shows the same phenotype and is reportedly not involved in HS mRNA export (Rollenhagen et al., 2007). When it comes to Sub2, another component of the TREX complex, and the members of the THO complex, things are not that clear. Some publications show that the THO complex is important for normal but not heat stress mRNA export (Rollenhagen et al., 2007). On the other hand nuclear accumulation of HS mRNAs in cells lacking THO complex proteins was observed (Strässer et al., 2002; Thomsen et al., 2008). Due to the fast and strong response of heat stress genes to elevated temperatures, HS mRNAs were often used as inducible reporter constructs to study the effect of THO/TREX mutants on mRNA maturation and export. Several experiments however, were not performed under severe heat stress conditions at 42 °C, but with a mild heat stress at 37 °C. As presented in the introductory part, only severe heat stress leads to the characteristic changes including bulk mRNA export block and splicing inhibition (Saavedra et al., 1996; Yost and Lindquist, 1986, 1991). Therefore, it is difficult to separate what effect is a general result of THO/TREX mutants and what can be observed in response to heat stress and also affects HS mRNAs.

Nevertheless, sumoylation of THO seems to be important for expression and stabilization of mRNAs in response to acidic stress (Bretes et al., 2014), and in surveillance of correct 3' end formation under 42 °C heat stress (Saguez et al., 2008). In contrast as presented in this study, the THO component Hpr1 dissociates from bulk mRNA in response to stress like all other mRNA binding factors (Figure 11) and another THO component, Mft1, is not involved in Mex67 binding to the transcription factor Hsf1 (Figure 25). This would suggest the THO complex is dispensable for the fast Hsf1-mediated recruitment of Mex67, but still might support transcription elongation and efficient 3' end formation and further prevent formation of R-loops (Huertas and Aguilera, 2003). In fact, severe disruption of HS mRNA production, like impairment of polyadenylation results in a retention of transcript that potentially is facilitated by THO/Sub2 components and the exosome component Rrp6 (Hilleren et al., 2001; Saguez et al., 2008). When these mutations are combined with a deletion of *RRP6* these un-polyadenylated HS mRNAs are exported and translated, indicating Rrp6 actively retains and degrades stress mRNAs that cannot leave their site of transcription (Hilleren et al., 2001; Thomsen et al., 2003).

However, even though Rrp6 is active under normal and stress conditions, a clear difference must be noted. Under normal conditions, deletion of Rrp6 leads to accumulation of faulty transcripts in the nucleus that are released to the cytoplasm when an additional deletion of adaptor proteins can no longer facilitate retention (Hackmann et al., 2014; Zander et al., 2016).

Under heat stress on the other hand, neither in an *RRP6* deleted strain, nor in cells mutated for the TRAMP component and exosome co-factor *MTR4* a nuclear accumulation of stress mRNAs was detectable (Zander et al., 2016). This strongly argues against an active retention of malformed HS mRNAs by factors of the quality control machinery under stress.

5.2.1 The promoter of a gene encodes for the route the transcript takes

Hence the exosome is active during stress, there must be a mechanism of discrimination between normal and heat stress mRNAs. The here performed promoter swap experiments show that a housekeeping gene can be transformed in a stress responsive construct by changing its promoter (Figure 27 and Figure 29). Already the addition of an HSE that serves as a binding platform for Hsf1 is sufficient to induce gene expression in response to heat stress. The importance of the promoter for the fate of a transcript was shown before under conditions of glucose starvation (Zid and O'Shea, 2014). Here mRNAs derived from genes under control of Hsf1 bound to HSE-sequences localize diffusely to the cytoplasm and are translated. In contrast, transcripts expressed under stress responsive element (STRE)-containing promoters are transcribed but accumulate in cytoplasmic granules. These transcripts encode mainly for proteins involved in glucose metabolism and are likely expressed and stored until starvation ends (Zid and O'Shea, 2014). Even though glucose shortage and heat stress represent different forms of hazards for the cell, this supports the importance of gene expression controlled by Hsf1 for efficient export and translation.

This Hsf1-controlled transcription seems in fact to be a route for the mRNA to bypass quality control. The data presented here show that transcripts under control of housekeeping promoter are retained in mutants of the degradation machinery (*rrp6Δ* and *mtr4-G677D*), as these transcripts cannot be eliminated. This is irrespective of the mRNA's coding sequence. Contrastingly, the same mRNA expressed from a Hsf1-controlled promoter is properly exported (Figure 28 and Figure 30). A general export block of all transcripts can only be detected in the export mutants *mex67-5* and *mtr2-21*. One reason for no detectable retention of stress transcripts in *rrp6Δ* and *mtr4-G677D* strains could be that no mistakes occur during HS mRNA transcription. This however is rather unlikely due to certain reasons. First, expression and export of stress mRNAs is fast and thus it is even more prone to errors. Second, elevated temperatures result in overall enhanced molecular movement and speeding up of processes, which could shift kinetic balances in an adverse way. Furthermore, is mRNA transcription a general process that should not become less error-prone depending on the sort

of transcript. In fact, the only global difference between these two kinds of mRNAs is their time of transcription before or after the stress.

While during normal transcription several mRNA-binding adaptor proteins are recruited to maturing mRNAs, these adaptors dissociate under stress and are not needed for efficient HS mRNA export.

5.2.2 Collaboration of quality control factors and the degradation machinery is crucial under normal but not under heat stress conditions

Export of stress mRNAs happens without the need for adaptor proteins, resulting in fast export and translation to proteins. However, association of several adaptor proteins to the nascent mRNA must have advantages as otherwise this would not be the mode of normal mRNA expression. The interaction partners of the adaptor proteins and the time point they assemble on the mRNA has led to the idea that these proteins are required at single pre-mRNA maturation steps (Baejen et al., 2014; Hackmann et al., 2014; Tutucci and Stutz, 2011). Thus, the adaptor Npl3 interacts with the RNAP II and the cap-binding complex, which is loaded directly after transcription initiation (Lei et al., 2001; Shen et al., 2000). This study shows genetic interactions of *NPL3* with both, *CET1* a component of the capping machinery and *RAT1* the nuclear 5' exonuclease involved in efficient gene expression and nuclear quality control (Figure 31 and Figure 32). In both cases mutation of *CET1* or *RAT1* alone was not sufficient to cause lethality indicating that defects that might occur due to these mutants can be compensated by other mechanisms. Additional removal of *NPL3*, in contrast, did cause cell death, which might be explained by a surveillance mechanism where Npl3 recruits the quality control and degradation machinery to aberrant transcripts. In case of Npl3's absence those faulty mRNAs could block the transcription machinery or leak into the cytoplasm and disturb efficient translation.

The importance of Gbp2 and Hrb1 in control of efficient splicing and retention of improperly processed mRNAs was already shown (Hackmann et al., 2014) and (Figure 33). To assure that only spliced mRNAs reach the cytoplasm is of even greater importance for higher eukaryotes where almost all transcripts are spliced. Thus, the SR-rich RNA-binding proteins in mammalian cells exhibit specialized binding profiles for certain sets of RNAs and are important to control alternative splicing events and their coupling with mRNA export by the Mex67 homolog NXF1 (Müller-McNicoll et al., 2016). Furthermore, correctness in gene expression is fundamental for development and health of an organism. Latest research shows that ZC3H14 and its ortholog in *S. cerevisiae,* Nab2, influence splicing, polyadenylation and overall quality control for which

reason they play a role in prevention of neuronal dysfunction and intellectual disability (Fasken and Corbett, 2016). The essential protein Nab2 is in general involved in formation and quality of the 3' end and the poly(A) tail (Schmid et al., 2012, 2015). Therefore, it seems likely that the adaptor proteins represent checkpoint marks for correct pre-mRNA maturation events.

If these proteins are dispensable for HS mRNA export they should also not be involved in their quality control and retention of false transcripts. Due to the diversity of errors that might occur, artificial stress responsive constructs with introns were used to visualize that indeed unprocessed mRNAs are properly exported under stress (Figure 35). These data elucidate a mechanism by which the promoter and concurrently the time of expression determine if an mRNA is quality controlled or not. Bypassing of these control steps results in a faster export and translation of HS mRNAs (Figure 36), which in turn might further support an immediate response to the stress condition. Beside the avoidance of adaptor-mediated quality control during HS mRNA maturation, easier export through the NPC should also fasten this process. Under normal conditions the amounts of adaptors and the receptor Mex67-Mtr2 on an mRNA kinetically determine export or retention upon interaction with the quality control factor Mlp1 at the NPC (Soheilypour and Mofrad, 2016). Under stress in contrast, Mlp1 is sequestered to nuclear foci (Carmody et al., 2010) and can no longer act in retention of erroneous transcripts. In conclusion, the cell has established a way to export HS mRNAs very fast even though this might include false transcripts that reach the cytoplasm. Albeit mRNA quality control is crucial for the cell during its normal growth, avoidance of this control under stress appears to be less hazardous. Faulty mRNAs that result in misfolded proteins presumably make up a very small fraction when compared to all those proteins that denature under stress. As the stress response in part aims to degrade destroyed proteins, some more should not be problematic. Another point might be that HS mRNAs without correct processing are even more prone to cytoplasmic degradation once the stress is over.

Hence, the overall survival of a cell under stress relies on a very reduced set of proteins including Mex67-Mtr2 and the NPC component Rip1/Nup42. Shuttling adaptor proteins, components of the quality control or degradation machinery on the other hand, do not contribute to the stress response and are dispensable under stress (Figure 37). All these findings suggest that adaptor protein-mediated quality control is avoided under stress to assure a fast expression of HS genes.

5.3 Model for mRNA maturation and export under normal and stress conditions

The data presented in this study give an explanation by what mechanisms the cell could discriminate between normal and stress mRNAs and allow their favored export. Furthermore, the importance of the shuttling mRNA-binding adaptor proteins in control of a maturing mRNA's quality is supported. All together the information gathered, leads to two modes of mRNA export, either under normal or under stress conditions. Normal mRNA transcription is coupled to control of every maturation step, which is mostly facilitated by the CTD of RNAP II (Figure 38). The adaptor protein Npl3 interacts genetically with the capping machinery and later physically the cap-binding complex (Cbp20/80) (Shen et al., 2000), while Gbp2 and Hrb1 control the splicing status of the transcript (Hackmann et al., 2014).

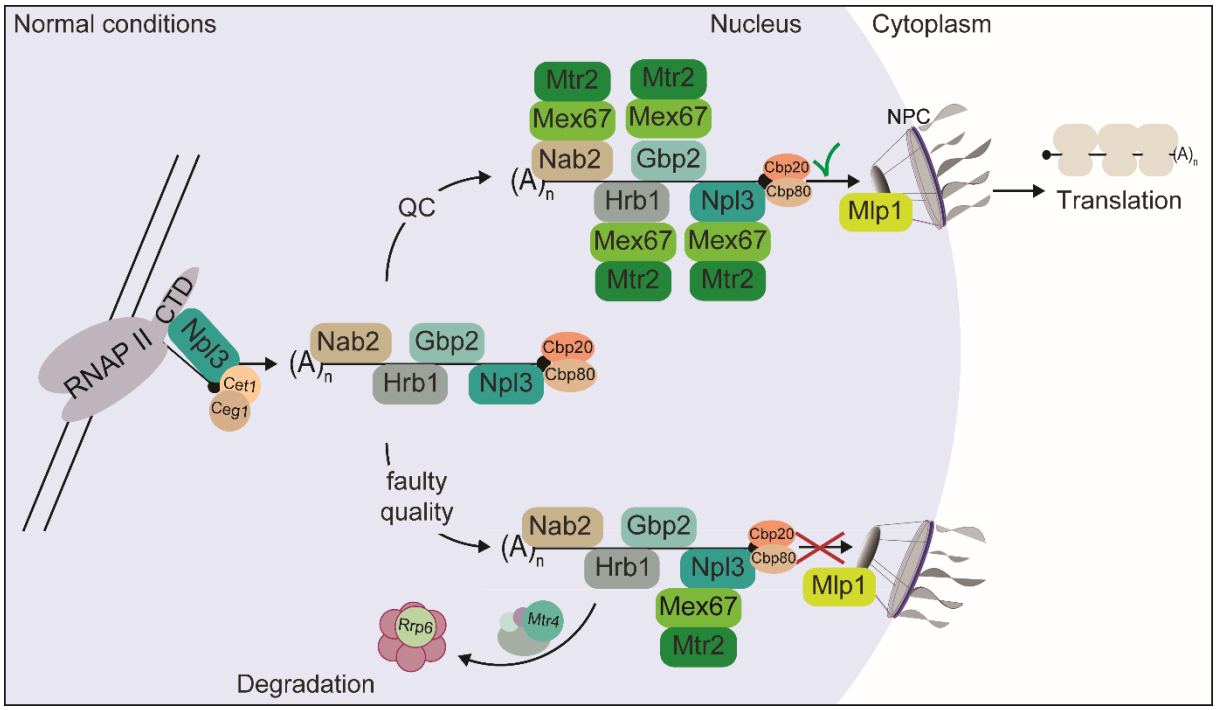

Figure 38: Export of mRNAs from the nucleus to the cytoplasm under normal conditions. The mRNA adaptor protein Npl3 associates early after transcription initiation with the cap structure of the mRNA. During maturation, the other shuttling adaptor proteins Gbp2, Hrb1 and Nab2 assemble on the transcript. In case every maturation step is performed accordingly, the mRNA passes quality control (QC) and the export receptor heterodimer Mex67-Mtr2 can associate to the mRNP, before export. Mlp1 at the NPC performs the last quality check and blocks transcripts, which do not have the right protein composition. These mRNAs are marked by the Mtr4-containing TRAMP complex and degraded by the Rrp6-containing exosome.

Nab2 controls the quality of the poly(A) tail (Soucek et al., 2012). Upon correct quality control the export receptor hetero-dimer Mex67-Mtr2 can bind the mRNP and after a final check at the NPC (Galy et al., 2004; Palancade et al., 2005) the transcript is exported and translated. In case

the mRNA is malformed and thus not packed with adaptors correctly, association with Mlp1 at the NPC is not efficient (Soheilypour and Mofrad, 2016) and the transcript will be degraded (Mühlemann and Jensen, 2012).

When the cell faces a stressful situation, normal mRNAs are no longer produced and already synthesized mRNAs lose their association with adaptors and the export factor (Figure 39). This likely is due to post-translational modifications or conformational changes and potentially is the reason for the mRNA export block (Saavedra et al., 1996). Several normal export factors are secreted in nuclear foci. On the other side, recruitment of the heat stress transcription factor Hsf1 to promoters that contain heat shock elements (HSEs) induces HS mRNA expression.

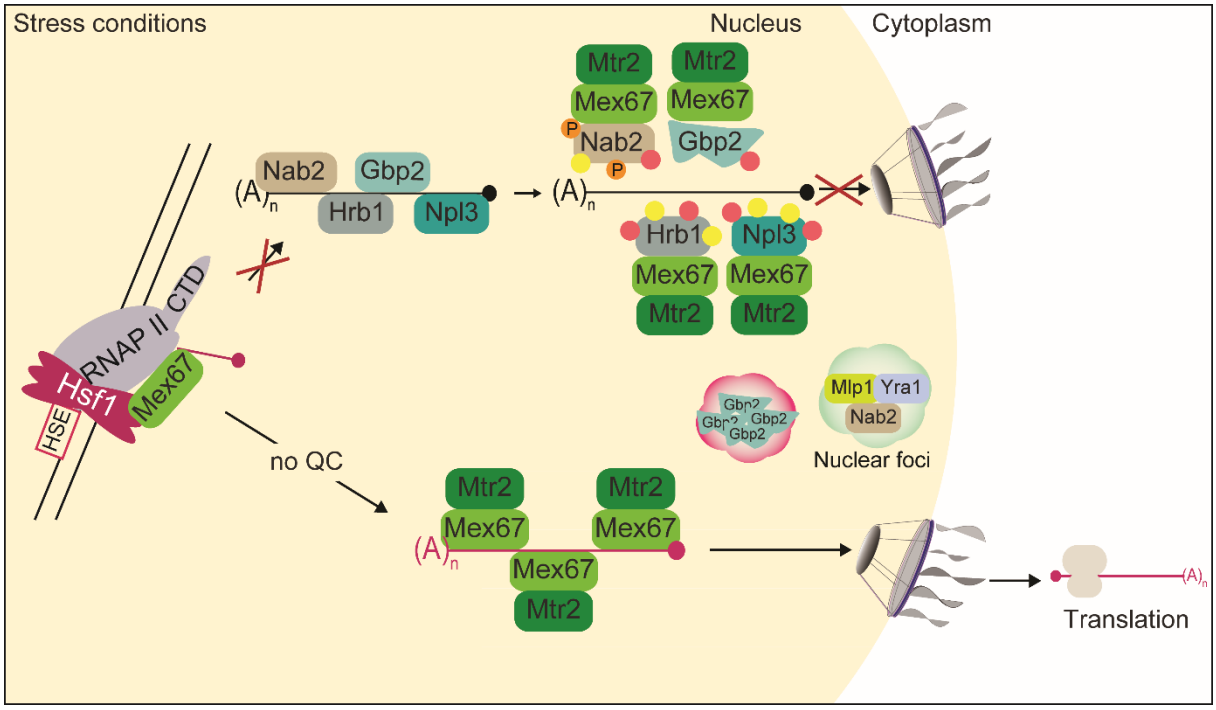

Figure 39: Stress responsive transcripts are exported directly by Mex67-Mtr2. Upon heat stress, housekeeping mRNAs (black) are no longer produced and the shuttling adaptor proteins dissociate from normal mRNAs together with the exporter heterodimer Mex67-Mtr2 causing an export block. Adaptor protein dissociation is presumably caused by post-translational modifications like phosphorylation, methylation or ubiquitinylation (orange, yellow, pink). Nab2 aggregates together with Yra1 and Mlp1 in nuclear foci, while Gbp2 forms foci as well. Expression of HS mRNAs (purple) is induced by binding of the transcription factor Hsf1 to the heat shock element (HSE) in the gene's promoter. Mex67 is directly recruited to the transcript by interaction with Hsf1 and Mex67-Mtr2 facilitates export of HS mRNAs directly.

The exporter Mex67 binds to RNAP II and Hsf1 and associates directly with the new mRNA. This HS mRNA is not controlled by the adaptor proteins for its quality allowing even faulty transcripts to omit the degradation machinery (Mtr4 and Rrp6). Thus, even though mRNA correctness is reduced, it enables a faster export of stress transcript and an enhanced response to heat stress.

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