Post-transcriptional regulation after stress in *Schizosaccharomyces pombe*

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2011

ISBN 978-91-628-8251-8

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Göteborg University, Box 462, SE-405 30 Göteborg

Printed by Chalmers reproservice
Göteborg, March 2011

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ABSTRACT

Post transcriptional regulation is part of the gene expression control and is important for many cellular processes. It influences how mRNAs are selected for translation, degradation or storage. In this thesis, I describe some of the known mechanisms for transcriptional regulation in *S. pombe* including MAP kinase (MAPK) signaling, translation and mRNA localization to cytoplasmic RNA granules. The MAPK Sty1 in *S. pombe* is activated in response to a wide range of stresses and regulates transcription as well as translation. In a screen for novel interaction partners to Sty1 we identify translation factors eEF2 and eIF3a. The Sty1-eIF3a interaction weakened upon stress treatment but Sty1-eEF2 remained unchanged. Translation initiation is impaired in response to stress in *sty1* cells and the Atf1 transcription factor, which is a known target for Sty1 contributes to translation recovery after osmotic stress but in no other stress investigated. Under conditions of nitrogen limitation we found that both interactions with eEF2 and eIF3a disappeared and that eIF3a is degraded at a time point correlating with the time of translation re-initiation. Both phosphorylation and protein levels of eIF3a in *sty1* cells were reduced. *S. pombe* forms cytoplasmic granules in response to stress positive for the RNA-binding and translation proteins Csx1, Dcp2, eIF4G, eIF3a, Pabp (polyA-binding protein), and mRNA. Pabp and Dcp2 almost exclusively co-localize after glucose starvation but not after osmotic stress. Ca^{2+} perturbations affect the formation of granules after glucose starvation and the Ca^{2+} chelator EGTA alone induced granules. Pathway regulating granules are under control of eIF2a and Protein kinase A (Pka1). eIF2α is not a requirement for granule formation but appear to be important for the disaggregation of granules after osmotic stress and EGTA but not after glucose starvation. *pka1* cells were unable to form Pabp positive granules after glucose starvation and EGTA. Ribosomes in *pka1* cells failed to fully dissociate in response to glucose starvation. In a whole genome mRNA stability analysis we find that mRNAs that are transcriptionally upregulated also become stabilized in the early response to oxidative stress and that this is largely Sty1 dependent.

Keywords: *S. pombe*, MAPK, stress, translation, RNA granules, Sty1, Pabp, mRNA stability
ISBN 978-91-628-8251-8

List of publications

This thesis is based on following papers:

I: **Fission yeast mitogen-activated protein kinase Sty1 interacts with translation factors**. Eva Asp[1], Daniel Nilsson[1], and Per Sunnerhagen. *Eukaryotic Cell 7:328-338 (2008)*
[1]Shared first authorship

II **Cellular stress induces cytoplasmic RNA granules in fission yeast**
Daniel Nilsson and Per Sunnerhagen.
RNA 17:120-133 (2011)

III **Impact of oxidative stress and the MAP kinase Sty1 on mRNA stability in *S. pombe*.** Eva Asp, Rebecka Jörnsten, Daniel Nilsson, Alexandra Jauhiainen, Olle Nerman, and Per Sunnerhagen.
Manuscript (2011)

Paper not included in this thesis

Ubiquitin protease Ubp3-Nxt3 complex is a component of the fission yeast stress granules but not required for their assembly. Chun-Yu Wang, Wei-Ling Wen, Hsiang-Ju Chen, Daniel Nilsson, Per Sunnerhagen, Tien-Hsien Chang, and Shao-Win Wang.
Manuscript, submitted (2011)

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Introduction

Introducing Schizosaccharomyces pombe

Schizosaccharomyces pombe (*S. pombe*) is a unicellular fungus, phylogenetically classified as an archaeascomycete in the ascomycete lineage. *S. pombe* is also known as fission yeast because of its mode of division. Rather than budding off a daughter cell like the well known *Saccharomyces cerevisiae* (bakers' yeast), mitotic *S. pombe* produces two equally sized cells. *S. pombe* was first isolated 1893 by P. Lindner from East African millet beer, and the name Pombe is derived from the Swahili word for beer. It was not until the 1950's that it was developed as model organism by Urs Leopold (*Die Vererbung von Homothallie und Heterothallie bei "Schizosaccharomyces pombe*).

S. pombe are rod shaped cells, 7-14 µM in length and 3-4 µM in diameter. The S. *pombe* genome is 13.8 Mb and dispersed on three chromosomes. The genome, which was sequenced in 2002 [1] contains 4,824 protein coding genes. The cell cycle under normal vegetative growth is about 2-5 hours depending on media and is characterized by a long G2 phase. *S. pombe* has no obvious G1 phase and roughly 10 % of cell the cycle is spent in G1. *S. pombe* cells septate as they enter the S-phase which means that cells enter the S-phase before cytokinesis. Meiosis is instigated when *S. pombe* enters unfavorable growth conditions, like nutritional depletion. Cells of opposite mating type, P (plus) and M (minus) conjugate and form zygotes and the resulting diploid undergoes meiosis which produces an ascus with four haploid spores. When the condition is favorable again, the spores germinate and cells return to vegetative growth. One common method to induce mating in S. *pombe* is by depleting the nitrogen sources, which results in G1 arrest and subsequent mating if the two mating types are present.

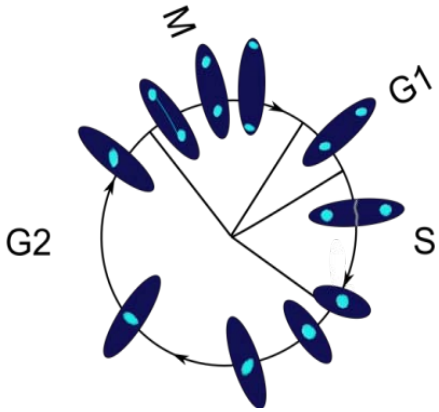

Figure 1. The cell cycle in S. pombe. *In vegative growth, S. pombe spends 2,5- 5 hours to complete the cell cycle which is characterized by a very short G1 phase.*

Why we use yeasts as model organisms

Multicellular animals, such as humans, are products of billions of cells cooperating and interacting. As a result, they are highly complex and complicated to understand and study. That is why many scientists sometimes try to boil down the whole complex organism into one tiny cell when studying specific cellular mechanisms. That cell is often a fungus, either budding yeast or fission yeast. How is that possible? How can you understand what is going on in a human cell by studying a yeast? The answer is simple. Many of the proteins and cellular mechanisms found in yeast and other simpler organisms have their mammalian counterparts. Maybe it is not as simple as to interpolate the results from experiments made on unicellular organisms to mammalian cells, but some puzzle pieces do fit and give at least partial information of how the cellular mechanisms you are studying works in mammalian cells. Perhaps the most important discovery made in *S. pombe* was the discovery of conserved protein factors regulating the cell cycle by Paul Nurse. This information has helped the scientific community to gain knowledge and understand mechanisms that contribute to cancer. The choice of model organism is often determined by what cellular mechanism you aim to study. For instance, *S. pombe* has been a popular model organism when studying the cell cycle because of more similarity to the mammalian cell cycle compared to the budding yeast *Saccharomyces cerevisiae*.

Stress

Most people deal with stress every day. Too little time on our hands and too much to fit in our busy schedule often results in stress that is typically controlled by hormones at the higher organism level resulting in physiological consequences. It is physically and mentally perceived by the organism itself and symptoms like elevated blood pressure, as well as increased pulse and breathing are typical. It is basically a good thing since it prepares the body for physical action, the fight or flight response. For the single cell, stress is environmental changes that could inflict damage to essential macromolecules, in particular DNA, RNA, and proteins, potentially leading to cell death or mutations. Macromolecules and biochemical processes often functions optimally under specific physiological conditions. Biochemical fluctuations can result in suboptimal physiological conditions and lead to molecular malfunction which make cells susceptible to damages. The array of toxic compounds and conditions that are potentially harmful is extensive. A few of them have been used in this thesis to study stress related mechanisms in fission yeast; *oxidative stress* is the

manifestation of Reactive Oxygen Species (ROS) which includes peroxides and free radicals. ROS is highly reactive and can inflict damages to cellular macromolecules such as DNA lipids and proteins. High levels of ROS in the cells are counteracted by enzymatic and non-enzymatic antioxidant systems which degrade or scavenge ROS species. *Hyperosmotic stress* is caused by conditions when water is drawn out from the cell by osmosis resulting in cellular dehydration. Cells defend themselves by leveling out the external and internal osmotic difference by increasing the internal omolarity by the production of inert compound called osmolytes. *Heat stress* is caused by elevated temperatures which results in protein aggregation, unfolding and misfolding. Proteins called heat shock proteins (HSP) sequester heat damage proteins and assist their refolding or degradation. HSPs are not exclusively induced by heat shock but are activated in any condition which leads to protein damages. *Nutritional stress*/*deprivation* is the depletion of carbon and nitrogen sources which cells utilize for growth. Single cell organisms can elude nutritional deprivation and other harsh conditions by producing spores that are far more resistant to unfavorable conditions and can stay dormant for long periods.

Single cell organisms are in general more exposed to environmental challenges than higher eukaryotes are. Unicellular organisms like yeast, in contrast to higher eukaryotes, are unable to escape unfavorable conditions and are not part of closed cell system. Higher eukaryotes have specialized organs to regulate body fluid homeostasis where conditions can be very harsh (kidney, liver) but individual cells are generally not exposed to extreme physiological change like stationary unicellular organisms are. The consequence of which environmental factors and selective pressure cells are faced with is notable in the transcriptional response to stress. In coordination with other stress specific regulated genes, *S. cerevisiae* and *S. pombe* execute a broad transcriptional response referred to as the Core Environmental Stress Response (CESR), also known as the core stress response (CSR) or Environmental Stress response (ESR) almost independently of the type of stress. Approximately 10 % of all genes are significantly up or down regulated in response to stress [2, 3] and result in a transcriptional shift from genes that are expressed for energy consuming processes like growth, to expression of genes that are involved in stress tolerance, which in addition, results in cross protection against other stresses [4]. In multicellular organisms, the set of CESR genes is considerably smaller [5, 6] and represents different genes compared to yeast.

Mitogen Activated Protein Kinase (MAPK)

Protein phosphorylation is a major regulatory mechanism used in intracellular signaling pathways to transduce extracellular and intracellular signals to the nucleus and other intracellular targets. Among these pathways are the Mitogen Activated Protein Kinase (MAPK) pathways. MAPKs belongs to a large and well conserved family of eukaryotic serine/threonine specific kinases [7] involved in pathways, regulating a plethora of cellular processes such as cellular differentiation and proliferation, development, cell cycle, cell death as well as stress tolerance. MAPKs are components of a three-layered kinase module [8], where the three kinases act sequentially within distinct pathways. The archetype of MAPK signaling, the three kinase canonical cascade was described and proposed as conserved phenomenon after studies in *S. cerevisiae* and *S. pombe* [9]. The cascade starts with the activation, often via cell surface receptors, of the first kinase in the module, called MAPK kinase kinase (MAPKKK). Activated MAPKKK then activates the MAPK kinase (MAPKK) via phosphorylation of two adjacent threonine and tyrosine residues [10-12]. Active MAPKK phosphorylates the MAPK at dual sites of the activation loop, commonly a tyrosine and threonine separated by a single variable residue [13-15].

MAPK's have unique activation properties; the MAPK and MAPKK are found in specific combinations in the MAPK module in contrast to MAPKKKs which can interact with several MAPK-MAPKK combinations. This avoids crosstalk between different pathway and for diversity of inputs that can feed into specific MAPKs. Inactivation of MAPK pathways is often carried out by dual specificity MAPK phosphatases (MKPs) and negative feedback loops [16-18]. The spatial organization of MAPKs increases the specificity by which the signals are transmitted and received into sites of action, *i.e.* distinct populations of MAPKs at confined regions in the cell such as the membrane, nucleus, or cytoskeleton limit their range of action.

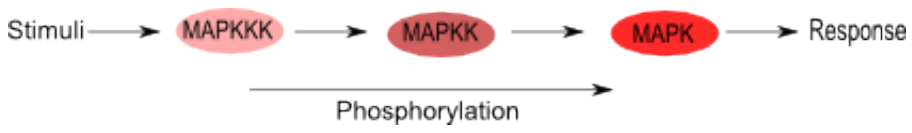

Figure 2. MAPK activation. *Sequential phosphorylation in the MAPK module activates the MAPK.*

MAPK Subgroups

All eukaryotic cells possess multiple MAP kinase pathways where three major MAPK cascades thoroughly have been investigated and described in mammalian cells, the ERK, p38 and SAPK/JNK pathways.

The Extracellular Signal Regulated Kinases (ERK) signaling pathway is one of the most studied MAPK cascades. It is often activated on mitogenic stimuli such as growth factors or cytokines. ERK pathway exerts control over cellular processes like transcription and translation making ERKs important regulators of cellular proliferation, differentiation and apoptosis. A large number of nuclear and cytosolic proteins are phosphorylated by ERK where the major direct or indirect substrates are transcription factors [19, 20].

The p38 MAPK pathway comprises at least four different isoforms of the p38 MAPK: p38-α (SAPK 2), p38-β (SAPK 2b), p38-γ (ERK 6/SAPK 3) and p38-δ (SAPK4) [21-24]. The p38 module consists of numerous MAPKKKs, including MKK 1 to 4, MLK2/3, DLK, ASK1, and Tpl2, but only two MAPKKs, MEK3 and MEK6. p38 is strongly activated by environmental stress such as UV light, heat, hyperosmolarity, and to some mitogenic stimuli like growth factors and cytokines. p38 signaling targets a broad range of targets, many of which are transcription factors but also other protein kinases such as MAPK effector kinases called MAPK-Activated Protein Kinases (MAPKAPs or MKs). Cellular events under p38 regulation include cell differentiation and development, cell cycle, apoptosis [25], and tumor suppression.

c-Jun N-terminal kinases/stress activated protein kinases (JNK/SAPK) are encoded by three genes, JNK MAPKs, JNK1, JNK2 and JNK3. Alternative splicing results in at least 10 different isoforms of JNKs [26]. The core JNK MAPK module is made up of several MAPKKKs, for example MEKK 1 to 4 TAK1and Tpl2. There are two MAPKK, MKK4 and MKK7 which *in vitro* also have the ability to phosphorylate p38 although JNK is the preferred substrate [27]. JNK like p38 is activated by many types of stress including UV, heat shock and hyperosmolarity. Targets of JNK are almost exclusively transcription factors such as c-Jun, ATF-2 and p53. The JNK pathway is involved in intracellular signaling pathways controlling cellular processes including, cell proliferation, differentiation and apoptosis.

Three known MAPK signaling cascades are known in *S. pombe,* the Spk1 cascade, the Sty1/Spc1 cascade, and the Pmk1 cascade. The Spk1 signaling module consists of the Byr2 MAPKKK, the Byr1 MAPKK and the Spk1 MAPK. It is involved in sexual differentiation, *i.e* conjugation and sporulation [9, 28]. The Spk1 MAPK pathway is functionally related to

the Fus3 MAPK signaling pathway in budding yeast, responsible for the mating response, and the ERK2 signaling pathway in mammalian cells. Functionally related to the mammalian JNK MAPK pathway, the MAPK Pmk1 pathway comprises Mkh1 (MAPKKK), Pek1 (MAPKK) and Pmk1 (MAPK), and is involved in regulation of cell wall integrity, cell shape, metabolism of ions and cell cycle cytokines [29-31].

The Sty1/Spc1 MAPK pathway

The Sty1 signaling pathway is the most studied and characterized of the fission yeast MAPK signaling pathways. As a member of the Stress activated Protein kinases (SAPK), it shares functionality with the mammalian p38 and SAPK/JNK signaling pathways. Sty1 was first isolated in a genetic screen where *sty1* cells were found to have severe defects in cell cycle control, exhibiting G2 cell cycle delay [32, 33]. Sty1 has also been implicated in the actin cytoskeleton mitotic checkpoint [34]. *sty1* cells lose viability in stationary phase and are sterile as a result of their inability to arrest at G1 upon nitrogen starvation [35].

The Sty1 MAPK signaling pathway serves as one of the key stress induced regulators in *S. pombe*. Sty1 is activated in response to a wide range of stress stimuli including osmotic stress, oxidative stress, heat stress, nutrient limitation, metal toxicity and UV irradiation. Sty1 is activated by phosphorylation on the Tyr-173 and Thr-171 residues by Wis1, which in turn is activated by Win1 and Wis4 [36-38]. In moderate oxidative stress Sty1 is regulated by a phosphorelay system comprising two peroxide-sensing histidine kinases Mak2 and Mak3 [39 , 40], the phosphorelay protein Mpr1 [41] and the response regulator protein Mcs4 which couples the phosphorelay system with the two MAPKKKs Win1 and Wis4 [37, 38, 42]. At high levels of oxidative stress, Sty1 is activated independently of Mak2 and Mak3 [43] by an unknown mechanism. Pyp1 and Pyp2 are dual phosphatases mainly responsible of keeping Sty1 inactive in unstressed cells as well as attenuating stress induced Sty1 activity by removing the activating phosphates at Thr-171 and Tyr-173 [33, 44]. In response to heat and As^{3+}, Pyp1 and Pyp2 are inhibited by an unknown mechanism, which is followed by a net increase of phosphorylated and activated Sty1 [36, 45, 46]. This could be accomplished by the fact that the cytoplasmic solubility of Pyp1 and Pyp2 are altered within minutes, possibly promoted by conformal changes causing their inactivation [46]. This contributes to Sty1 activation independent of Wis1, which is only weakly induced. Although the Sty1 MAPK signaling pathway is well characterized it is unknown how the Sty1 MAPK module is activated in response to other stresses such osmotic stress, and UV irradiation.

In mammalian cells, the p38 and JNK SAPKs respond to a number of environmental insults by phosphorylating the AP-1 like bZIP transcription factor ATF-2 which initiates a transcriptional stress induced response. In *S. pombe*, Sty1, like its mammalian counterparts, phosphorylates and activates the transcription factor Atf1 [35, 47]. Atf1 is structurally and functionally similar to mammalian ATF-2 [48] and is required for the regulation of several stress-induced genes. Atf1 binds to and functions together with the transcription factor Pcr1 by forming a heterodimer [49] The exact function of this heterodimer is not clear since binding of the Atf1/Pcr1heterodimer to stress gene promoters seem redundant in some cases [50]. Upon stress activation, Sty1 accumulates in the nucleus [51, 52] where it regulates expression of CESR genes essential for the stress response [3]. For the majority of Sty1 regulated CESR genes, Sty1 dependent phosphorylation of Atf1 are also required for proper regulation. Recent studies suggest that Sty1 phosphorylation of Atf1 serves the purpose of stabilizing Atf1 rather than to activate it [53], as an Atf1 mutant lacking all MAPK phosphorylation sites still retains wt activity but is highly unstable.

Sty1 acts on downstream kinases called Mkp1 and Mkp2, who are functional homologues to mammalian MAPKAPs. Sty1 binds to and activates Mkp1 by phosphorylation after stress. Mkp1 regulates mitosis by deactivating the Cdc25 phosphatase which is a Cdc2 activator. Mkp1 has also been shown to be transcriptionally up-regulated in a Sty1 dependent manner and belongs to the CESR genes which are induced in response to several stresses. Mkp2 is poorly characterized but has also been implicated in cellular stress response and cell cycle regulation.

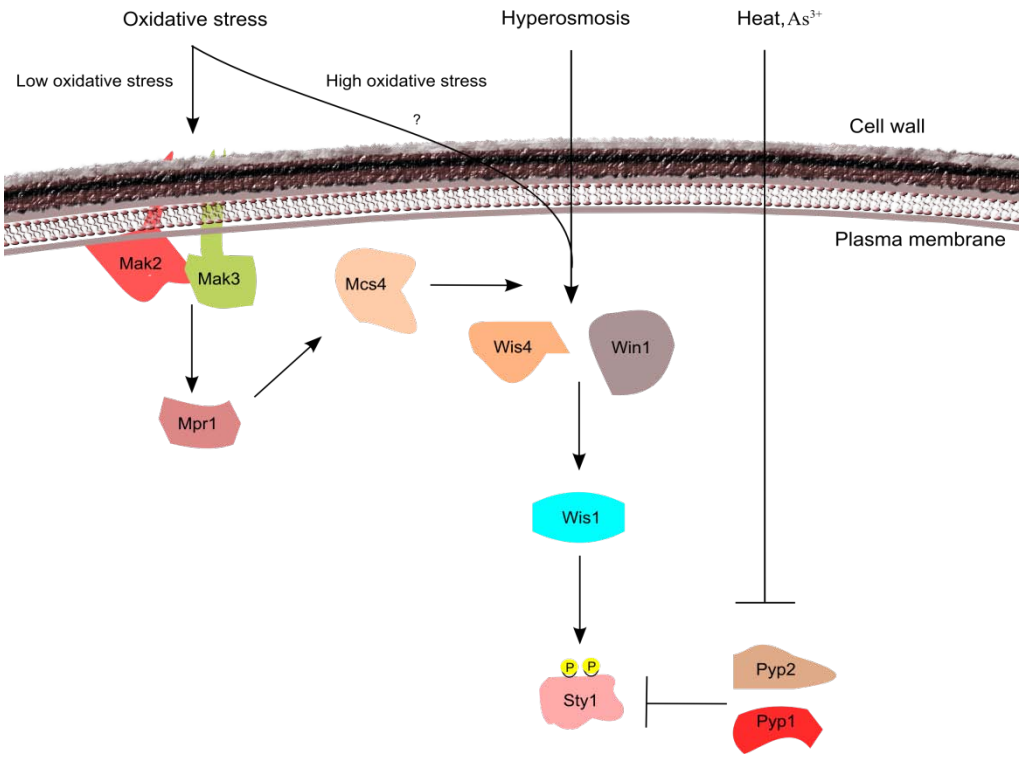

Figure 3. Activation of Sty1 MAPK pathway. *Sty1 is activated within the MAPK module after oxidative stress and hyperosmosis. Oxidative stress is signaled to the Sty1 MAPK module via the phosphorelay system. Sty1 is activated after heat and arsenite by inhibition of the Sty1 phosphatases Pyp1 and Pyp1 independently of Wis1*

Post-transcriptional regulation

Regulation of gene expression is a fundamental process which basically occurs at the transcriptional or the post-transcriptional level. To put it simply, regulation at the transcriptional level provides a frame work for adjusting mRNA levels but does not directly influence how the mRNAs are prioritized in terms of stabilization/destabilization and translation. On the other hand, post-transcriptional regulation acts on the RNA level after transcription but before or during translation. Ultimately, it influences how mRNAs are differentially translated into proteins by controlling steps that determine the mRNA distribution, stability and translation.

Overview of translation and translation regulation in eukaryotes

Translation, protein synthesis, is one of the most energy consuming processes in the cell and is therefore highly regulated. It can be divided into three specific stages; initiation, elongation and termination. In the initiation phase, a 40S ribosomal subunit is loaded onto an mRNA. Scanning of the mRNA locates an appropriate start codon to which the initiatior – methionyl transfer RNA (Met-tRNA$_i^{Met}$) is positioned. The initiation phase ends when the 60S

ribosomal subunit assembles onto the correctly positioned 40S producing an 80S ribosome. The initiation phase is facilitated by proteins called initiation factors 1-5 (eIFs 1-5). In the elongation phase, aminoacyl tRNAs (aa-tRNA) are stepwise recruited to the ribosome and polymerized into a growing polypeptide chain in a codon specific manner. This process requires proteins termed elongation factors 1-2 (eEFs 1-2). The translation terminates when the ribosomal complex reach a stop codon which induces the release of the ribosome from the mRNA which additionally requires release factors 1 and 3 (eER1and eRF3). *The translation termination will be excluded from further discussions it has little relevance to this thesis.*

Cap-dependent initiation. (reviewed in refs. [54-57]). The ternary complex comprising eIF2-GTP-Met-tRNA$_i$, binds to the 40S ribosomal subunit. This is facilitated by initiation factors eIF1, eIF1A, eIF3 and eIF5 which also bind to the 40S subunit, resulting in a 43S pre-initiation complex (PIC). The 43S PIC is assembled onto the mRNA via the 5´ M^7-GppppG (M^{7}G) cap which is mediated by the 5´ cap associated eIF4F complex. The eIF4F complex consists of eIF4A, eIF4E and eIF4G. eIF4A is a DEAD RNA helicase which unwinds secondary structures in the 5´ end of the mRNA important for AUG scanning, the ATP dependent RNA unwinding activity of eIF4A is promoted by eIF4B. The cap binding protein eIF4E physically interacts with 5´ cap, thereby recruiting the eIF4F complex to the 5´cap. The 43S PIC assembly onto mRNAs involves interaction with the scaffold protein eIF4G and IF3. The eIF4G-eIF3 interaction is not found in *S. cerevisiae*, it is thought that eIF4G interacts with other proteins to recruit the 43S subunit to the mRNA. eIF4G also promotes circularization of mRNAs by interactions with 5´ bound eIF4E and 3´ bound poly(A) binding protein (Pabp). The 43S PIC scans downstream of the 5´- end of the mRNA for a proper match with an AUG start codon. The 43 PIC arrests the scanning when it encounters and identifies a start codon and forms a 48S PIC. This also triggers the irreversible hydrolysis of the GTP moiety of the eIF2-GTP-Met-tRNA$_i$ by eIF5, producing GDP. eIF2-GDP releases the Met-tRNA$_i$ into the P-site of the 40S subunit and releases from the 48S PIC. eIF5B-GTP then binds to the 48S and the subsequent hydrolysis of eIF5B-GTP catalyses the release of other initiation factors and joining of the 60S ribosomal subunit to yield an 80S initiation complex. For another round of successful initiation, the GDP bound to eIF2 is exchanged for a GTP to form active ternary complex. This is carried out by the Guanine Exchange Factor (GEF) eIF2B, which catalyses the exchange of GDP for GTP bound to eIF2.

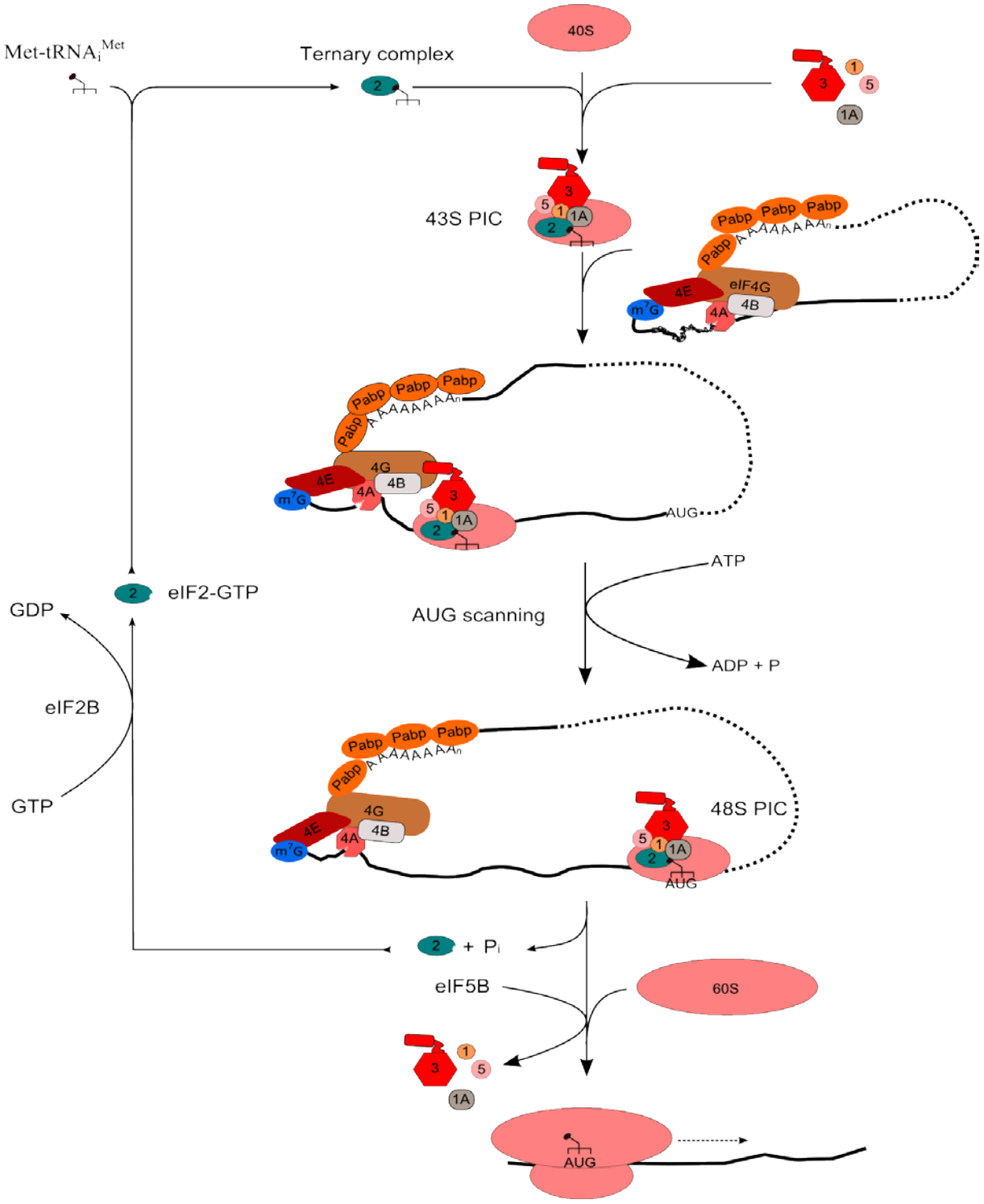

Figure 4. Schematic overview of translation initiation events. *(1,2) Active ternary complex (TC) and eIFs 1, 1A, 3 and 5 binds to a 40S ribosomal subunit producing a 43S pre-initiation complex (PIC). (3) The PIC is loaded onto an mRNA which is facilitated by eIFs 4A-E. (4, 5) The 43S PIC forms 48S PIC when it reaches a start codon after mRNA scanning.(5,6) eIFs releases from the mRNA as the 80S ribosome assembles.(7) The eIF2 GDP-GTP exchange is catalyzed by eIF2B and an active ternary complex can regenerate.*

Translation elongation. (reviewed in refs. [54, 58]). A polypeptide chain is produced in translation elongation. An aminoacyl-tRNA (aa-tRNA), the GTPase eEF1A and GTP is brought to the A-site of the ribosome as a ternary complex, aa-tRNA-eEF1A-GTP. Codon-anticodon base pairing between the mRNA and incoming aa-tRNA results in a mRNA-tRNA duplex which interacts with ribosomal rRNA and activates the GTPase activity of eEF1A. The subsequent hydrolysis of eEF1A-GTP releases the aa-tRNA to the A-site. Inactive eEF1A-GDP is re-activated by the GEF eEF1B by GDP-GTP exchange. A peptide bond between the incoming amino acid and the peptidyl tRNA is catalyzed by the peptidyl transferase center which involves deacylation of the peptidyl tRNA. eEF2 catalyzes the translocation of the ribosome relative to the mRNA which translocates the new peptidyl tRNA to the P-site and the deacylated tRNA to the E-site where it releases the ribosome. This continues until the ribosome reaches a stop codon. Several ribosomes can be engaged in translation of a single mRNA and are then referred to as polysomes.

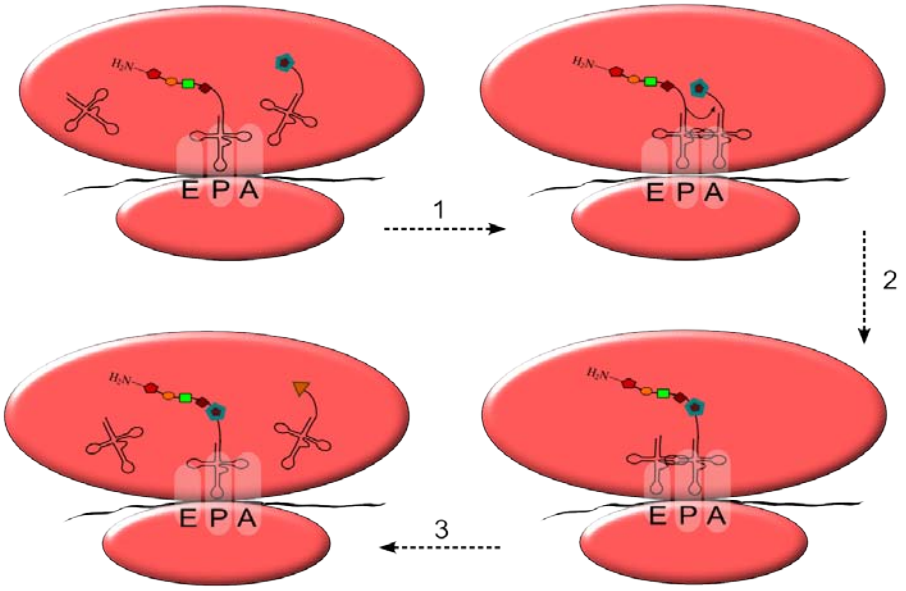

Figure 5. Schematic overview of translation elongation events. *A polypeptide is produced by making peptide bonds between an incoming aminoacyl-tRNA and a peptidyl tRNA at the A and P sites of the ribosome (1, 2). Unloaded tRNAs are released from the ribosome as it enters the E site of the translocating ribosome (3).*

Along the protein synthesis process there are several mechanisms that regulate the rate, specificity and selectivity of mRNA translation. Translational control of gene expression can roughly be divided in two groups; global regulation which mainly occurs via modifications of translation factors, and specific regulation of translation which depends on intrinsic elements in the mRNA recognized by regulatory protein complexes [59].

Translation initiation regulation is generally part of the global control of protein synthesis where the phosphorylation state of initiation factors and regulatory proteins determine the translational status of the cell. There are two main and well characterized targets for initiation regulation, eIF2α and eIF4E. The eIF2 complex comprises three subunits, α, β, and γ. The conditional phosphorylation of the Ser51 residue in the eIF2α subunit blocks the GDP-GTP exchange of the eIF2γ. Phosphorylated eIF2-GDP has higher affinity for eIF2B than unphosphorylated eIF2 which makes it a competitive inhibitor that sequesters the guanine exchange factor eIF2B. The inactive eIF2-eIF2B complex cannot regenerate an active ternary complex [59-62]. So far, four eIF2α kinases have been characterized in mammalian cells. Heme regulated inhibitor (HRI), is activated by conditions of heme deficiency and stress [63]. Double-stranded RNA-dependent protein kinase (PKR) is stimulated in response to double stranded RNA during viral infection. General control non-derepressible-2 (GCN2) is activated by amino acid depletion, UV irradiation and viral infections [64-66]. PKR-like endoplasmic reticulum kinase (PERK) is activated by unfolded proteins in the ER. *S. pombe* has three eIF2α kinases; Hri1 and Hri2, both related to mammalian HRI, and Gcn2. Each kinase responds to different subsets of stress signals, although is some cases they can work in coordination. Sty1 has been shown to support the general translation during stress. *sty1* cells exhibit reduced levels of translating ribosomes polysomes after osmotic and oxidative stress and are severely impaired in polysome recovery following osmotic stress compared to wild type [67, 68]. There is evidence to suggest a pivotal role for Sty1 in maintaining proper translational initiation, at least during oxidative stress, balancing the action of eIF2α kinases demonstrated by eIF2α hyper-phosphorylation in *sty1* cells [68].

Another way cells regulate general translation rates is to inhibit cap-mediated translation. Phosphoproteins called 4E binding proteins (4E-BP) are able, when hypo-phosphorylated, to competitively displace eIF4G in the eIF4F complex, thereby disrupting it resulting in translational repression. There are no known 4E-BP sequence homologues in *S. pombe*, but two isoforms of eIF4E; eIF4E1 and eIF4E2, with different affinity for eIF4G [69, 70]. The fact that eIF4E2 has a 100-fold lower affinity for eIF4G compared to eIF4E1 suggests that it could be a functional homologue to 4E-BP proteins, regulating translation rates by translation initiation attenuation.

Translation elongation is also regulated in response to unfavorable conditions, foremost by regulating elongation factor 2 (eEF-2) activity. It has been shown in mammalian cells that phosphorylation on eEF-2 Thr-56 inhibits its association with the ribosome resulting in a decreased protein synthesis rate. The main kinase responsible for EF-2 phosphorylation is

referred to as eEF-2 kinase [71, 72] due to its monospecificity for eEF-2. eEF-2 kinase in turn can be regulated by protein kinase A and different isoforms of p38. How conserved the phosphorylation of eEF-2 is in yeast is still unknown. From all yeast species sequenced, no apparent eEF-2 kinase homologue has been found so far. In *S. cerevisiae* eEF2 is phosphorylated on Thr-57 by the MAPKAP Rck2 [73] in the p38 homolog Hog1 pathway.

mRNA stability and decay

mRNAs have two elements co-transcriptionally integrated which determine the intrinsic stability of the specific mRNA, the 5′ M^7G cap and the 3′ poly A tail. These elements help to protect the mRNA from degradation by exonucleases and enhance translation. The M^7G cap interacts with eIF4E (discussed above) and the poly (A) tail interacts with PABPs. For exonuclease to gain access to the mRNA, at least one of these structures must be removed. The mRNA can be degraded by two major pathways; the 5′→ 3′ degradation pathway and the 3′→ 5′ degradation pathway. The degradation pathways, initially described in *S. cerevisiae*, can work in conjunction or separately and are initiated by the shortening of the poly (A) tail by deadenylases and removal of the 5′cap by two proteins called decapping protein 1 and 2 (Dcp1 and Dcp2) [74, 75] exposing the transcript to Xrn1 ribonuclease dependent 5′→ 3′ degradation [74, 76, 77] or 3′→ 5′ degradation by a multi-protein complex of exonucleases called the exosome. Several pathways governing mRNA surveillance are coupled to mRNA decay pathways. The nonsense mediated decay pathway (NMD) is best understood. In yeast, the NMD complex consists of the core proteins Ufp1, Ufp2 and Ufp3 that detects and acts on transcripts that contain premature termination codons (PTC) which would result in truncated proteins with abnormal functions. The main rerouting of mRNAs detected by NMD involves a deadenylation–independent mechanism in yeast [78] but in mammalian cells, deadenylation-dependent NMD seems to be favored [79].

One of the most studied control elements are AU rich elements (AREs) which are found in many mRNAs. Based on the number and context of the AUUUA pentamer in the 3′ -UTR, AREs are classified into several groups which influence the mRNA stability and translation. AREs destabilize mRNAs by mechanisms that involve interactions with the exosome or mRNA decay factors either direct or indirect via ARE-binding proteins. mRNAs can also be stabilized by ARE-binding proteins, possibly by competing for ARE binding sites or removing the mRNA from the site of decay [80-82]. Phosphorylation of ARE-binding proteins can alter either the function, localization or the affinity for the substrate which results

in destabilization or stabilization of mRNAs. For instance, the p38 MAPK pathway is known to regulate the stability of certain mRNAs by phosphorylation of ARE-binding proteins [83-86]. In *S. pombe*, Sty1 has been implicated in regulating mRNA stability as well. Sty1 together with the RNA binding protein (RBP) Csx1 regulates *atf1*$^{+}$mRNA stability after oxidative stress [87]. Moreover, Csx1 undergoes Sty1 dependent phosphorylation induced by oxidative stress, though the physiological reason for this phosphorylation is not yet resolved. Sty1 is also responsible for the stabilization of the *uvi15*$^{+}$ transcript after UV irradiation which also requires an AU rich 54 nt element present in the 3´ region [88].

Stress granules and P-bodies, the whereabouts of mRNAs after stress.

When protein translation is impaired, generally caused by stress, cells make a decision regarding already synthesized mRNAs; should it be translated, degraded or stored in a translational repressed state. Studies in mammalian cells and yeast have shown that stress-induced translation repression causes dense messenger ribonucleoprotein particles/complexes (mRNPs), composed of translational repressed mRNAs and various RNA binding proteins (RBPs), to aggregate in the cytoplasm referred to as processing bodies (PBs) and stress granules (SGs).

Functions of PBs and SGs

Several functions of PBs have been suggested, including mRNA degradation, translation repression, and mRNA storage. One of the main functions of PBs is likely to be mRNA degradation, as indicated by the presence of mRNA decay factors and proteins of the decapping machinery. mRNA degradation is normally preceded by irreversible steps of deadenylation and decapping. Inhibition of these processes leads to loss of P-bodies [89-91] and impairing the catalytic steps of mRNA degradation results in increased volume of PBs [91-93]. Although mRNA degradation occurs in PBs, organization into large PBs is not a requirement for basal mRNA degradation. Yeast cells that are defective in PB formation are still able to repress translation and degrade mRNA [94]. One explanation would be that mRNA turnover rate is increased when mRNA decay factors and mRNAs are compartmentalized, due to higher concentrations relative to the cytoplasm [94-96]. In addition, this could also limit “unspecific” interactions between mRNA decay factors with other proteins and “normal” mRNAs ensuring effective degradation [97].

Another function ascribed to PBs is mRNA storage. Observations that mRNAs can re-enter the polysomal pool from a translationally repressed state in PBs in response to shifting conditions [80, 98] demonstrate that not all mRNAs are degraded in PBs. Further, keeping untranslated mRNAs compartmentalized in PBs could serve as protection against exosome degradation and unwanted interference with the translational apparatus.

The function of SGs is yet to be fully established. SGs have been proposed to function as repositories for stalled 48S initiation complexes, or a place for mRNA triage. Depending on interacting RBPs, mRNAs are believed to be sorted for degradation is PBs, stored or sent for translation initiation [99, 100]. One other possibility is that SGs represent hot spots for translation initiation [95, 101]. Increasing the local concentration of initiation factors would increase the assembly rate of initiation complexes, which functionally would relate to nuclear structures called Cajal bodies. These nuclear structures are involved in the assembly of small nuclear ribonucleoproteins (snRNPs) and have been proposed to increase the assembly rate of snRNPs by 10-fold [102].

PBs and SGs transiently dock and/or form in conjunction to each other in both metazoans and yeast [103-107]. Moreover, Fluorescence Recovery After Photobleaching (FRAP) studies have revealed that certain PB and SG components shuttle [106-110], and some of these proteins are components of both PBs and SGs suggesting that there is crosstalk between PB and SGs.

Components of PBs and SGs

Core constituents of mammalian stress granules are components from 48S translation initiation complexes. This includes small ribosomal subunits and early initiation factors eIF3, eIF4E and eIF4G, and PABP [111-113]. Additionally, SGs contain numerous RBPs including HuR, G3BP, TIA-R, TIA-1, and Ataxin-2, exonucleases such as Xrn1, and members of the RNA-induced silencing complex (RISC). In S. *cerevisiae* eIF4E, eIF4G, and RBPs Ngr1, Pabp1, Pbp1 and Pub1 accumulate in granules, named EGP bodies or yeast stress granules [105, 114], upon glucose starvation. It is not clear if EGP bodies should be defined as “real” SGs since these granules lack certain mammalian SG markers such as eIF2 and eIF3. Yeast SG core constituents seem to vary depending on condition. For instance, glucose starvation induced EGP bodies /yeast SGs are negative for eIF3 components in contrast to cells exposed to heat where SGs are positive for eIF3 components [104]. Stress-induced granules containing components of eIF3 complex and eIF4E induced by heat stress and osmotic stress were

observed in *S. pombe* [115]. Although they were suggested by the authors to be sites of translation during stress, recent work has further demonstrated the existence of SGs in fission yeast that share features with both mammalian cells and *S. cerevisiae* [116, 117]. Several proteins from the RNA interference (RNAi) pathway are also constituents of mammalian SGs. An RNAi pathway in *S. pombe* was recently described [118, 119], in contrast to *S. cerevisiae* which lacks an RNAi pathway. Whether these components also are enriched in SGs has to be determined by future work.

Components of PBs have been defined in both mammalian cells and *S. cerevisiae*. Dcp1, Dcp2, Xrn1 and GW182 were the first PB components to be described [89, 120-122]. PBs and SGs share some components like Xrn1 and eIF4E and also Pab1, although exclusively for yeast. Dcp1 and Dcp2 are considered to be exclusive markers for PBs, at least in mammalian cells, and are therefore used in many studies to separate PBs from SGs while components of the 48S initiation complex like eIF3, eIF4G and subunits of the small ribosomal complex remain exclusive for SGs. Additional PB components include several proteins implicated in the mRNP assembly, mRNA decay and RNAi pathway. Little is known about which specific mRNA transcripts are included or excluded in SGs and PBs since there are no large scale studies of mRNAs localization in PB and SGs.

Assembly and disassembly of PBs and SGs

In mammalian cells, the phosphorylation of eIF2α is the main initial step for SG assembly. This reduces the availability of active ternary complexes resulting in initiation inhibition. Cells expressing mutants of eIF2α that are phosphomimetic (S51D) or non-phosphorylatable (S51A) either induce or prevent SG assembly respectively [112]. Although eIF2α phosphorylation is a major regulatory step in SG assembly, it is not a requirement for induction of SGs *per se*. Conditions when energy is depleted and active TCs are reduced can induce SGs without elevated eIF2α phosphorylation [111, 123]. It is therefore likely that it is the availability of active TC following eIF2α phosphorylation that induces SGs, not the eIF2α phosphorylation itself. Also, SGs can be induced independently of eIF2α phosphorylation by drugs that inhibit translation initiation by inactivating eIF4A, which consequently perturbs eIF4E function [124-126]. eIF2α phosphorylation as a major regulatory step in SG induction does not apply in yeast. Although assembly of SGs in *S. cerevisiae* is enhanced by eIF2α phosphorylation, it is not a hallmark of SG formation. The consequences of eIF2α

phosphorylation in *S. pombe* are even less where little or no effect can be seen on SG formation [116, 117].

When translation initiation is inhibited, prion like RBPs with Q/N rich domains such as TIA-R and TIA-1 and G3BP in mammalian cells are able to bind the stalled mRNP and subsequently self aggregate. Homotypic and heterotypic interactions are thought to drive oligomerization of RBPs which crosslinks individual mRNPs and promote aggregation/nucleation into larger microscopically visible SGs, which is further assisted by piggyback recruitment of non-core constituents of SGs [101, 109, 112, 127]. Overexpression of nucleating proteins causes SG formation in the absence of stress while knockdowns impair SG assembly [112, 127, 128]. Several post-transcriptional modifications have also been shown to influence SG assembly and composition. Phosphorylations seem to represent a major regulatory type of modification; many ARE-binding proteins implicated in SG function and assembly is regulated by phosphorylations. For example, phosphorylation of G3BP, and BRF1 and TTP decrease their association with SGs [127, 129, 130] and mRNA destabilization activity. Other types of post-transcriptional modulations that influence SG assembly include O-Glc-NAc glycosylation [131] and methylations [132, 133].

The core mechanism for assembly of PBs is similar to SG assembly because it requires proteins with QN-rich domains that are capable of self-aggregating and driving the aggregation. In contrast to SGs assembly, PBs is not induced by eIF2α phosphorylation and it may require steps of deadenylation [134] and recruitment of the mRNA decapping machinery before mRNAs are sequestered into PBs. In yeast, a core set of proteins have been identified as essential for PB assembly. They are not exclusively required for PB assembly but interdependent for the recruitment of PB components and PB formation. Edc3, Dhh1, Lsm4 and Pat1 have all been shown to influence PB assembly and are thought to exist in two separate complexes each contributing to PB assembly. One complex consists of at least Edc3 and Dhh1and Dcp2. The other complex consists of Lsm1-7 and Pat1 as a minimum. Edc3 is an enhancer of decapping [135] which via two domains is able to promote PB assembly. Edc3 recruits components of the decapping machinery, such as Dcp2 and Dhh1, and crosslinks individual mRNPs which enhance formation of small PBs to microscopically visible aggregates [94, 136, 137]. The DEAD box helicase Dhh1 is a translational repressor and activator of mRNA decapping [90, 138]. Dhh1 promotes the release of mRNAs from polysomes which is necessary for the decapping machinery to gain access to the mRNA. Lsm4 is one of the subunits of the Lsm1-7 complex consisting of Lsm protein 1-7 which is involved in decapping and subsequent 5′→ 3′ degradation [139, 140]. Lsm4 facilitates PB

formation via its aggregation prone Q/N rich domain [94]. The Lsm1-7 complex is recruited to the mRNA by the bifunctional protein Pat1 which serves as both a translational repressor [90] and an anchor for the Lsm1-7 complex [141].

It is not really established how mRNAs are selected for PBs or SGs, but interruption of 5´- 3´ interaction in mRNAs could be one factor that determines if mRNAs assembles into PBs or SGs. mRNAs that assembles into PBs undergo mRNP modifications that precedes general mRNA degradation and NMD. For instance, deadenylation which is an important step in the mammalian NMD pathway, have also been shown to be a prerequisite for PB formation in mammalian cell [134]. In both mammalian cells and yeast, the NMD involves decapping of mRNAs, in yeast however, decapping can occur independently of deadenylation [78, 142]. Moreover, NMD components that accumulate in PBs can also target normal mRNAs for PBs in yeast [143]. Both deadenylation and decapping would interrupt the 5´- 3´ interaction in mRNAs and potentially lead to a step by step recruiting of PB factors and ultimately PB assembly. In this respect, SG assembly could be viewed as a “passive” aggregation of mRNPs. SG assembly as it seems, does not require any major mRNP reorganizations and may represent an intermediate state to mRNPs in PBs.

The preference for deadenylation dependent or independent decapping in the NMD pathway could explain why Pabp accumulates in PBs in yeast but not in mammalian cells. In mammalian cells, Pabp would not be able to bind deadenylated mRNAs and therefore not accumulate in PBs. In yeast however, a subset of the mRNAs entering PBs from the NMD pathway, with intact poly (A) tails, could potentially be bound by Pabp.

The disassembly of both PBs and SGs are likely to be regulated by molecular chaperones. Prion-like proteins can assume different conformations which are either soluble or aggregation-prone [144]. The conformation is regulated by chaperones such as HSP40, HSP70 and HSP90 [144]. The Q/N rich domains of mammalian TIA-1 and yeast Lsm4 have been shown to specifically influence the aggregation of SGs and PBs [94, 128] and HSP70 in mammalian cells is responsible for regulating TIA-1 aggregation [128].

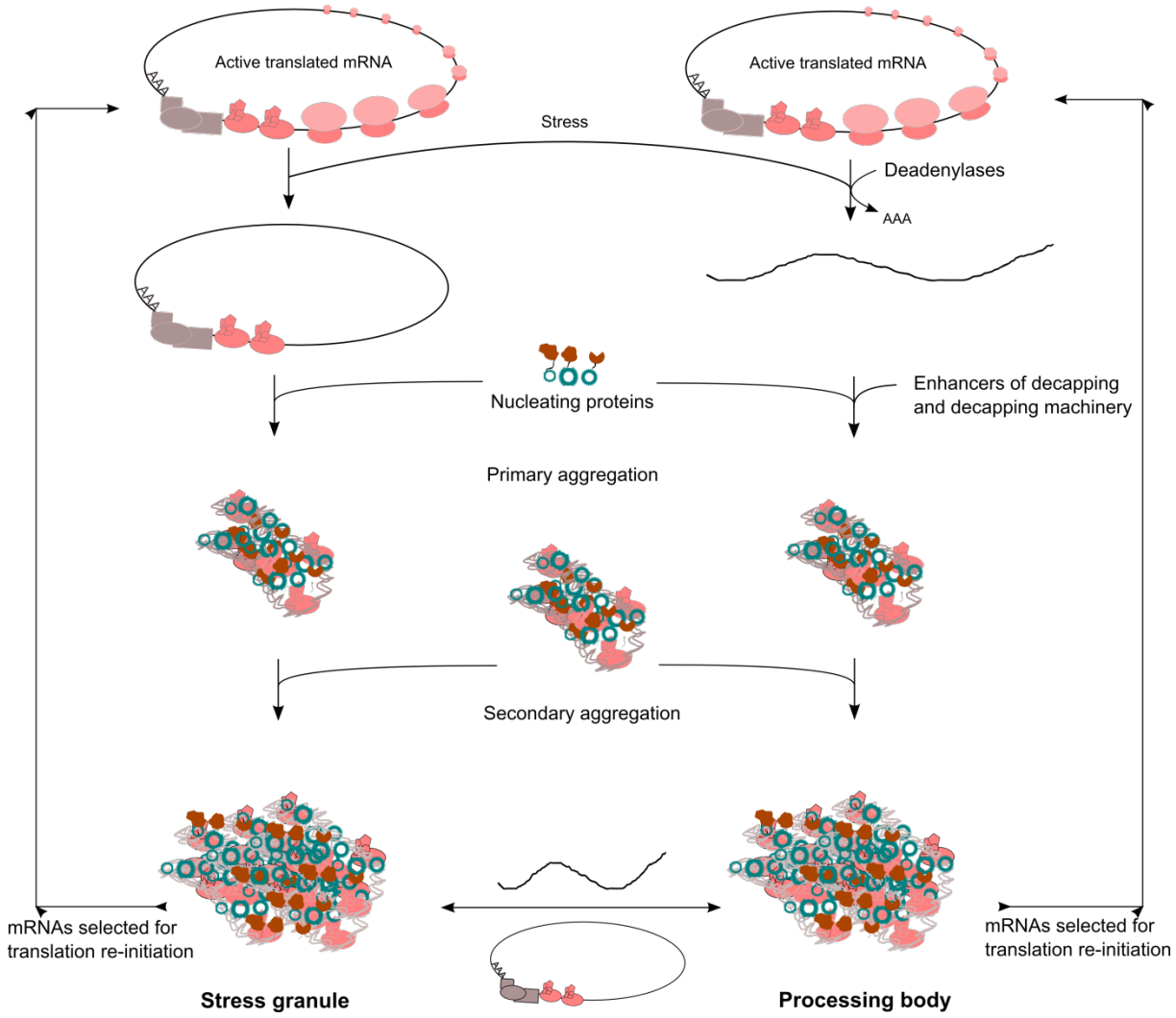

Figure 6. PB and SG assembly. *Stress induces translational repression which either leads to dissociation of translation initiation factors (right) and breakage of the 5´-3´ interaction of mRNAs or stalled translation initiation complexes (left). In the right pathway, mRNP reorganization facilitated by mRNA deadenylation and decapping recruit PB factors and ultimately leads to PB formation. In the left pathway, SGs are formed without any major mRNP reorganizations. Nucleating proteins such as mammalian TIA proteins cause a primary aggregation of mRNPs. Crosslinking of individual mRNPs causes secondary aggregation into larger aggregates. mRNAs shuttle between PBs and SGs and can also be returned to translation.*

Present study

Fission yeast mitogen-activated protein kinase Sty1 interacts with translation factors - Paper I

In a screen to find novel interactions partners to the MAPK Sty1 and the MAPKAPK Mkp1, we performed a Tandem Affinity Purification (TAP). Several proteins, including translation factors eEF2 (eft201), eEF3 (tef3), and eIF3a (tif32), were found to co-purify with Sty1 and Mkp1. Based on previous findings that the Hog1 MAPK pathway influences the phosphorylation status of eEF2 in *S. cerevisiae*, and Sty1 impacts the general protein synthesis in *S. pombe* after stress [68], we proceeded to verify interactions of eEF2 and eIF3a by co-immunoprecipitations (Co-IPs). Since eEF3 is yeast-specific and therefore less interesting from a broader cross-species perspective, we left it out from further analysis.

In co-IPs with extracts from undisturbed cells, we concluded that both eEF2 and eIF3a interact with Sty1 above the background level, but interactions with Mkp1 were no longer observed. Interactions between translation factors and Sty1 were further characterized by co-IPs under different environmental stresses. The Sty1-eEF2 interaction remained quantitatively unchanged relative to unstressed conditions when cells were exposed to oxidative and hyperosmotic stress, but the Sty1-eIF3a interaction did weaken upon the same stress treatments. To find out if this was caused by a reduced protein level of eIF3a we ran western blots of eIF3a under the same conditions, but the eIF3a protein level remained unchanged.

Other than stress signaling, Sty1 is also involved in nutrient sensing and the meiotic program. Nitrogen limitation is a potent inducer of the meiotic program in fission yeast and Sty1 becomes maximally activated at 30 to 60 min after nitrogen withdrawal [35]. To see if this influenced the interactions with eEF2 and eIF3a, we performed additional co-IPs when cells where starved for nitrogen. Both interactions with eEF2 and eIF3a were maintained up to 30 minutes upon nitrogen withdrawal where the interactions also peaked, but completely disappeared after 60 minutes with a small decrease after 45 minutes. The protein level of eEF2 was relatively stable with a small decrease after 120 minutes, as shown by western blots. At the same time, the eIF3a protein level decreased, starting at 30 minutes, to be completely vanished at 90 minutes. To investigate if autophagy, *i.e.* targeted degradation of cellular components with the purpose of recycling nutrients, was responsible for the decrease of eIF3a protein levels, we performed western blots of Cdc2 and tubulin from same protein

preparations. No significant changes in protein levels in Cdc2 or tubulin protein could be detected, which suggests that eIF3a is selectively targeted for degradation under conditions of nitrogen depletion. Fission yeast eIF3a is a subunit of the core eIF3 complex which exists in two different forms; one complex is required for global protein synthesis while the other complex is specifically recruited to a subset of mRNAs [145]. Polysomal profiles and metabolic labeling revealed that cells are translationally active. Clearly, eIF3a is dispensable for translation during nitrogen starvation, indicating a novel compositional eIF3 complex during such conditions. The eIF3a homolog in mammalian cells p170 is not required for global synthesis, but influences the translation of specific mRNAs [146]. It is not known if fission yeast eIF3a is required for global protein synthesis or if it regulates the re-direction of translation to a subset of mRNAs, but a similar role as its mammalian counterpart is possible. Since Sty1 and eIF3a physically interact, or at least are components of the same protein complex, we wanted to study the status of eIF3a in *sty1* cells. Protein levels as well as phosphorylation of eIF3a were reduced in *sty1* cells. This is perhaps caused by instability of the unphosphorylated species; eIF3 has been shown to interact with proteasomal proteins [147] Sty1 dependent eIF3a phosphorylation may stabilize eIF3a and confer resistance to proteasomal degradation. The amount of Sty1 occupied in interaction with eIF3a was estimated to ~ 20% of total Sty1 and the amount of Sty1 is considerably less than eIF3a, therefore it is unlikely that Sty1 serves as stabilizing binding partner to eIF3a.

Mammalian eEF2 and *S. cerevisiae* eEF2 are phosphorylated on Thr56 or Thr57, respectively, in response to stress. To investigate if fission yeast eEF2 undergoes post translational modifications, we resolved purified eEF2-HA on 2-D gels before and after stress and performed western blotting. Although stress introduced different spot migration patterns compared to untreated cells, no major difference could be seen between *sty1*, *mkp1*, or wt cells. The migration pattern of *S. pombe* eEF2 after stress resembles the ones seen of mammalian eEF2 after stress, but further experiments are required to identify the different eEF2 isoforms. To further investigate the role for Sty1MAPK pathway in protein synthesis, we performed polysomal profile analysis of *sty1, mkp1, mkp2, mkp1 mkp2* and *atf1* cells after osmotic shock, oxidative stress and nitrogen starvation. *sty1* cells were defective in translational recovery in all conditions tested compared to wt cells. *sty1* cells also seemed unable to re-initiate translation after being translationally arrested, seen by the loss of the ribosomal 80S peak. Deletion of Mkp1 or Mkp2 had little or no effect on protein translation. *atf1* cells were unable to recover from translational arrest only after osmotic shock which may explained by higher transcriptional redundancy after oxidative stress. It has been shown that

the transcriptional response to oxidative stress is dose dependent and involves different sets of transcription factors [43]. At low or moderate levels, (< 0.25 mM of H_2O_2); the H_2O_2-induced gene expression requires the transcription factor Pap1. Increased levels of H_2O_2 (> 1 mM), shifts the requirement from Pap1 to Atf1. At intermediate H_2O_2 levels either Pap1 or Atf1 can regulate transcription. Osmotic stress also requires Atf1 for stress induced transcription. It regulates the majority of KCl induced transcription as a heterodimer together with Pcr1 with a subset of genes solely dependent of Atf1. Specifically which Atf1-regulated genes support protein translation after osmotic stress but not after oxidative stress is not clear. One plausible explanation would be that Atf1 dependent transcription is necessary at the time of translational recovery in stress adaptation which may reflect the different polysomal characteristic induced by oxidative stress and osmotic stress. As seen by polysomal profiles, oxidative stress and osmotic stress are different in terms of translational recovery. KCl at a concentration of 0.6 M causes a transient drop in translational activity, but translation starts to recover between five and ten minutes. Oxidative stress produces a different translational response; polysomes gradually decrease and translation does not recover, at least within the timeframe of our experiment.

Cellular stress induces cytoplasmic RNA granules in fission yeast - Paper II

Processing bodies (PBs) and stress granules (SGs) have been well characterized in mammalian cells and in *S. cerevisiae,* but little is known about these structures in fission yeast. We wanted to investigate if fission yeast indeed form stress induced RNA granules, and if so, characterize granules in terms of basic composition and formation.

A number of markers for PBs and SGs are used to specifically visualize the respective granule type. In mammals, Pabp and translation initiation factors are often used as selective markers for SGs, while Dcp1 or Dcp2 are markers for PBs. In our study, we used GFP or RFP tagged versions of Pabp, Dcp2, eIF3a, eIF4G, and Csx1 (a protein sequence homolog of TIA-1) with Pabp as the main reference granule marker. All strains were observed under the microscope, untreated or treated with either 1 M KCl or with media without glucose. KCl at 1 M induced granular structures in all strains, reaching maximum intensity and quantity around 30 minutes. All proteins examined were cleared from granules by 60 minutes except Dcp2 which localized to granules even after 60 minutes. Glucose starvation induced granules positive for Pabp, Dcp2, eIF4G, and Csx1, but not for eIF3a. This time, granules were not cleared until glucose was re-added to the media. One explanation would be that KCl induces transient stress followed by an adaptation phase (production of osmolytes) whereas glucose withdrawal causes a permanent stress condition which does not cease until nutrients are available. Interestingly, polysomal profiles revealed that KCl causes translational arrest which remains even after Pabp-granules are dissolved with little or no translation re-initiation. This suggests that formation of RNA granules induced by hyperosmosis serves to protect mRNAs rather than to repress translation. Or, the bulk of Pabp associated mRNAs dissociates from Pabp-granules and gets degraded or stored in Dcp2 granules which can be seen even after 60 minutes of hyperosmosis. Moreover, Pabp co-localized with each protein investigated, but we were particular interested in the Pabp-Dcp2 co-localization given the differences between mammalian cells and yeast. Similar to what has been observed in *S. cerevisiae* [103], fission yeast Pabp co-localizes with Dcp2. Even though there was clear co-localization of Pabp and Dcp2, there was also separation of the two. Individual granules positive for either Pabp or Dcp2 could be seen which were more evident in KCl treated cells compared to glucose-starved cells. This is also seen in *S. cerevisiae*; in the course of time of glucose starvation there is a gradual separation between Dcp2 and Pabp which ultimately produces granules, postulated to be yeast SGs, consisting of Pabp, eIF4E and eIF4G as a minimum [103, 114].

A hallmark of regulation of SG formation in mammalian cells is the phosphorylation of eIF2α. To find out if fission yeast eIF2α has the same role as mammalian eIF2α, we proceeded to tag Pabp with RFP in a strain background expressing non-phosphorylatable eIF2α (*eIF2α- S52A*). In contrast to mammalian cells, phosphorylation of fission yeast eIF2α is dispensable for granule formation; there were no apparent defects in granule formation, but a slight delay in KCl-treated cells compared to wt cells. However, *eIF2α- S52A* mutants had a remarkably reduced ability to dissolve granules under hyperosmotic conditions, which implies that phosphorylation of eIF2α supports disassembly of granules more than being an obligatory event in granule formation in fission yeast. This notion is also reaffirmed in mammalian cells where the phosphomimetic eIF2α (S51D) is excluded from nascent SGs but is later on recruited to SGs in the recovery phase [111]. Glucose starved *eIF2α- S52A* cells behaved much like wt cells, which indicates that there is pathway separation between osmotic stress and nutrient stress induced granule assembly.

Fission yeast protein kinase A (Pka1) is involved in glucose signaling and is therefore a candidate for regulating granule formation. *pka1*Δ mutants were unable to form Pabp granules after glucose starvation, whereas Dcp2 granules still formed as in wt. Glucose starvation in wt cells caused an immediate shutdown of protein synthesis with no visible polysomes or monosomes indicating that ribosomes are fully dissociated and translation initiation is inhibited. By the same time, *pka1*Δ mutants had a substantial fraction of ribosomes still engaged in polysomes. To clarify if this was caused by failure to inhibit initiation or elongation defects in *pka1*Δ mutants, we measured the global protein synthesis. If translation initiation resumed without elongation being affected we would expect a clear difference between wt and *pka1*Δ mutants, where *pka1*Δ mutants still produce proteins. If elongation was impaired, it would result in ribosomes which are trapped in polysomes in an unproductive state. Notably, the global protein synthesis rate declined to similar extent in *pka1*Δ mutants compared to wt cells upon glucose withdrawal, which points to elongation defects. Likewise, in *S. cerevisiae* cells expressing the dominant-negative *rck2-kd* allele, ribosomes are trapped in unproductive complexes even if global protein synthesis is reduced [148]. A third possibility is that Pka1 regulates translation termination under conditions of stress. In *S. cerevisiae*, the termination factor Sup35 has been shown to be phosphorylated by Pka1 *in vitro* [149] but the biological relevance remains obscure.

Connections between Ca^{2+} signaling and glucose have been found in yeast [150, 151]. We wanted to explore if there was a functional link between Ca^{2+} signaling and granule formation in fission yeast. No effect was seen on cells subjected to 100 mM $CaCl_2$, but

glucose deprivation combined with 100 mM $CaCl_2$ clearly increased the number of granules compared to glucose starved cells alone. Exposing *pka1*Δ mutants to the same treatment did produce granules, but this was only seen after long incubations and the number of granules was considerably less than in wt cells, which again emphasizes the importance of Pka1 in glucose induced granule formation. We added the Ca^{2+} chelator EGTA to see if a reduction of the Ca^{2+} concentration would trigger granule formation. EGTA alone resulted in granules which were readily reversible by adding Ca^{2+} to EGTA treated cells. EGTA in combination with glucose starvation produced even more granules which possibly is an additive effect since both conditions trigger granule formation. *pka1*Δ mutants were resistant to EGTA treatment while *eIF2α- S52A* again was defective in granule disassembly.

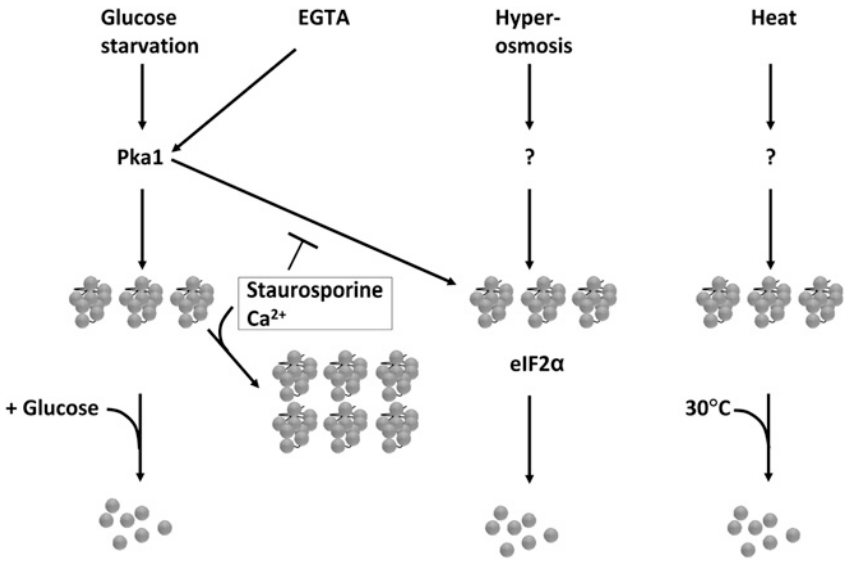

Figure 7. Pathways governing RNA granule assembly and disassembly in S. pombe. *Pka1 is required for RNA granule formation positive for Pabp after glucose starvation and EGTA. eIF2α is required for the disassembly of granules after hyperosmosis and EGTA. Staurosporine and calcium have an additive effect on granule formation after glucose starvation but inhibits EGTA induced RNA granules. It is unclear what controls RNA granule formation after hyperosmosis and heat.*

Impact of oxidative stress and the MAP kinase Sty1 on mRNA stability in S. pombe - Paper III

Different events of post-transcriptional regulation such as mRNA turnover and translation regulation account for significant variations in global protein abundance seen in yeast and mammalian cells which cannot be explained by mRNA transcription rate [152, 153]. The Sty1 SAPK/MAPK pathway has been implicated in post-transcriptional regulation in response to stress by influencing global translation, and mRNA decay after oxidative stress via the RBP Csx1.

To further investigate the role for Sty1 in mRNA stability after oxidative stress, we studied the mRNA decay rates after transcriptional shut-off by whole genome microarray analyses in wild type and *sty1* cells. To estimate the relative stabilities of mRNAs, we compared the levels of mRNA in unstressed cells and in cells at 0, 15 or 60 minutes of exposure to 1 mM H_2O_2 at 5, 15 and 60 minutes after transcriptional inhibition.

In unstressed wild type cells we found the profile of transcript stabilities to be similar to previous studies in *S. cerevisiae* [154]. 649 mRNAs were defined as the most stable and includes functional groups such as mRNAs coding for proteins involved in cellular metabolic processes, transcripts coding for oxidoreductases, ER to Golgi vesicle-mediated transport and mitochondrial transport.

528 mRNAs were defined as the least stable. In this group mRNAs coding for proteins involved in ribosome biogenesis and assembly corresponding to the previous identified “RiBi” regulon [155] were overrepresented concurrent with previous findings. Ribosome biogenesis accounts for more than 50 % of the total transcription in yeast and mammalian cells [156, 157] and is highly regulated, which may reflect the need for cells to have capacity to quickly redirect transcription and resources when needed for energy conserving purposes.

Among the 128 genes that had significantly increased stability after 15 minutes of H_2O_2 stress were genes involved in the cellular response to stress. Among these genes we find the mRNA of the stress induced transcription factor Atf1, known to be stabilized by the RBP Csx1 in response to oxidative stress, and glycerol-3-phosphate dehydrogenase Gpd2 which is implicated in stress survival. In addition we find genes that are involved in amino acid metabolism, above all cysteine and methionine, and genes involved in biosynthesis of lysine and aspartate. Biosynthesis of glutathione is tightly coupled to metabolism of sulfur-containing amino acids, cysteine and methionine. It is therefore not surprising that genes

involved in sulfur containing amino acids metabolism becomes stabilized as the biosynthesis of glutathione increases in response to oxidative conditions. The diaminopimelate pathway in yeast uses aspartate as substrate for lysine biosynthesis. Further, lysine residues become carbonylated under oxidative conditions [158]. It is thus reasonable that the stabilization of genes involved in biosynthesis of these amino acids reflects the requirement for cells to replenish the cellular pool of lysine after oxidative damage.

Although the steady state levels of the initial stabilized genes remained high, only 3% remained stabilized after 60 minutes of H_2O_2. One interpretation would be that the immediate stabilization of mRNAs we observe after 15 min of oxidative stress is shifted to transcription induction at the later timepoint similar to observations in *S. cerevisiae* [159].

Already before stress a substantial fraction of stress stabilized mRNAs in wild type cells are significantly stabilized in *sty1* cells. A possible explanation would be that *sty1*cells are already stressed caused by impairment of biological functions which is normally under the control of Sty1. *sty1* cells show severe defects in mRNA stabilization after oxidative stress, many of the stabilized mRNAs in wild type cells is abolished in *sty1* cells which further underlines the importance of Sty1 after stress. By comparing the stress-stabilized mRNAs to the Csx1 dependent increase of steady state levels after oxidative stress [87], we find that there is a strong correlation between Csx1 dependent mRNAs and mRNA stability after oxidative stress which is dependent on Sty1. In line with previous studies, this suggests that Sty1 and Csx1 coordinately regulate a subset of mRNAs after oxidative stress.

In addition, Sty1 seems to regulate the stability of transcripts in the “RiBi” regulon. In contrast to wild type cells, these transcripts are stabilized in *sty1* cells which indicates that Sty1 directly regulates the stability of these transcripts.

Acknowledgements

First, I would like to take the opportunity to say that I have really appreciated my years as a PhD student. Although it has been kind of an emotional roller coaster throwing you between a mental state of despair and euphoria, I managed to keep my sanity through the years (at least I think so). So, I would like to thank the people, former and present, at lundberg laboratory for giving me valuable scientific input, support and what's more important, friendship and laughs. I don't think I've had a dull moment! Well I've had, but the good spirit at the lundberg laboratory and all the friends I've made during the years have really made it easier to shape up in unfavorable conditions. I am sincerely going to miss you all when I eventually leave.

To my supervisor Per. Obviously, thank you for taking me on as a PhD student! You are a person with great knowledge, not just in science, but in many other areas as well. You have given me valuable feedback and encouragement in my research and just as important, you are really committed to your PhD students which I feel is equally important as scientific support.

Eva, thank you. To phrase you "we both know that I wouldn't have made it without you, practically, sanely or in such a good mood" I can only say the same!

I would also like to thank the other group members for all the help, support and company in the lab.

To my family. Well, none of you have really contributed scientifically but you are nonetheless all very important to me. Thank you for just being there!

My own little family, Eleine, Molly and Wilmer. I love you all very much and I enjoy every day I get to spend with you.

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