mRNA Degradation and Decay

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Chapter 7: mRNA degradation and decay

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Abstract

Why is it important to understand mRNA degradation in a cell ? First, RNA degradation has a clearing function and removes RNAs arising from transcription, splicing, export or translation "accidents" to ensure robust gene expression (see Chapter 8). Second, while regulation of gene expression has a very important transcription component, mRNAs must be turned over rapidly for fast changes in transcriptome composition. Coordinated destabilization of an entire class of mRNAs can promote major physiological changes in a cell. Third, specific mechanisms of mRNA decay can serve to regulate gene expression through feed-back control. Research on these topics has been frequently done first with yeasts and led to a better understanding of gene expression in eukaryotes. We start with an overview of the methods for measuring mRNA decay on a large scale with an emphasis on how technical issues affect the current picture of global mRNA decay in yeast. Next, we describe the importance of nuclear degradation in shaping the stable transcriptome. Once in the cytoplasm, mRNAs are exposed to translation and we provide an overview of the complexes and individual enzymes that ensure progressive deadenylation, mRNA decapping and 5' to 3' or 3' to 5' exonucleolytic RNA degradation. Finally, how organelle transcripts are degraded in mitochondria is briefly exposed.

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1 Introduction

As a preliminary to describing how RNA is degraded in yeasts, we will first define what messenger RNA (mRNA) is. Probably one of the simplest definition is based on the mRNA potential to code for a functional protein, unlike non-coding RNAs such as tRNA or rRNA that

are direct effectors in cellular machineries. To infer the protein coding potential and thus the inclusion of a transcript detected in a cell in the mRNA category, initial definition of open reading frames (ORFs) was done on the basis of length, starting at 100 codons. A powerful technique to go beyond such initial annotation is the comparison of genome sequence for many related species to identify conservation of predicted amino acid sequences (see, for example Dujon et al., 2004). The increase in the number of related genomic sequences leads to continuous improvements and reannotations of coding sequences in yeast (for an example of the evolution of *Saccharomyces cerevisiae* strain genomic sequence annotations over time, see Engel and Cherry, 2013).

A pragmatic definition of an mRNA is based on its association with translating ribosomes. Such association can be tested by several methods, including measurement of where an RNA sediments in a sucrose gradient (Arava et al., 2003), the extent of association with affinity purified ribosomes (Halbeisen et al., 2009), and the identification of ribosome-protected fragments (Ingolia et al., 2009). The final proof of coding potential and actual translation of an RNA is detection of the newly translated protein by mass spectrometry analysis (see, for example Menschaert et al., 2013). This operational view of an mRNA also includes transcripts that do not produce functional polypeptides.

Studies of the factors affecting RNA decay and degradation allowed the discovery of novel concepts in transcription, nuclear export, and protein synthesis. Since the RNA degradation factors can have an action that is limited to one or another of the different cellular compartments, our description will follow the cellular organization of yeast cells. Nuclear, cytoplasmic and mitochondrial processes will be described in separate sections.

2 Large scale methods for mRNA decay measurements

What is the average half-life of each mRNA species in a yeast cell ? In an era of deep RNA sequencing and refined methods for mRNA quantitation (see chapter 15), the answer to this question could look trivial, but it is not. We will thus first take a look at the results of several methods used for mRNA decay measurements genome-wide.

2.1 RNA labelling with modified nucleotides

It would be highly convenient to be able to obtain statistics on the stability of individual RNA molecules in individual cells, and then calculate average values for an entire population of molecules and cells. Following the decay of single RNA molecules is possible but not yet at a

large scale (Trcek et al., 2011). A traditional way for looking at molecules and their synthesis and decay uses radioactive labeling of living cells. A pulse of radioactively labeled compound that is incorporated in RNA can be used to follow the synthesis and, during a "chase" period, the disappearance of molecules over time. Such an approach has been instrumental, for example, in describing rRNA biogenesis intermediates in early studies done in the 70s (Trapman et al., 1975; Udem et al., 1971).

To establish the kinetics of synthesis and degradation of individual mRNA molecules in an organism, several criteria need to be met. First, labelling should not influence the process that is under study. This condition is very demanding since cell walls need to be removed or made permeable if labeled NTPs are to be incorporated. Second, labeled molecules should be easy to quantify and distinguish one from the other. Third, when cells grow fast, as many yeasts do, the dilution of the labeled molecule will impact the ability of the method to estimate long half-life values. Dilution through active dividing cells during the assay needs to be taken into account (see Pelechano et al., 2010). Last, the localization of the degradation processes can affect the half-life estimations.

One of the first attempts to use a pulse labeling method to estimate indirectly mRNA half-life has been described as a genomic run-on strategy (García-Martínez et al., 2004). The method was based on previously described run-on experiments for transcription rate estimations (Hirayoshi and Lis, 1999). Pulse labeling of newly synthesized RNA in yeast was done through a five-minute incubation with ^{33}P-UTP. Cells had been depleted of nucleotides and rendered permeable to UTP by detergent treatment (García-Martínez et al., 2004). Incorporation of UTP in specific RNA molecules was estimated by the use of nylon membranes on which PCR products encompassing annotated ORFs had been deposited. By a comparison between pulse labeling of RNA molecules and known steady-state levels of the corresponding mRNAs, estimates of half-life for thousands of yeast transcripts could be obtained. Enhancements and further corrections of the original data have been published a few years later (Pelechano et al., 2010). Nuclear degradation of some transcripts could affect the amount of newly synthesized RNA that reaches the cytoplasm (Gudipati et al., 2012). Thus, the stability of mature mRNA is likely to be underestimated by genomic run-on experiments. If a fraction of newly synthesized transcripts is degraded in the nucleus and another fraction in the cytoplasm, the kinetics of degradation could appear bimodal. Thus, real half-life values for cytoplasmic mRNAs could be longer than predicted from observed synthesis and steady-state levels.

Non-radioactive labeling of RNAs is a clever alternative to ^{33}P-UTP labeling. The incorporation

of 4-thio-UTP in newly synthesized RNA (Fig. 7.1A and 7.1B) provides "hooks" that allow their isolation through biotinylation and affinity purification (Cleary et al., 2005; Dölken et al., 2008). The 4-thio-UTP precursor 4-thiouridine does not penetrate readily in yeast cells but its entry can be enhanced by the expression of the human nucleoside transporter hENT1 (Miller et al., 2011). Further analysis of the enriched RNA is performed by sequencing or DNA microarrays. 4-thiouridine based methods are not devoid of problems. Two studies performed in two different laboratories (Munchel et al., 2011; Sun et al., 2012) have shown no correlation between the half-life estimates of yeast transcripts. Among several explanations for this lack of correlation, it is possible that the levels of 4-thiouridine used for pulse labelling RNAs were high enough to induce a stress response similar to the one observed in mammalian cells (Burger et al., 2013). Once the right conditions are set up, it is likely that 4-thiouridine labelling will turn out to be currently the most flexible method available for large scale mRNA decay studies.

2.2 General transcription inhibition for global mRNA decay tests

A straightforward and widely used method to assess mRNA stability is to follow what happens with an mRNA after transcription shut-down. Transcription inhibition is done either by using yeast strains with point mutations in an RNA polymerase II component (*rpb1-1*, Nonet et al., 1987) or by adding chemicals that are believed to specifically block RNA polymerase II, thiolutin (Jimenez et al., 1973) or ortho-phenantroline (Grigull et al., 2004) (Fig. 7.1C). An inherent problem of using a mutant strain defective for a major cellular pathway is that even at permissive temperature, transcription of the *rpb1-1* strain is reduced in comparison with a wild-type strain (Sun et al., 2012). It was even suggested that the measurements of half-life based on the study of such mutants are more likely to reflect changes in RNA stability due to heat shock. The very good correlation between the results of half-life estimates based on a temperature shift of an *rpb1-1* strain, or addition of thiolutin or phenantrolin (Grigull et al., 2004) indicate that these conditions affect transcripts stability by a common mechanism. Thiolutin treatment of the cells also inhibits mRNA degradation in a dose dependent manner (Pelechano and Pérez-Ortín, 2008), an effect that needs to be taken into account if estimates of half-life are done with this method.

For a more in-depth review on mRNA decay strategies, including a comparison with methods used for half-life estimations in other eukaryotes, see (Perez-Ortin et al., 2012). Half-life estimates of mRNA on a large scale need carefully crafted controls and independent methodological validation in different laboratories. Caution should be the rule when using any kind of half-life estimates for mRNA in yeast and probably in other species as well.

2.3 Linking mRNA degradation with global transcription changes

What knowledge can be gained from the study of mRNA half-life estimates and their changes in different environmental conditions? It was this type of studies that pioneered the idea that mRNA steady-state levels are a poor reflection of the relative role played by RNA degradation factors in cellular metabolism (Dori-Bachash et al., 2012; Shalem et al., 2008; Sun et al., 2012, 2013). Deleting genes for factors involved in mRNA degradation was accompanied by global transcription inhibition, leading to constant relative levels of mRNA in mutant strains. Contrary to the expected result, even if some classes of mRNA were affected differentially, the steady state levels of mRNA were little affected by the absence of any given RNA degrading protein. A notable exception is the 5' to 3' exonuclease Xrn1, a major degradation factor in yeast cells (Sun et al., 2013). Why Xrn1 has an effect that is different from other degradation factors on the relationship between transcription inhibition and mRNA decay remains unknown.

An explanation for how RNA degradation and synthesis could be linked comes from the observation that mRNAs for transcription repressors, like Nrg1, increase in degradation deficient strains (Sun et al., 2013). As a consequence, the steady-state level of the protein is likely to rapidly increase, which leads to general repression of transcription. *NRG1* mRNA is known to be under tight degradation control since its levels increase in mutants depleted for the major deadenylase Ccr4 (Lo et al., 2012). *NRG1* transcript levels also increase upon translation inhibition with cycloheximide (Sun et al., 2012). Thus, deletion of genes for RNA degradation factors could mimic physiological situations that modulate translation. The tight coupling between translation, translation regulation and mRNA degradation of transcription factors could ensure robust responses of yeast cells to environmental changes and stress. Transcription rate of thousands of genes can be affected by changes in translation or degradation of a few key mRNAs. As a consequence of yet unknown secondary effects, steady-state levels of transcripts are a poor predictor of the direct effect of a degradation factor or complex perturbation on RNA. Tests of direct binding of degradation factors to RNA, as explained in the following section, are likely to be more effective in finding physiological roles for proteins involved in RNA stability.

3 mRNA stability, associated proteins and the RNA operon concept

The first experimental observations that identified a potential role of RNA binding proteins as global gene expression regulators were made in human cells (Tenenbaum et al., 2000). Based on these data and on previous knowledge on the properties of RNA binding proteins and especially their ability to change mRNA turnover, Keene and Tenenbaum proposed the RNA operon

concept (Keene and Tenenbaum, 2002; reviewed in Keene, 2007), which states that a given protein can be part of a large number of mRNPs and can play key roles in affecting the turnover of a large number of mRNAs that code for functionally related factors. While these ideas started from work done with mammalian cells, an experimental confirmation of this idea came later from yeast experiments performed in Brown's laboratory. The development of affinity based purification of RNAs in association with specific proteins coupled with microarrays allowed some of the first large scale estimations of the complex picture of the mRNP world in yeast (Gerber et al., 2004; Hogan et al., 2008).

The best known cases of RNA operons involve proteins of the Puf family. Described initially in *Drosophila melanogaster* and *Caenorhabditis elegans*, there are six Puf proteins in *S. cerevisiae* (reviewed in Quenault et al., 2011; Wickens et al., 2002). The Puf proteins share eight repeats of the Puf motif that forms an arc like structure allowing interaction with both RNA and other protein cofactors. Subtle amino acid changes allow specific interaction with slightly different RNA motifs for each Puf protein (Qiu et al., 2012). While some Puf proteins overlap in their specificity of binding to 3' UTR regions of yeast transcripts (Puf1, Puf2 for membrane proteins mRNA), others are highly specific to particular mRNA classes (Gerber et al., 2004). Puf4 prefers mRNAs for ribosome biogenesis factors while Puf5 is mostly devoted to chromatin modification factors mRNAs.

Puf3 binds to many transcripts coding for proteins that are imported co-translationally into mitochondria. Their 5’ regions of translated polypeptides contain a sequence of 60 amino acids coding for a mitochondrial targeting sequence, which contributes to mRNA targeting to mitochondria. The Puf3 protein is required for the specific intracellular localization of one of two classes of mitochondria targeted transcripts (Saint-Georges et al., 2008). While more than 200 transcripts are localized to mitochondria in a Puf3 dependent manner and contain consensus binding sequences for Puf3 in their 3' UTR region, other mRNAs that are translated in the vicinity of the mitochondria are not affected by Puf3. Puf3 binding to mitochondrial transcripts contributes not only to their localization but also stimulates deadenylation and modulates the stability of the bound mRNA (Foat et al., 2005; Jackson et al., 2004; Olivas and Parker, 2000). An MFA2-COX17 chimeric transcript bearing the 3' UTR of *COX17*, coding for a mitochondrial protein, thus sees its half-life increased from 3 to 10 minutes in a *puf3*Δ strain. Whether Puf proteins directly activate deadenylation by interacting with Pop2 and recruiting the Pop2-Ccr4-Not complex (Goldstrohm et al., 2006, 2007), modify decapping or inhibit translation, the net effect of these mechanisms is a change in the stability of the class of mRNA to which these proteins bind.

The RNA operon concept is not limited to Puf proteins. Many other abundant RNA binding proteins potentially affect the stability of classes of mRNA. The formation of RNA protein complexes in the nucleus and the factors that affect mRNA export can influence whole classes of transcripts. Hrp1 (Nab4), an RNA biding shuttling protein that is required for mRNA export was found to strongly bind to transcripts encoding proteins involved in amino acid metabolism (Kim Guisbert et al., 2005). Changes of a single protein, like Hrp1, can thus affect the availability of a large number of mRNAs for cytoplasmic translation and their stability.

Analysis of mRNP composition by affinity purification of RNA bound to RNA binding proteins is not without technical biases and can lead to erroneous results. The use of porous agarose based affinity matrices leads to a heavily shifted representation of various classes of transcripts. Very large mRNPs are excluded from binding to chromatographic beads, unless those beads are small and compact and only allow affinity binding on their surface (Halbeisen et al., 2009). Fortunately, a more fine-grained image of mRNP composition became possible through the development of cross-linking and sequencing methods, best illustrated in yeast studies by the CRAC technique (Granneman et al., 2009), a variation of the widely used CLIP approach (Ule et al., 2003)(more details in chapter 14). Recent data on sets of RNA binding proteins and their RNA targets have further enriched our knowledge of mRNP composition at different stages of mRNA maturation or destruction (Klass et al., 2013; Mitchell et al., 2013; Tuck and Tollervey, 2013). Various methods have been used to analyse either the protein composition of poly(A) bound material (Garland et al., 2013; Mitchell et al., 2013) or the RNA composition of protein associated complexes stabilized through UV cross-linking (Tuck and Tollervey, 2013). These recent data are rich in information and are likely to vastly expand the knowledge on mRNA-protein interactions. Correlations between the various large-scale data sets are expected to bring more detail and mechanistic insights into which RNAs bind which proteins and in what physiological context these interactions are relevant.

4 Nuclear degradation of nascent mRNA

The mRNA cap and poly(A) tail are the most important determinants of mRNA stability. Uncapped nascent transcripts, intermediates in mRNA formation and even mRNA that are not efficiently exported from the nucleus are the subject of degradation mechanisms that are confined to the nucleus. In addition to nuclear quality control of RNA, co-transcriptional recruitment of RNA binding proteins can affect the cytoplasmic stability of the corresponding mRNA (Bregman et al., 2011; Trcek et al., 2011).

4.1 mRNA capping and nuclear quality-control of the 5′ end

mRNA capping is one of the first co-transcriptional modifications that occurs when nascent pre-mRNA reaches 22 to 25 nucleotides in length (in human cells, Moteki and Price, 2002). The capping complex containing RNA 5' triphosphase and guanyltransferase activities was first purified from vaccinia virus (Ensinger et al., 1975). Forty years later, mechanistic details about three successive steps are available (Fig. 7.2). Cet1, an RNA 5' triphosphatase, hydrolyses the gamma-phosphate from the 5' end of the nascent transcript. This step is followed by a transfer of GMP to the diphosphate 5' end by Ceg1, an RNA guanyltransferase. Finally, Abd1, an RNA N^7 Guanine methyltransferase transfers a methyl group on the guanine base at the N^7 position (reviewed in Shatkin and Manley, 2000). The Cet1/Ceg1 heterodimer interacts with the Ser5 phosphorylated form of the CTD of RNA polymerase II. Based on structural studies of the capping complex (Gu et al., 2010), it was proposed that Cet1/Ceg1 are recruited by the Ser5 phosphorylated CTD near the transcription start site. Recruitment of Abd1 is maximal at about 100 nt downstream transcription start and is almost concomitant with the binding of the cap binding complex (CBC). Both Abd1 and CBC are required for the recruitment of the kinases Ctk1 and Bur5, which release the capping enzymes and promote RNA polymerase II elongation (Lidschreiber et al., 2013). If the capping process is erroneous, the resulting nascent transcripts are degraded from the 5' end in a process involving the exonuclease Rat1 and the associated factor Rai1 (Jiao et al., 2010).

RAT1 was identified through a genetic screen for factors affecting RNA export from the nucleus (Amberg et al., 1992). The protein is a nuclear 5' to 3' exoribonuclease, which is similar in sequence with the cytoplasmic exonuclease Xrn1 and is partially functionally redundant in yeast (Johnson, 1997). Rat1 actively degrades RNA with a monophosphorylated 5'-end. Such an end can be generated by the decapping activity of Rai1, a Rat1 cofactor, that can remove the dinucleotide cap, especially if it is not methylated (Jiao et al., 2010) (Fig. 7.2). Completion of the capping reaction is affected under amino acid or glucose starvation, physiological conditions that might require rapid changes in mRNA transcription, export and degradation. Biochemical data also indicate that Rai1 stabilizes and stimulates Rat1 activity, which alone is relatively unstable (Xue et al., 2000), probably through the formation of heterodimers (Xiang et al., 2009). In addition to the involvement of Rat1 in the 5' to 3' degradation of partially capped or uncapped nascent transcripts, the enzyme also participates in transcription termination. Active degradation of RNA fragments synthesized by an RNA polymerase II after the cleavage step could serve in the removal of the polymerase from the DNA template ("torpedo" termination model, Luo et al., 2006; more details in chapter 1). The importance of the catalytic activity of Rat1 in this context

is not clear (Pearson and Moore, 2013).

4.2 Nuclear retention and transcript degradation

Recognition and degradation of aberrant or intermediate mRNA forms that fail to mature properly is essential for the control of gene expression. Both nuclear and cytoplasmic degradation mechanisms are required for quality control of RNA. The balance between degradation into the nucleus or cytoplasm depends on export or retention of RNAs. Unspliced pre-mRNAs that are exported to the cytoplasm are degraded by nonsense mediated mRNA decay (NMD) (see chapter 8), but aberrant pre-mRNAs retained in the nucleus are efficiently degraded. One of the first observations of nuclear retention of unspliced pre-mRNA was done with beta-galactosidase reporters designed to distinguish exported unspliced pre-mRNA from the spliced form (Legrain and Rosbash, 1989). The involvement of the exosome in the degradation of pre-mRNA in the nucleus and functional links between 3' to 5' intranuclear degradation of transcripts and splicing were demonstrated later on endogenous RNAs (Bousquet-Antonelli et al., 2000). In *Cryptococcus neoformans*, an organism in which all mRNAs are generated from spliced transcripts, splicing seems an absolute requirement for mRNA export. Transcripts without introns are retained and degraded in the nucleus in a process dependent on *C. neoformans* Pab2 (Goebels et al., 2013), a protein related to *Schizosaccharomyces pombe* Pab2 (Lemieux and Bachand, 2009) and to *S. cerevisiae* Sgn1 (of unknown function).

If nuclear degradation is not effective, the yeast cells are able to degrade pre-mRNAs in the cytoplasm through NMD. Studies of cells with defective or absent *RRP6*, a nuclear 3' to 5' exonuclease and *NAM7* (*UPF1*) a major NMD factor, indicate that nuclear exosome and cytoplasmic NMD pathways could act sequentially. Blocking RNA export in a *mex67* mutant strain leads to an accumulation of unspliced mRNA in the absence of *RRP6* (Sayani and Chanfreau, 2012). Some pre-mRNAs are degraded predominantly by nuclear mechanisms while others are exported and degraded in the cytoplasm.

4.3 Nuclear degradation from the 3' end: the exosome

The nuclear exosome is a conserved complex of proteins mentioned in the previous section as a key player in the 3’ to 5’ exonucleolytic degradation of RNA synthesized by all the eukaryotic RNA polymerases (tRNA, rRNA, nascent mRNA). The core exosome is present both in the nucleus and the cytoplasm and consists of nine subunits (Rrp4, Rrp40, Ski6/Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, Csl4 and Mtr3) with which Dis3/Rrp44 is strongly associated (Mitchell et

al., 1997). The nuclear and cytoplasmic forms of the exosome differ in their binding partners and post-translational modifications of the components. Phosphorylation of Csl4 at Ser94 was found to be specific for the nuclear version of the exosome, associated with Rrp6, and is less present in the cytoplasmic version, associated with Ski7 (Synowsky et al., 2009). While the core exosome components are related to RNAses, only Dis3/Rrp44 is an active enzyme (Dziembowski et al., 2007; Schneider et al., 2007) through two catalytic domains: one that belongs to the RNAse II 3' to 5' exonuclease family and a second, endonucleolytic PIN domain (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). In addition to Dis3, Rrp6 is a strictly nuclear exonuclease of the 5' to 3' DEDD family of RNases (reviewed in Arraiano et al., 2013) associated with the nuclear core exosome.

The nine subunits of the core exosome are essential for yeast viability and form a ring structure (Liu et al., 2006). Mutations occluding the ring channel inhibit endo- and exonucleolytic activities suggesting that the core exosome modulates Rrp6 and Dis3 activity (Wasmuth and Lima, 2012). Unwound RNA substrates enter into the internal chamber formed by the 9 inactive subunits organized in a barrel-like structure (Makino et al., 2013a) and progress through the chamber up to the catalytic site of Dis3, which interacts with the bottom of the core exosome. Rrp6 is associated with the side in proximity of the mRNA entry point in the barrel-like structure and its catalytic site is exposed at the surface of the enzyme. The unwinding activity for the entry of mRNA into the internal chamber of the exosome is probably provided by Mtr4, a helicase of the DExH family that is similar to Ski2, a cytoplasmic RNA helicase associated with the exosome (see Ski complex, section 5.2).

4.3.1 Mpp6 and Rrp47

Deletion of either *MPP6* or *RRP47/LRP1* has a negative effect on the growth of strains depleted for Rrp6 (Milligan et al., 2008 and CS, unpublished). Both proteins are also physically associated with Rrp6 and their function is partially redundant since deletion of the corresponding genes leads to a growth defect (Milligan et al., 2008). Rrp47 concomitantly interacts with Rrp6 and RNA through its N-terminal and C-terminal domains, respectively (Costello et al., 2011; Stead et al., 2007). In contrast to Mpp6, which recognizes pyrimidine-rich sequences, Rrp47 binds to structured RNA molecules suggesting that Rrp47 could promote the binding of Rrp6 to substrates (Butler and Mitchell, 2011 for review; Garland et al., 2013).

Mpp6 function and role in RNA degradation remain elusive. Deletion of the corresponding gene leads to growth defects specifically in association with concomitant deletion of genes for components of the TRAMP complex. Its association with Rrp6 in the nucleus as well as the

effects of its deletion, revealed that Mpp6 is involved in surveillance and degradation of nuclear pre-mRNAs and pre-rRNA (Milligan et al., 2008). It was proposed that Mpp6 could also promote the activity of Dis3 and be involved in the functional coupling between Rrp6 and the TRAMP complexes (for review, Butler and Mitchell, 2010).

4.3.2 TRAMP complexes

The nuclear exosome is helped by additional factors for degradation of its RNA substrates. A major functional nuclear co-factor of the exosome that adds poly(A) tails to nuclear RNA is the TRAMP complex. TRAMP complexes are formed of a poly(A) polymerase (Trf4 or Trf5), an RNA helicase of the DexH family (Mtr4) and of a Zn-knuckle RNA binding protein (Air1 or Air2) (LaCava et al., 2005; Vanácová et al., 2005; Wyers et al., 2005). At least two TRAMP complexes with different substrate specificity have been described. A major difference between these complexes is the Trf component, with Trf5-TRAMP most likely located to the nucleolus (Wery et al., 2009) and Trf4-TRAMP located into the nucleoplasm. A comparative transcriptome analysis of *trf4*Δ or *trf5*Δ mutants indicates that the two proteins affect the expression of distinct sets of genes (San Paolo et al., 2009). In addition, Air1 is mostly present in the Trf5-TRAMP complex while Air2 is mainly present in Trf4-TRAMP (reviewed in Houseley et al., 2006). These differences correlate well with recent RNA sequencing data obtained with *air1*Δ and *air2*Δ mutants, which revealed, as expected, a different global effect of each mutant on transcripts levels (Schmidt et al., 2012). A TRAMP complex would bind the RNA targets through its RNA-binding subunit Air1 or Air2 and add a poly(A) tail through its Trf4/5 polymerase subunit (Holub et al., 2012). The addition of poly(A) tails allows better access of the target to the nuclear exosome. The TRAMP complex is also able to enhance RNA degradation by Rrp6 independently of the presence of exosome *in vitro* (Callahan and Butler, 2010).

4.3.3 The Nrd1/Nab3/Sen1 complex (NNS)

Deciding whether an mRNA precursor will be stable until export to the cytoplasm or not was shown to depend in most instances on the way transcription by RNA polymerase II ends. Early transcription termination can occur by a pathway that is linked with nuclear processing or degradation of the corresponding RNA. Acting upstream of the nuclear exosome and TRAMP complexes, this pathway depends on a transcription termination complex and marks the corresponding RNAs as exosome targets (Fig. 7.3). The NNS complex is formed of Nrd1 and Nab3, two RNA binding proteins, which preferentially recognize short RNA motifs (such as

GUAA and UCUUG, Porrua et al., 2012) and Sen1, an RNA helicase. In contrast to the cleavage and polyadenylation complex that is required for transcription termination of most mRNAs (see chapter 3), the NNS complex is involved in transcriptional termination of cryptic unstable non-coding RNA (CUT) and of stable non-coding RNA like snoRNA and snRNA (reviewed in Jensen et al., 2013). Nrd1/Nab3 binding sites are underrepresented in mRNAs, which are thus less sensitive to the NNS-TRAMP-exosome dependent termination degradation pathway.

4.3.4 Nuclear mRNA degradation and regulation

NNS termination can play an important role in regulating mRNA levels. *RPL9B,* a gene coding for a ribosomal protein of the large 60S subunit has a choice between the two modes of transcriptional termination that depend on the level of nuclear Rpl9 protein. The protein binds a stem-loop located in the 3' UTR of the primary transcript of *RPL9B* and inhibits normal transcription termination. Alternative termination through NNS leads to efficient nuclear degradation of the transcript and effectively regulates mRNA levels for *RPL9B* (Gudipati et al., 2012).

NRD1 expression is regulated by premature transcription termination through the interaction of the Nrd1 protein with its own mRNA (Arigo et al., 2006). High-throughput analyses through cross-linking, protein purification and RNA sequencing revealed that Nrd1-Nab3 complexes are recruited during transcription of a large number of mRNAs, suggesting that the NNS complexes could be widely involved in mRNA down regulation (Schulz et al., 2013; Wlotzka et al., 2011).

Transcript retention or export play an important role in deciding which degradation pathway a given molecule will take. Polyadenylation of transcripts plays an important role in nuclear export. Co-transcriptional recruitment of Nab2, a nuclear poly(A) binding protein (Anderson et al., 1993) is one of the molecular events that shape the export competent mRNA protein complexes (Green et al., 2002; Hector et al., 2002). Nab2 interaction with Mex67 and Yra1, mRNA export factors, could ensure recruitment of the Mex67-Mtr2 complex (Iglesias et al., 2010), and further interactions with the nuclear pore proteins. In addition to a general role in mRNA export, Nab2 regulates its own expression levels by acting at the level of the 3' end formation of its own mRNA. This process depends on a genome encoded repeat of 26 adenosines found downstream the stop codon for *NAB2* (Roth et al., 2005). The autoregulation of *NAB2* depends on the encoded poly(A) sequence and also requires the nuclear exosome and TRAMP complexes (Roth et al., 2009). *NAB2* is thus an example of auto-regulation mechanism that uses nuclear degradation of a transcript to reduce gene expression.

5 Cytoplasmic mRNA degradation

Cytoplasmic mRNA decay occurs mainly from both the 5' and the 3' end, with little or no endonucleolysis occurring in yeast. Whatever the pathway, deadenylation is considered to be the first step in mRNA turnover. In 1975, Darnell's lab observed a correlation between poly(A) tail shortening and mRNA turn-over in HeLa cells (Sheiness et al., 1975). Using stable and especially the *MFA2* unstable mRNA, it was later established that deadenylation is the first step required for mRNA decay in *S. cerevisiae* (Muhlrad et al., 1994). Two complexes, Pan2/Pan3 and Ccr4/Not, are involved in the deadenylation process. Once deadenylated, mRNA is predominantly degraded by the 5' to 3' degradation pathway, which is initiated by the removal of the cap structure by the Dcp2/Dcp1 complex with the help of enhancers of decapping. After decapping, the 5' end of mRNA is accessible to the cytoplasmic exonuclease Xrn1, related to the nuclear exonuclease Rat1. Cytoplasmic mRNA degradation and translation are tightly related through translational repressors that also act as activators of decapping. After deadenylation, mRNA can also be degraded by the 3' to 5' pathway. This degradation is done by the cytoplasmic exosome with help from specific cytoplasmic cofactors, the Ski complex and Ski7. An overview of the cytoplasmic degradation pathways is presented in Fig. 7.4.

5.1 Cytoplasmic mRNA deadenylation

5.1.1 Poly(A) binding proteins Pab1 and Pub1

Pab1, for poly(A) binding protein, was first isolated and its gene identified by Sachs and Kornberg (Sachs et al., 1986). This protein binds specifically poly(A) through four RNA recognition motifs (RRM); however the first two RRMs are sufficient for most *in vivo* functions of the protein. A minimum of 12 adenosines is required for the association and multiple associations define a coverage unit of 27 adenosines (Sachs et al., 1987). One of the best characterized roles of Pab1, which decorates the 3' end of transcripts like beads on a string (Baer and Kornberg, 1980, 1983), is to assist translation initiation through specific interactions with the translation initiation factor eIF4G (Tif4631/Tif4632 in yeast). eIF4G binds the cap binding protein eIF4E (Cdc33 in *S. cerevisiae*). These interactions lead to the formation of a "closed loop" structure, which was proposed to promote translation initiation and ribosome recycling (Amrani et al., 2008; see chapter 5). Depletion of Pab1 leads to an inhibition of poly(A) tail shortening and has strong functional interactions with factors involved in 60S ribosomal subunit biogenesis (Sachs and Davis, 1989). Thus, paradoxically, Pab1 both protects the polyA from degradation and contributes to the controlled 3' end trimming, presumably by recruiting the

Pan2/3 complex (Mangus et al., 2004b) and by inhibiting the deadenylase activity of Ccr4/Not (Tucker et al., 2002).

In addition to Pab1 and Nab2, which are mainly cytoplasmic and nuclear respectively, Pub1 is a third polyA binding protein present in both cellular compartments but mainly located in the cytoplasm (Matunis et al., 1993). Pub1 also recognizes poly(U) stretches and interacts with 5' and 3' UTR regions in several mRNAs. In contrast to Pab1, Pub1 is not associated with translationally active mRNAs (Anderson et al., 1993b). Pub1 is abundant but not essential for growth of cells under laboratory conditions. The protein is involved in mRNA stability and translational control under environmental stress and colocalizes with Pab1, eIF4G1 and eIF4G2, *inter alia*, in granules in glucose deprivation stress and is required for the formation of these granules (Buchan et al., 2008). Of the three RRM motifs found in Pub1, at least one is required for an interaction with eIF4G. It was thus proposed that Pub1 could act cooperatively with Pab1 to simultaneously interact with eIF4G (Santiveri et al., 2011).

5.1.2 The Pan2/Pan3 deadenylase

PAN stands for poly(A) nuclease and corresponds to a cytoplasmic enzymatic activity that shortens poly(A) tails of yeast transcripts only in the presence of the poly(A) binding protein Pab1. The identification of the PAN complex components was the result of a purification procedure that enriched a Pab1 dependent deadenylase activity (Sachs and Deardorff, 1992). Initially, a co-purifying protein, called Pan1, was thought to be the enzyme required for the observed Mg^{2+} dependent exonuclease activity. However, an increase in the amounts of purified complex and further analyses around the role of Pan1 in the catalytic activity of the purified fraction have shown that the isolated deadenylase consisted of two subunits: Pan2, the enzyme (Boeck et al., 1996) and Pan3, a cofactor (Brown et al., 1996). While the catalytic center belongs to Pan2, Pan3 is required to an equal extent for the deadenylase activity of the complex. The Sachs group, involved in the initial identification of the Pan proteins demonstrated later that the Pan2/Pan3 complex plays a role in the modulation of poly(A) tail length distribution in yeast (Brown and Sachs, 1998).

Pan2 is a 3' to 5' exonuclease of the DEDD family (ribonucleases reviewed in Arraiano et al., 2013) that slowly removes 5' AMP from the 3' end of a poly(A) tail only in the presence of Pab1 but will not proceed to lengths inferior to 20 nucleotides *in vitro* (Lowell et al., 1992). The enzyme generates *in vitro* the entire range of oligo(A) intermediates, showing a distributive mode of action. The *in vivo* role of Pan2/Pan3 in deadenylation of mRNA remains poorly characterized.

5.1.3 The Ccr4/Not deadenylase

Deadenylation of mRNA in the cytoplasm requires the Ccr4/Not complex, also called the major mRNA deadenylase complex. Ccr4/Not complex is formed of nine subunits (Not1, Not2, Not3, Not4, Not5, Ccr4, Pop2, Caf40 and Caf130). Not1 is the scaffold protein on which various partners bind: the C-terminal part recruits Not2, Not3, Not4 and Not5 (the Not module) and the N-terminal part recruits Ccr4 and Pop2 (the nuclease module) (Basquin et al., 2012). Ccr4 bears the main catalytic activity (Tucker et al., 2002) while Pop2 contains a RNAse D domain and has an exonuclease activity *in vitro* (Daugeron et al., 2001). To what extent Ccr4 and Pop2 cooperate in the deadenylation process remains unclear. The deletion of the other genes of the complex only weakly slows down deadenylation of a model substrate. It was proposed that the Not module could adapt the deadenylase complex to mRNA according to the cellular context. To gain a better understanding of the role of each component of the Not module, genome wide analyses using deletion mutants suggested that the Ccr4/Not complex is involved in a number of other cellular functions (for review, see Collart and Panasenko, 2012).

The Pan2/3 and Ccr4/Not complexes in association with Pab1 are involved in 3' end deadenylation. It is believed that Pan2/3 acts first to shorten the poly(A) of newly synthesized mRNA. Next, it is the Ccr4 complex that deadenylates mRNAs until the tail reaches a length of 10 to 12 residues, and can be bound by the Lsm complex and Pat1 that trigger mRNA decapping (for a review, Parker, 2012).

5.2 Cytoplasmic degradation from the 3' end: the exosome with Ski

The cytoplasmic exosome contains the same factors as the nuclear version but associates with the cytoplasmic Ski complex and Ski7, instead of nuclear Rrp6 or the TRAMP complex. The Ski complex is formed of three proteins and was initially identified for its role in the degradation of viral RNA in yeast. Yeasts deficient for *SKI* genes have a super-killer phenotype because a virally encoded toxin is expressed at higher levels and kills neighbouring susceptible yeasts (Toh-E et al., 1978; Widner and Wickner, 1993). While Ski2 is an RNA helicase related to nuclear Mtr4, Ski3 and Ski8 contain structural motifs that allow protein-protein interactions; tetratricopeptide repeats (TPR) for Ski3 and WD40 repeats for Ski8. Native mass spectrometry experiments showed that the Ski complex is a hetero-tetramer composed of two molecules of Ski8 and one copy of Ski2 and Ski3 (Synowsky and Heck, 2008). Recently, the crystal structure of the complex revealed its organization in which Ski3 plays a scaffold role. RNase protection assays on RNA in presence of exosomes with or without Ski7 and the Ski complex revealed RNA fragments of 43-44 nt or 31-33 nt respectively, indicating that fragments of RNA are

protected in the exosome channel (Halbach et al., 2013). Interestingly, the large size of the protected fragments was compatible with the sum of the size of the channel of the Ski complex and the exosome channel suggesting that the two complexes are stacked in close proximity. Therefore, the structural organization of the exosome and Ski complex bears similarities with the proteasome structure. The Ski complex would play the role of the regulatory 19S proteasome subunit and the core exosome would be structurally similar to the 20S proteasome (Makino et al., 2013b).

The role of Ski7, which strongly associates with the cytoplasmic exosome remains elusive. No structural data exists on Ski7 but an association of the protein with the Ski complex (Araki et al., 2001; Wang et al., 2005) and with the Csl4 subunit of the exosome (Schaeffer et al., 2009) were shown. Ski7 homologues cannot be found outside yeasts in contrast to the components of the Ski complex and exosome, which are highly conserved in eukaryotes. It has been proposed that yeast Ski7 resulted from divergent evolution of a duplicated ancestral Ski7/Hbs1 gene (Marshall et al., 2013). Hbs1 is a conserved protein related to translation termination factors and involved in solving the problem of ribosomes stalled on mRNA. Subfunctionalization of Hbs1 and Ski7 was potentially influenced by the requirement for maintaining yeast viral systems that have no equivalent in other eukaryotes (see Drinnenberg et al., 2011).

In contrast to *S. cerevisiae*, where only one version of *DIS3* exists, *S. pombe* cells contain a related enzyme called *DIS3L2*. This exonuclease is cytoplasmic, shows preference for poly(U) tailed transcripts and does not require the rest of the exosome subunits for its activity (Malecki et al., 2013). Poly-urydilated substrates in *S. pombe* could results from the action of Cid1, an enzyme related to Trf4 and Trf5 (Wang et al., 2000). Cid1 is cytoplasmic and shows both a poly(A) polymerase activity and a significant poly(U) polymerase activity on a model RNA substrate *in vitro* (Read et al., 2002). Uridylation of substrates does not require prior deadenylation and facilitates the binding of the Pat1/Lsm complex for decapping and mRNA degradation (Rissland and Norbury, 2009). The specific role of Dis3l2 and the number and importance of substrates that are poly-uridylated for degradation in *S. pombe* remains to be investigated.

5.3 Cytoplasmic degradation from the 5' end

5.3.1 The cytoplasmic decapping enzyme (Dcp1-Dcp2)

In 1976, J. Warner's laboratory published the observation that yeast mRNA contains a modified 5' end with either m7G(5')pppAp or m7G(5')pppGp (Sripati et al., 1976). A correlation between

the 5' end structure and mRNA stability was later described by experiments with viral mRNA injected into *X. laevi*s oocytes or incubated with cellular extracts from mouse cells (Furuichi et al., 1977). An important conclusion of this study was that capped mRNAs were not degraded from 5' to 3' and their stability was independent of translation. In 1980, a decapping activity was detected from a high concentration salt wash ribosomal fraction by Audrey Stevens who purified a decapping enzyme from *S.cerevisiae* (Stevens, 1980, 1988). A decade later, the first decapping enzyme gene, *DCP1* was identified by Parker's laboratory (Beelman et al., 1996). Genetic screens using a *dcp1-2 ski8*Δ strain, allowed the isolation of a second decapping gene, *DCP2* (Dunckley and Parker, 1999). Human Dcp2 was shown to bear the decapping catalytic activity (van Dijk et al., 2002) and in yeast it was also shown that Dcp1 plays the role of an auxilliary, albeit important, factor for the Dcp1/2 heterodimer (Steiger et al., 2003). The binding of Dcp1 to the N-terminal domain of Dcp2 was later shown to promote the catalytic activity without affecting the binding of RNA to the C-terminal Nudix domain of Dcp2 (Deshmukh et al., 2008).

Dcp2 is not the only decapping enzyme in yeast. Dxo1, sharing a weak homology with Rai1 was identified as a novel decapping enzyme, which also has a 5' to 3' exonuclease activity (Chang et al., 2012). Global GFP-fusion protein localization indicates that Dxo1 is mainly present in the cytoplasm. However, additional studies are required to determine whether Dxo1 is strictly cytoplasmic or could be also involved in nuclear decapping processes.

5.3.2 Activators of decapping: Edc1, Edc2, Edc3

Two regulators or "enhancers" of decapping, Edc1 and Edc2 were isolated from a genetic screen looking for genes whose overexpression could restore the viability of a *dcp1-2 ski8*Δ strain (Dunckley et al., 2001). These proteins bind RNA and directly interact with Dcp1 *via* their proline-rich regions to stimulate the activity of the decapping enzyme (Schwartz et al., 2003; Borja et al., 2011).

Edc3 was originally selected in two-hybrid screens as physical partners of Lsm proteins, Dcp2 and Xrn1 suggesting that the protein could play a role in mRNA decay (Fromont-Racine et al., 2000). Unlike Edc1 and Edc2, Edc3 is a conserved protein in most eukaryotes and its sequence contains an Sm-domain (Albrecht and Lengauer, 2004). In the absence of *EDC3,* Dcp1 and Dcp2 defective strains show lower decapping levels (Kshirsagar and Parker, 2004). These effects of Edc3 can be explained by its direct interaction with Dcp2 (Harigaya et al., 2010; Nissan et al., 2010).

While Edc3 participates in general decapping, it has an essential role in specific degradation mechanism that ensure autoregulation of *RPS28B* and *YRA1* expression. The presence of a conserved stem-loop structure in the long 3' UTR of the transcript for the ribosomal protein Rps28b triggers rapid mRNA decay through a mechanism that bypasses deadenylation and directly activates decapping (Badis et al., 2004). *YRA1* auto-regulation occurs by a mechanism in which the protein Yra1 acts to inhibit its own pre-mRNA splicing. The degradation of the unspliced precursor is stimulated in the presence of Edc3 (Dong et al., 2007). Initial deadenylation is thus not an absolute pre-requisite for decapping activation, at least in a few known cases.

5.3.3 The Lsm complex – RNA chaperone and decapping activator

The Lsm proteins are characterized by the presence of two Sm motifs that are similar to protein sequences found in snRNP particles components. A search for like-Sm domains identified a family of related Lsm proteins (Fromont-Racine et al., 1997; Salgado-Garrido et al., 1999). Two-hybrid screens with each Lsm protein revealed that they were highly connected to each other. These screens also indicated potential roles for the Lsm proteins in two different processes, the nuclear splicing pathway and cytoplasmic mRNA degradation involving Dcp1, Dcp2, Pat1, Xrn1 and Yel015 later known as Edc3/Lsm16 (Fromont-Racine et al., 2000). Lsm1 was shown to facilitate mRNA decapping (Boeck et al., 1998). Affinity purifications and functional experiments demonstrated that the Lsm1-7 cytoplasmic complex was involved in mRNA degradation whereas the Lsm2-8 nuclear complex was involved in splicing. In *lsm* mutant strains, lsm1 to lsm7, mRNAs are still capped but their 3’ ends are oligoadenylated (10 to 12 residues) indicating that the Lsm complex was required for mRNA decapping (Bouveret et al., 2000; Tharun and Parker, 2001; Tharun et al., 2000).

The Lsm1-7 and Lsm2-8 complexes form ring-shaped heptameric assemblies, which directly bind to the 3’ end of mRNAs. *In vitro* experiments revealed that Lsm complexes preferentially bind oligoadenylated rather than polyadenylated mRNA and that the presence of a U-rich stretch of nucleotides near the 3’ end facilitates the binding (Chowdhury et al., 2007). Binding of Lsm1-7 to the RNA protects the 3’ end of an mRNA from the degradation by the exosome (Chowdhury et al., 2007; He and Parker, 2001). Therefore Lsm complexes could have a role as both enhancers of decapping and protectors of oligoadenylated 3’ end trimming. Unlike many of the factors described here that are specific for eukaryotes, Lsms have equivalents in prokaryotes: the Hfq proteins. Hfq form multimeric rings that bind and affect the function and stability of many non-coding regulatory RNAs in bacteria (for review, see De Lay et al., 2013).

5.3.4 Pat1, Dhh1 and Scd6 - linking translation and mRNA decay

Translation and mRNA decay are tightly linked through the action of proteins that have roles in both processes. Three such proteins stand out: Pat1, Dhh1, a DEAD box helicase and Scd6 that is endowed with an Sm domain variant and was also called Lsm13 (Albrecht and Lengauer, 2004). These proteins affect both mRNA degradation and translational repression (Coller and Parker, 2005). Furthermore, these factors as well as most of the factors involved in mRNA decapping co-localise with translation repression proteins in P bodies (for review, see Eulalio et al., 2007a).

Pat1 is a key player in mRNA degradation because it serves as a scaffold for decapping and activator of decapping components (Nissan et al., 2010). The N-terminal part of the protein interacts with Dhh1 whereas the middle and C-terminal domains interact with the Lsm complex, Dcp1/2, Xrn1 and the Ccr4/Not complex. Pat1 was originally identified as interacting with all the Lsm proteins in two-hybrid screens (Fromont-Racine et al., 2000). Surprisingly, the binding of Pat1 (via the C-terminal part of Pat1) to the cytoplasmic Lsm complex is not provided by Lsm1, which is the specific cytoplasmic subunit, but by Lsm2 and Lsm3, as shown by recent structural work (Sharif and Conti, 2013; Wu et al., 2013). Mutations in the C-terminal domain of Pat1 or in the C-terminal helix of Lsm2 or in the N-terminal helix of Lsm3, which impede the interactions between Pat1 and the Lsm complex affect mRNA decapping and 3' to 5' degradation (Wu et al., 2013).

The DEAD box helicase Dhh1 functions in mRNA decapping and interacts with both decapping and deadenylase complexes (Coller et al., 2001). Dhh1 binds to RNA and also associates with Edc3 or the N-terminal part of Pat1. There is a competition between Pat1 and Edc3 for binding to Dhh1, which interferes with the RNA binding capacity of Dhh1. Competition for binding could thus lead to variable remodelling of the corresponding mRNP (Sharif et al., 2013). It was proposed that Dhh1 is especially present on slowly translated mRNA molecules and that slow translation could favour mRNA decapping (Sweet et al., 2012).

In addition to its Sm domain, Scd6 contains an RGG motif that allows its association with the eIF4G subunit of eIF4F translation initiation complex and participates to translation repression. Edc3 and Scd6 compete through their Lsm motifs for the same HLM domains of Dcp2 (Fromm et al., 2012). *In vitro* decapping assays revealed that both Edc3 and Scd6 are able to stimulate Dcp1/Dcp2 decapping activity. However, Scd6 has a relatively low affinity for Dcp2 and Scd6-mediated activation is weak. Moreover, since Dcp2 contains several HLM motifs, it can bind concomitantly to different partners and it is not clear in which physiologically relevant

conditions the competition between different binders that can affect decapping is important.

5.3.5 Additional factors in mRNA decay: Pbp1, Pbp4 and Lsm12

Pbp1, Pab1 binding protein 1, was identified through a two-hybrid screen in association with Pab1 (Mangus et al., 1998). The same group identified later Pbp4, a factor that interacts with Pbp1 (Mangus et al., 2004a). Together with Lsm12, identified through its Sm motif, Pbp1 and Pbp4 are part of the same complex that interacts with ribosomes (Fleischer et al., 2006). The absence of any of these three factors has a negative effect on growth of yeast strains that also contain a deletion of *EDC3* (Decourty et al., 2008). Moreover, these factors colocalize together with Pab1 in stress granules in glucose deprived cells (Shah et al., 2013). Pbp1 promotes the formation of stress granules that contain Lsm12 and Pbp4, whereas Pbp4 has no effect on the stress granules containing Lsm12 or Pbp1 (Swisher and Parker, 2010). Pbp1 was also proposed to be involved in the regulation of poly(A) length through its interaction with Pab1 that could inhibit the recruitment of the Pan2/3 complex (Mangus et al., 2004b). While the Pbp1/Pbp4/Lsm12 complex is functionally linked with mRNA decay, the cellular role of these proteins remains unclear.

5.4 The 5′ to 3′ degradation of unprotected RNA: Xrn1

Xrn1 is the major 5’ to 3’ exonuclease in the cytoplasm and hydrolyses RNA that starts with a 5' monophosphate. Pioneer work leading to the discovery and initial characterization of Xrn1 came mostly from A. Stevens laboratory. The existence of a 5' to 3' enzymatic activity in eukaryotic cells was first suspected at the end of the 1970s because it was observed that uncapped mRNA were hydrolysed by crude extracts or by purified enzymatic yeast fractions whereas capped RNA were not (Furuichi et al., 1977; Stevens, 1978). Ten years later, the gene coding for Xrn1 was cloned from yeast (Larimer and Stevens, 1990). While yeast cells could adapt to the absence of the gene, their growth rate was severely affected. It was later shown that the absence of Xrn1 leads to accumulation of poly(A) deficient mRNA that lack the cap structure (Hsu and Stevens, 1993). Moreover, looking at endogenous mRNA in pulse-chase experiments highlighted a decrease of the mRNA turnover rate in *xrn1* mutants, which was the first evidence for a global role of Xrn1 in mRNA decay. The use of mRNA reporters containing a stretch of Gs (guanosine residues), that Xrn1 cannot degrade, allowed a description of RNA degradation species, leading to current models for mRNA decay (Muhlrad et al., 1994).

RNA sequencing performed with an *xrn1*-deficient strain identified a subgroup of non-coding transcripts, which were called XUT for Xrn1-sensitive unstable transcripts (van Dijk et al.,

2011), probably derived from pervasive transcription products that escape to the cytoplasm. In addition to a role of Xrn1 in mRNA turnover, the enzyme is thus also very important for its clearing function for cytoplasmic non-coding RNA.

Xrn1 is involved in lithium toxicity in yeast. It has been shown that lithium inhibits Hal2, an enzyme that converts adenosine 3',5' bisphosphate (pAp) into AMP. The accumulation of pAp inhibits Xrn1 and the RNase MRP (Dichtl et al., 1997). Whether these effects in yeast could have a counterpart in the mechanism of action of lithium salts, an effective treatment of psychiatric disorders in humans, remains unknown. However, lithium treatment of yeast cells is an effective tool for the study of otherwise unstable RNA species that are substrates of the ribonucleases.

5.5 Recycling of RNA degradation byproducts: Dcs1/Dcs2

When a capped RNA is degraded from 3' to 5', the last product of the enzymatic hydrolysis is the dinucleotide m7GpppN or oligonucleotides of the form m7GpppNNN. A "scavenger" mRNA decapping enzyme that can degrade these end products of mRNA decay, DcpS, was first identified in mammalian cells (Liu et al., 2002; Wang and Kiledjian, 2001). The enzyme contains a HIT motif (His-X-His-X-His-X, where X is a hydrophobic amino acid) essential for the cap hydrolysis activity. Recombinant DcpS is able to hydrolyse analogues of methylated cap or capped mRNA inferior in length to 10 nucleotides, suggesting that DcpS is unable to bind intact mRNA but degrade the residual cap structure after 3' to 5' exosome degradation. A sequence search in *S. cerevisiae* database identified two orthologues of human DcpS, Dcs1/Dcs2 (Liu et al., 2002). Despite the strong homology between Dcs1 and Dcs2, only Dcs1 seem to have a catalytic activity. Both proteins form a heterodimer and it was suggested that Dcs2 could be a modulator of Dcs1 activity (Malys et al., 2004). Deletion of Dcs1 leads to an accumulation of uncapped mRNA, which is the result of a decrease of 5' to 3' exonuclease activity showing that Dcs1/Dcs2 are not only involved in the clearance of the cap structure but also participate in the 5' to 3' mRNA decay by facilitating Xrn1 activity (Liu and Kiledjian, 2005). It has been recently shown by *in vitro* assays that Dcs1 directly activates Xrn1 and that Dcs1 is an *in vivo* cofactor of Xrn1 important for respiration in yeast (Sinturel et al., 2012).

5.6 P bodies are large aggregates of proteins involved in RNA decay

An estimated 15% of yeast proteins form aggregates in the cytoplasm of starved cells, as discovered from systematic analyses of GFP fusion yeast strain collections (Narayanaswamy et al., 2009; Noree et al., 2010; as reviewed by O'Connell et al., 2012). Many of the described

aggregation prone proteins are involved in glucose, amino acid or nucleotide metabolism. Proteins involved in mRNA decay, like the decapping enzyme Dcp2 and exonuclease Xrn1 had been also known for some time to form visible foci in the cytoplasm of mammalian (Bashkirov et al., 1997; van Dijk et al., 2002; Ingelfinger et al., 2002) and yeast cells (Sheth and Parker, 2003). Several experiments were used to propose that the Dcp/Xrn1 aggregates, called processing bodies or P bodies were sites where mRNA decapping and 5' to 3' degradation occurs (see also Cougot et al., 2004 for mammalian cells experiments). Treatment of cells with cycloheximide, which blocks translation, is followed by a redistribution of P body components, like Dcp1, to the entire cytoplasm (Sheth and Parker, 2003).

While P bodies could be places of mRNA degradation, the bulk of mRNA degradation was proposed to occur in polysome complexes (Hu et al., 2009). In addition, P body formation was found to be uncoupled from mRNA degradation both in yeast and in *D. melanogaster* cells (Decker et al., 2007; Eulalio et al., 2007b; Sweet et al., 2007). The number of proteins capable of entering P bodies has increased recently as a result of a systematic search for RNA binding proteins (Mitchell et al., 2013). The list of proteins present in distinct cytoplasmic foci when cells are deprived of glucose is long and includes: Dhh1, Eap1, Gis2, Hek2, Pat1, Pbp1, Pub1, Puf2-3-4-5, Sro9, Tif4631, Upf1, Upf3, Xrn1 among others. A historical perspective of P body discovery thoroughly describes both the complex protein composition of these aggregates and the relationship with other cytoplasmic large aggregates like stress granules (Jain and Parker, 2013).

The main difference between stress granules and P bodies is that they form as a results of a different stress and contain 40S ribosomal subunit components together with translation initiation factors. Due to this difference in composition, it was proposed that stress granules serve as sites of mRNA storage (reviewed in Yamasaki and Anderson, 2008).

P bodies could be a result of the propensity of RNA binding proteins to aggregate and their formation is increased by stress. It has been proposed recently that Pat1, one of the factors that could link mRNA translation and decay, could be the promoter of P body formation under glucose starvation (Ramachandran et al., 2011; Shah et al., 2013). Protein kinase A (PKA) signaling in yeast is important for the response of cells to glucose concentration. PKA is activated in response to glucose and leads to major changes in the yeast transcriptome (Zaman et al., 2009). Pat1 phosphorylation by PKA was shown to affect P body formation and point mutations of the phosphorylated residues influence the number of foci and their dynamics (Shah et al., 2013). At least another signaling pathway, involving protein kinases Pkh1/2 and Pkc1

affect P body formation and mRNA decay (Luo et al., 2011). The role of P bodies, how they form and the extent to which they serve as foci of RNA degradation or storage remains under very active investigation.

6 Mitochondrial mRNA degradation

A relatively small group of 19 protein-coding RNAs in *S. cerevisiae* are synthesized in mitochondria (Turk et al., 2013) and are subject to intra-organellar decay through mechanisms that are radically different from the nuclear derived mRNAs. Formation of mature mRNA sequences depends on processing of multigene transcripts. The mRNAs are not polyadenylated but can contain large untranslated regions. An interesting feature of mitochondrial mRNA 3' end is the presence of an A-rich sequence called dodecamer 5'-AAUAAUAUUCUU-3' that serves both as an endonucleolytic mark and as a protective sequence (Hofmann et al., 1993; Osinga et al., 1984).

mRNA degradation is mainly achieved by a complex named mitochondrial degradosome (mtEXO) that has two components: Dss1, a 3’ to 5’ exonuclease related to the RNAse II-like family (Dmochowska et al., 1995; Min et al., 1993) and Suv3, an ATP-dependent RNA helicase related to the Ski2 DExH/D superfamily (Stepien et al., 1992). Both components of the mtEXO are coded by the nuclear genome. The absence of Suv3 leads to a respiratory phenotype and to the accumulation of aberrant mitochondrial RNA. Point mutations in the gene for the mitochondrial RNA polymerase (Rpo41) or its essential cofactor Mtf1 partially restore the phenotype of a *suv3*Δ strain. It was proposed that maintenance of the balance between mRNA synthesis and degradation is essential for mitochondrial function (Rogowska et al., 2006). In contrast to the related bacterial degradosomes (reviewed in Bandyra et al., 2013), mtEXO degrades mRNAs that are not polyadenylated in *S. cerevisiae.*

Mitochondrial gene regulation seems to be highly different among eukaryotes. As an example, in *Arabidopsis thaliana*, mitochondrial mRNAs are polyadenylated and it was recently shown that two proteins regulate the poly(A) tail length. Interestingly, expression of the poly(A) polymerase *AGS1* from *A. thaliana* in yeast results in polyadenylation of the *COX3* mitochondrial mRNA, which is not normally polyadenylated (Hirayama et al., 2013). In *S. pombe*, the equivalent of mtEXO is composed of two proteins, Pah1 for the helicase and Par1 for the RNase, but RNA degradation is significantly different from *S. cerevisiae*. *SUV3* overexpression is able to restore the defect of *pah1*Δ whereas Dss1 is not functional in a *par1*Δ strain. In *S. pombe*, this complex was proposed to be mainly involved in 3’ end mRNA

maturation and was accordingly named a “processosome” (Hoffmann et al., 2008).

The existence of a 5’ to 3’ degradation pathway for mitochondrial mRNAs is still questionable. Since mitochondrial mRNAs are synthesized multigenic transcripts, their 5' end needs processing to become mature. In the absence of Pet127, precursor mRNAs accumulate. Moreover, when the 5’ ends of intermediate mRNAs are not protected they accumulate in *pet127*Δ mutant strain (Wiesenberger and Fox, 1997). These results suggest that processing and degradation could be coupled. Pet127 is a potential candidate for a 5’ to 3’ mRNA degradation factor, but there is no direct evidence of an enzymatic function of the protein, which does not possess any detectable RNase signature. However, overexpression of *PET127* can suppress the deletion of *SUV3* or *DSS1* suggesting that 5’ to 3’ and the 3’ to 5’ redundant pathways could exist in mitochondria (Wegierski et al., 1998).

7 Conclusion

While many factors involved in mRNA degradation and turnover are now known to exist, how they collaborate, on which substrates they act and under what environmental conditions, remain open questions. It will be interesting to see to what extent general models of mRNA decay proposed on the basis of studies of individual reporters will stand the test of time and the “storm” of large-scale data that are growing exponentially.

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9 Figure legends

Figure 1. Tools for large-scale analysis of mRNA turnover. **A.** Structure of the modified U base that can be used in pulse-chase experiments **B.** Incorporation of 4-thioU in RNA by yeast cells during a pulse period is followed by the purification of newly synthesized RNA at different time points of “chase”. The amounts of newly synthesized RNA are estimated through sequencing or DNA microarray analyses. **C.** General blockers of mRNA transcription, including a temperature

sensitive mutant of an RNA polymerase subunit and two toxic chemicals used for turnover estimations.

Figure 2. Formation and degradation of capped mRNA and *S. cerevisiae* enzymes involved in the process.

Figure 3. Nrd1-Nab3-Sen1 play an important role in an alternative transcription termination pathway that is linked with poly(A) addition by the TRAMP complex and degradation by the nuclear exosome. Such termination is most efficient on short transcripts.

Figure 4. Pathways for the cytoplasmic degradation of mRNA. Known functions and substrates of the different decay factors are discussed in the text.

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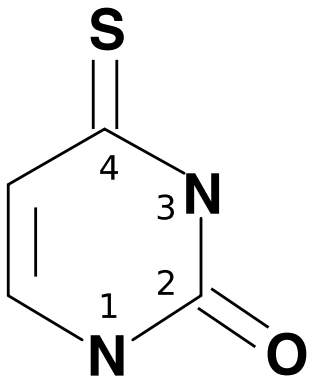
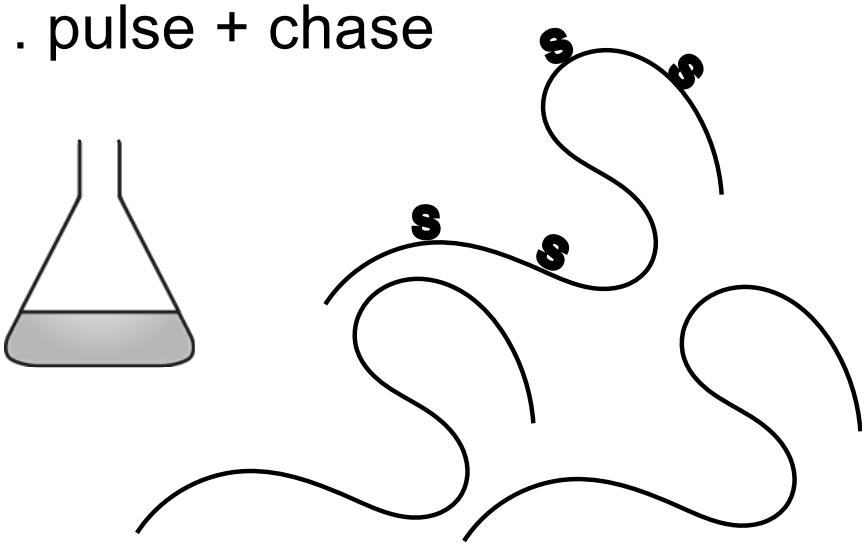
(a)



4-thio Uracil

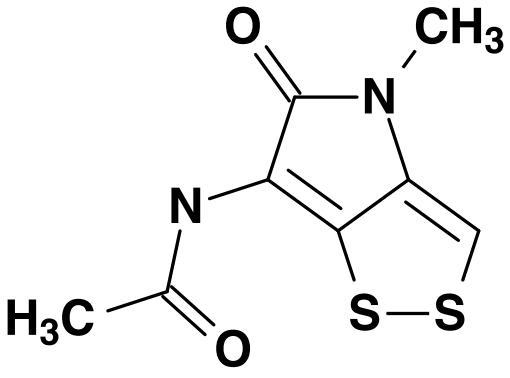
(b)

1. pulse + chase

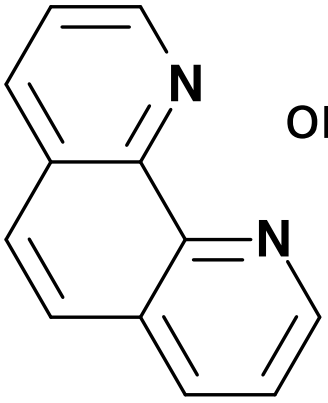


2. purification of «new» RNA
3. sequencing or microarray

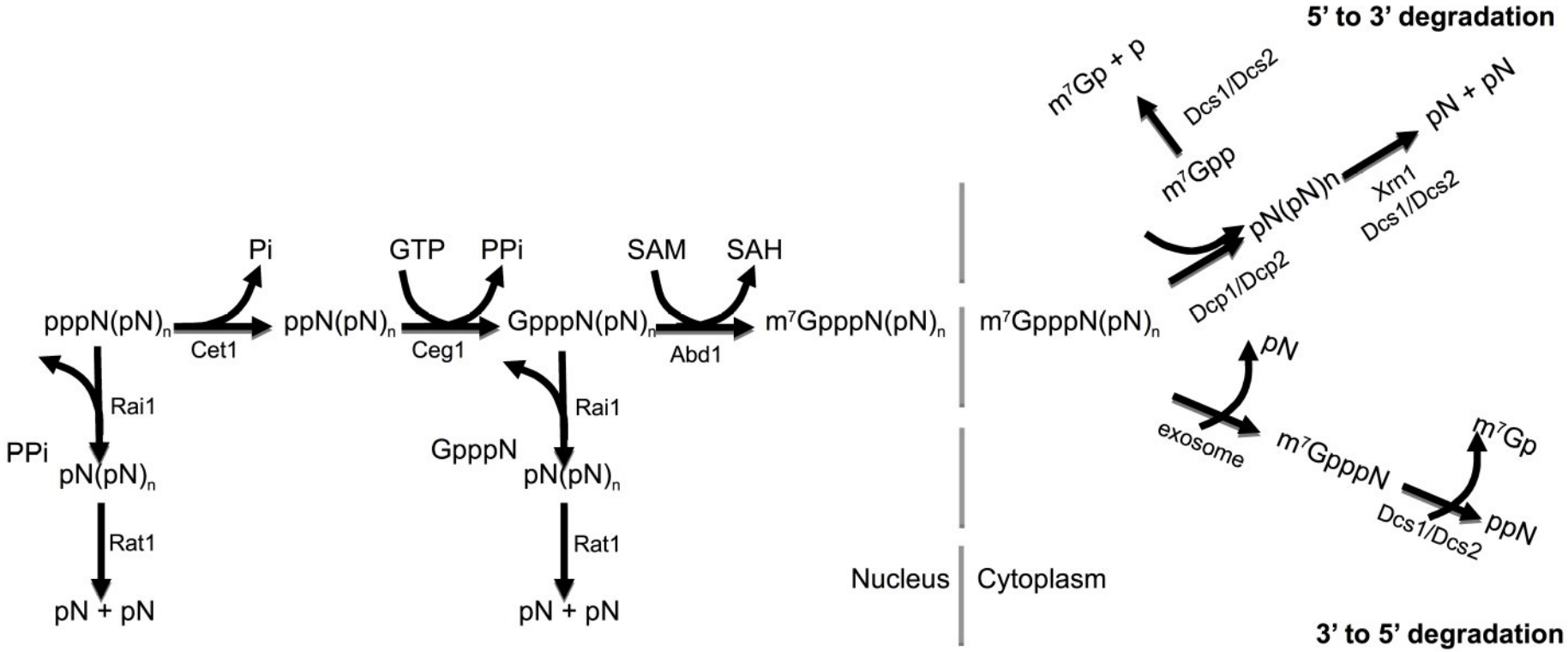
(c) ***rpb1-1 (36-39°C)***



Thiolutin



ortho (1, 10) - Phenantrolin


5' to 3' degradation
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m7Gpp
pN(pN)n
Xrn1
Dcs1/Dcs2
pN + pN
Dcp1/Dcp2
Pi
GTP
PPi
SAM
SAH
pppN(pN)n
Cet1
ppN(pN)n
Ceg1
GpppN(pN)n
Abd1
m7GpppN(pN)n
m7GpppN(pN)n
Rai1
PPi
pN(pN)n
Rat1
pN + pN
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GpppN
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Rat1
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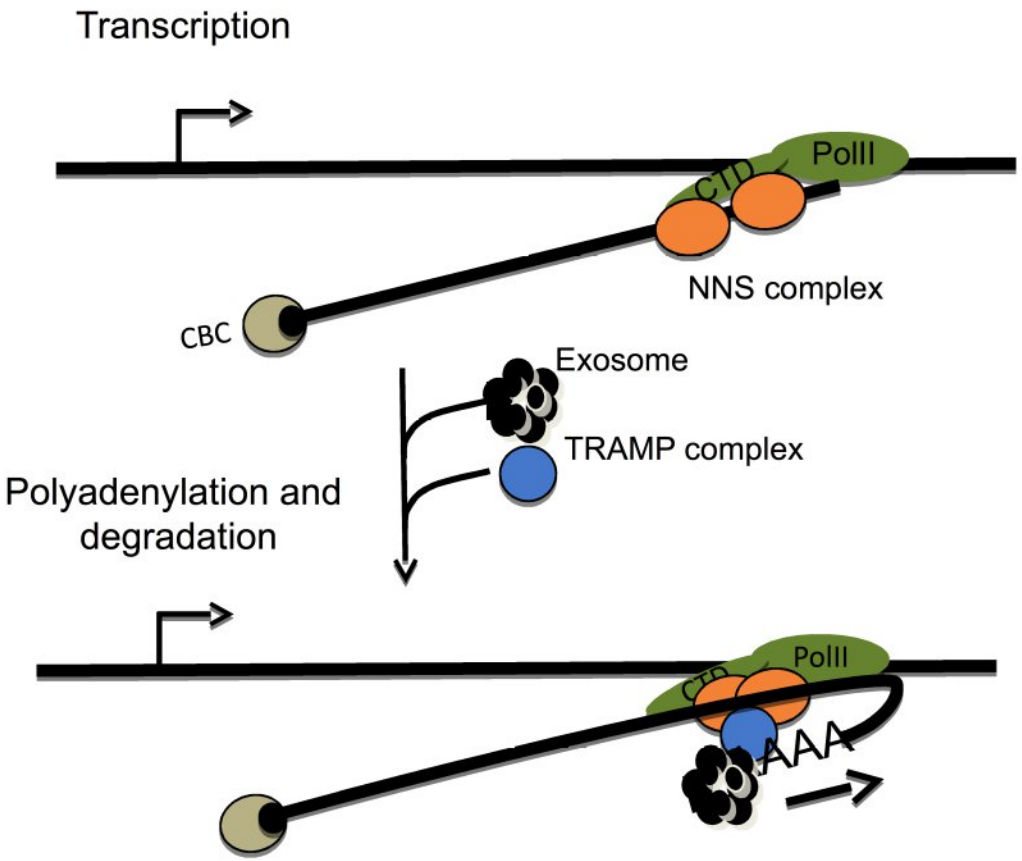
3' to 5' degradation


Transcription
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CTD
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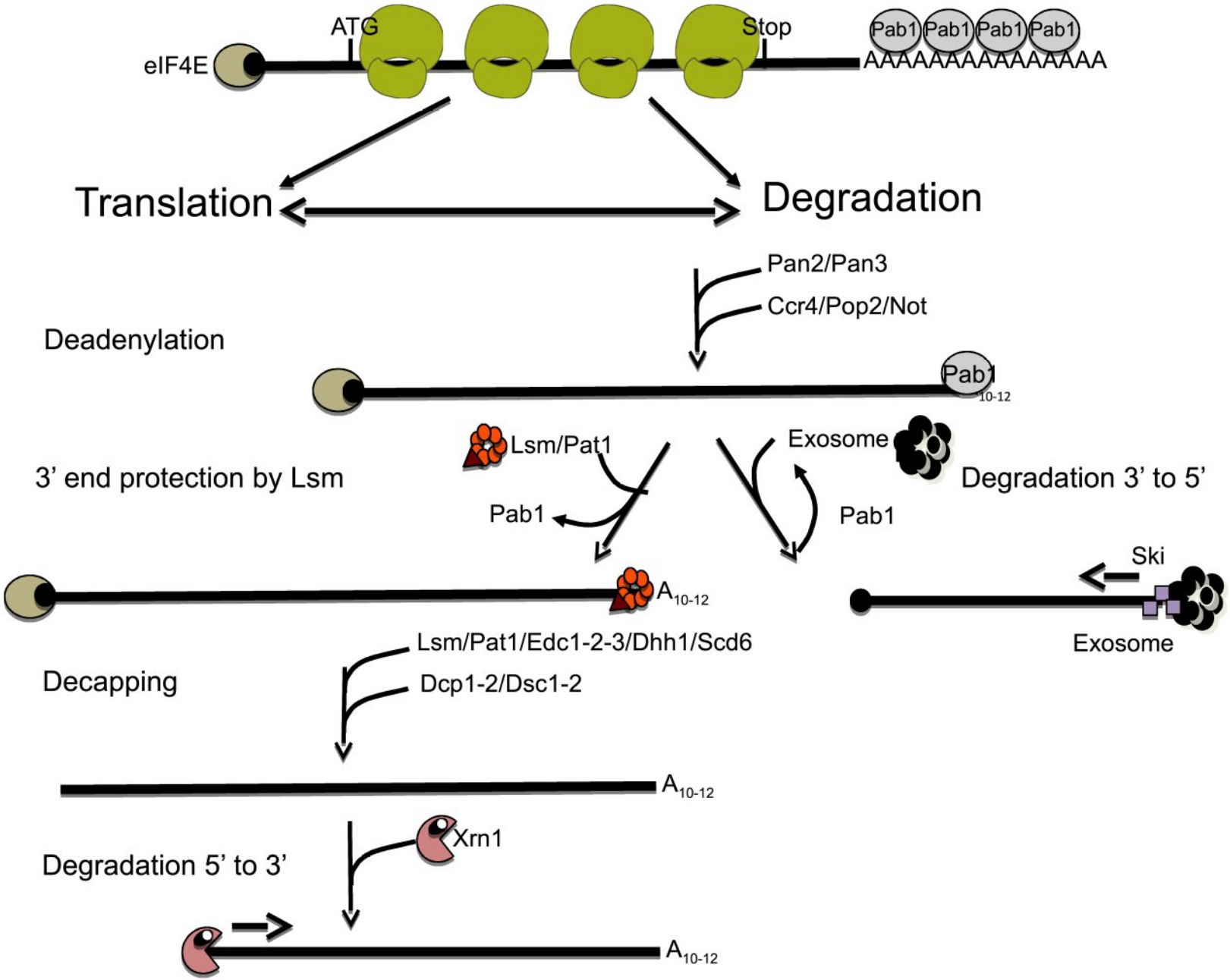
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Lsm/Pat1
Exosome
3' end protection by Lsm
Degradation 3' to 5'
Pab1
Pab1
Ski
A10-12
Exosome
Lsm/Pat1/Edc1-2-3/Dhh1/Scd6
Decapping
Dcp1-2/Dsc1-2
A10-12
Xrn1
Degradation 5' to 3'
A10-12