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Ways and Means of Eukaryotic mRNA Decay

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Abstract

Messenger RNA degradation is an important point of control for gene expression. Genome-wide studies on mRNA stability have demonstrated its importance in adaptation and stress response. Most of the key players in mRNA decay appear to have been identified. The study of these proteins brings insight into the mechanism of general and specific targeting of transcripts for degradation. Recruitment and assembly of mRNP complexes enhance and bring specificity to mRNA decay. mRNP complexes can form larger structures that have been found to be ubiquitous in nature. Discovery of P-Bodies, an archetype of these sort of aggregates, has generated interest in the question of where mRNA degrades. This is currently an open question under extensive investigation. This review will discuss in detail the recent developments in the regulation of mRNA decay focusing on yeast as a model system.

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1. **Introduction to mRNA decay**

Gene expression is a complicated multi-step process in eukaryotes that involves transcription, translation, mRNA and protein degradation. The predominant emphasis in gene expression has been on the role of transcriptional control for much of its history. Besides the early discovery of transcriptional regulation, it was clear from genome sequencing that transcriptional regulation was important since more than five percent of our genes were predicted to encode transcription factors [1]. mRNA is generally unstable and often associates with proteins to form heterogeneous mRNP complexes with diverse properties. These factors, along with the absence of effective ways to purify mRNPs, made it more challenging to study gene expression at the mRNA level [2]. Therefore, the perceived pre-eminence of transcriptional control coupled with the technical difficulty in studying mRNA stability slowed its recognition as a significant player in gene expression.

The relevance of mRNA stability in regulating steady-state mRNA levels and hence gene expression has been recognized for a while [3-5]. Pioneering attempts to understand the global decay rate of transcripts were made as early as the seventies and start of the eighties, which calculated the average half-life of mammalian mRNAs to be in the order of several hours [6,7]. Several messages with much shorter half-lives (less than two hours) were detected suggesting possible regulation at the level of mRNA decay.

This review will concentrate on the mechanisms of mRNA degradation and its importance in gene expression with an emphasis on the results obtained in yeast. Therefore, unless otherwise indicated, studies should be assumed to be performed on yeast or yeast proteins. We will discuss genome-wide studies and what they reveal about the ways mRNA decay affects how and when genes are expressed. While the genome-wide studies give a good picture of the global significance, they provide little insight into the actual mechanism of regulation of mRNA degradation. This understanding, rather, comes from studies on the proteins and mechanisms of the mRNA decay pathways in the last twenty years. We will predominantly use examples from the yeast system to illustrate the mRNA decay pathways

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and recent developments in the specific cellular localization of mRNA degradation.

1.1 Determination of global transcript stability

Accurate measurements of mRNA decay at a global level were made possible with the advent of microarray technology. Most of the early experiments used microarrays to assess global gene expression by measuring total cellular RNA at steady state. The balance between the rate at which it is produced and degraded determines the abundance of each mRNA in the cell. But in the absence of efficient means to quantify the global changes in mRNA decay and translation rates, total cellular RNA was often assumed to represent RNA production with mRNA degradation assumed to occur at a constant rate. More recently, a variety of experimental approaches have emerged that allow dissection of the contribution of mRNA degradation and transcription to gene expression.

Several genome-wide studies have attempted to measure global mRNA half-lives in several organisms from bacteria [8] to yeast [9,10], plants [11] and mammalian systems [12]. Three major strategies have been used to quantify the role of transcript stability in global gene expression:

1) Direct measurement of mRNA decay rates after inhibition of transcription.

2) Direct measurement of mRNA stability by *in vivo* labeling with modified uracil and pulse-chase experiments and

3) Indirect measurement of mRNA decay from measurements of total mRNA levels and transcription rate.

The first strategy is perhaps the most straightforward. Direct measurement of mRNA decay rates is a simple and elegant technique that was originally used to determine the half-lives of individual messages and later scaled up for global measurements. It is performed by inhibition of transcription using either chemical or genetic means followed by measurement of mRNA over a time course. However, there are several caveats to transcriptional shut-off experiments. The process of transcriptional shut-off is achieved by either a temperature shift

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or drug addition. This in itself is enough to change the cell physiology, create stress and alter the expression of several genes thereby resulting in large variability in measured half-lives [10]. Several physiological effects of the shut-off include: a) Reduction in the availability of labile mRNA decay machinery, which might affect the measurement of mRNA stability [13] b) Sudden increase in the abundance of stress response gene messages before the general shut-off, which interferes with the decay curve calculations especially for stress regulated messages [9,10] and c) At later time points where secondary effects play a major role in influencing the decay rates skewing the measured half-lives especially for long lived mRNAs [14].

In any time lapse experiment the half-lives are calculated from data over a course of time. Hence, the determined half-life is an average over a temporal window, concealing rapid fluctuations, especially for fast cellular responses. The number and choice of the collection time-points becomes crucial in determining the level of error in the calculation of half-lives. The fewer and further apart the time points, the higher the chances of error in obtaining the true half-lives especially with very short or very long-lived RNAs [9].

Furthermore, some genes have different mRNA half-lives depending on the stage of the cell cycle at which they are measured, for example for Histone [15], Topoisomerase II [16] and Cyclin [17] mRNAs. Half-life measurements are rarely performed on synchronized cell populations and hence do not factor in this effect.

A second strategy is a direct approach to measure mRNA decay under less perturbed conditions. Here, the mRNA in the cell is briefly pulsed with labeled uracil followed by a prolonged chase by addition of excess unmodified UTP. mRNA is then collected over a time course and subjected to microarray analysis or sequenced to measure mRNA degradation [18]. Although this method is less invasive than transcriptional shut-off, it is a more complicated method and still suffers from similar disadvantages because of the measurement of RNA at different time points over a course of time [14,19].

The third approach is more indirect but circumvents many difficulties by inferring decay rates indirectly from total cellular mRNA and transcription rates. This technique involves

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labeling the nascent mRNA followed by immunoprecipitation. The amount of total mRNA and transcription rates are then determined by microarray analyses or RNA sequencing. Since RNA sequencing is more sensitive than microarray technology, it reduces the time of pulse labeling, thus decreasing secondary effects. Assuming a steady state of mRNA (i.e. the transcription rate equals the decay rate), decay rates are then calculated from these values. Computational models provide a clearer picture of the contributions of RNA production and degradation to estimate changes in degradation rates between genes and over time [20,21]. This technique has the advantage of being able to conduct the experiments under *in vivo* conditions and not requiring a time lapse. A limitation to this method is that the mathematical calculation of decay rates assumes steady-state conditions for the mRNAs tested which is not often the case in the cell. More complex calculations are required to assume non-steady-state conditions, which results in experimental error being mathematically amplified (Reviewed in [22,23]).

One of the difficulties of the transcriptional rate analysis has been the method of labeling the mRNA to obtain a read-out of the transcriptional status of the cell. These data were originally obtained by nuclear run-on assays, which involved the isolation of nuclei and labeling nascent mRNA with radiolabeled nucleotides. This is a technically challenging experiment and the removal of nuclei could lead to experimental bias. Later developments allowed genomic run-ons on intact yeast cells using sarkosylation, however with the caveat of potential secondary effects on transcription [14].

Metabolic labeling of RNA using the naturally occurring nucleoside analog 4-thiouridine (4sU) is the least invasive technique currently available. 4sU is readily taken up by eukaryotic cells and incorporated into mRNA in place of uridine, is non-toxic, can be affinity purified, and does not affect transcription [14,24-27].

1.2 Importance of mRNA stability in gene expression: lessons from genome-wide studies

Data obtained from the global studies have led to several insights about mRNA decay and its role in gene expression. What is clear from these studies is that transcript turnover is

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an important point of control for overall gene expression. Several lines of evidence can be cited in support of this argument.

First, metabolic labeling and pulse-chase experiments show that protein and mRNA levels correlate and about 40% of the variation in protein levels is accounted for by changes at the mRNA level. The source of this can be mostly ascribed to changes in the mRNA transcription or decay rate. However, changes in transcription rates are not sufficient to explain the effect on mRNA abundances [20,21,28,29]. For example, in a study that used nuclear run-on analyses of nascent transcripts globally to monitor the effect of stimulation of T-cells resulted in more than one half of the genes showing changes in mRNA abundances while not having a corresponding change in transcription rate [30].

Second, mRNA half-lives show a wide distribution but mRNA stability is often conserved between cell types and species [14,18]. For example studies on murine fibroblast and human B cell lines have shown that half-lives of individual messages ranged from a few minutes to over 24 hrs but the median half-lives of mRNA calculated for both species were not significantly different from each other (274 minutes for murine fibroblast versus 315 for human B cells). Also the distribution of half lives appear to be similar. A comparison of more than 4500 transcripts between the human and mouse cell lines revealed that the half-lives of transcripts between the two species did not differ by more than two-fold [14].

Finally, it was also noted that clusters of functionally related genes showed coordinated changes in mRNA stability. Examples include immune regulators such as cytokine and chemokine mRNAs, histone mRNAs and ribosomal protein transcripts [9]. This suggests a hypothesis of regulation at mRNA decay levels referred to as mRNA-decay operons or regulons [31].

1.3 Decay appears to be important in stress response and regulation of transcriptional processes

It is clear that regulation of decay of transcripts plays an important role in the control of gene expression. Two logical questions that follow include: 1) Is this true for all transcripts or

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are some subsets of transcripts more likely to be regulated at the decay level and 2) Is mRNA decay regulation important at all times or only under certain conditions?

We can try to understand the physiological logic of mRNA decay by classification of genes by their gene ontology. Almost all of the studies so far have noted that constitutive genes involved in housekeeping processes, such as respiration and general metabolism have long half-lives. In contrast, regulatory genes, such as transcription factors, cell cycle processes, and mRNA binding and processing factors, have exceptionally short half-lives [9,14,20,21]. Such a relationship has been proposed from studies on individual mRNAs and this is supported by these genome-wide studies. It appears that mRNA degradation is tightly regulated and critical for regulatory genes.

Since regulatory genes are responsible for stress response, a plausible hypothesis is that transcript stability plays an important role in the adaptation of the cell to a new condition. Several global gene expression studies have been conducted under stress conditions and have hinted at the importance of transcript stability. For example, one of the studies, which examined the change in mRNA levels under different stress conditions, revealed that the genes transcript abundance changes were transient although large and constant changes were observed in transcription rates [32]. This is further supported by the observation that an almost global reduction in mRNA stability was detected within 15 minutes of mild osmotic stress for over 80% of the genes [33].

1.4 Transcription shapes the net adaptive response but mRNA decay is a modulator of the kinetics of the response

How does mRNA decay influence changes in gene expression? Stress response or adaptation to new conditions are often characterized by sudden, large, unidirectional changes in gene expression [33]. Thus, transcript stability could be important for any of the three factors: the direction of the change of mRNA levels (either an increase or decrease), the net change in gene expression, and/or the kinetics of the change (the speed of increase or decrease). We will describe four approaches to understand how transcript stability

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influences gene expression upon stress response: one theoretical, two experimental and one re-analyzing existing genome-wide data.

Pérez-Ortin and coworkers have predicted mathematically that the final mRNA concentration of the new steady state might depend primarily on the transcription rate, but mRNA stability is key for the time required to transition to a new mRNA expression level [34]. They analyzed the cellular cost and time required for the various strategies of adaptation to provide mathematical support to a role for degradation in gene expression. For example, for a five-fold increase of a gene, they enumerate a variety of mRNA half-life and transcription rate responses and their advantages to cell. These range from the rapid (and energetically expensive) solution of decreasing the mRNA half-life while increasing the transcription rate to the slow (and cheap) method of simply increasing mRNA half-life. The hypothesis put forth is that decay may not be important for the net change in gene expression, but it definitely plays a role in the kinetics of the response. The strongest experimental evidence for this hypothesis comes from three studies.

The Pilpel group used stresses with different kinetics of mRNA response to provide experimental evidence for this hypothesis [35]. In the case of DNA damage response where induction is slow, the cells use a slow but energetically cheap strategy of stabilizing induced genes. On the other hand, when a rapid response is required, such as in oxidative stress, an energetically expensive strategy of transcription induction coupled with mRNA destabilization is adopted.

Elkon et al (2010) analyzed published time course expression sets of inflammatory response in human and murine cells and noted a striking negative correlation between response time and mRNA stability [36]. In simpler terms, genes that show rapid induction (i.e. early induced genes such as Fos, Jun, Ier3) were characterized by very short mRNA half-lives [36].

Rabani et al (2011) reached similar conclusions from metabolic pulse labeling studies upon lipopolysaccharide stimulation of dendritic cells. They observed that changes in total RNA levels correlate with corresponding changes in transcription and only a minority of the

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genes (17%) showed significant degradation rate changes. These genes seemed to make the response "sharper" (by a very rapid induction and then shut-off) and included many immediate-early genes or "first responder genes" identified in Elkon et al (e.g., Fos, Jun, Egr1) [20].

In conclusion, it appears that transcription rather than stability changes may determine the direction of the net response but transcript stability regulation appears to be important for the acceleration or braking required for adaptation to new conditions.

2. Mechanisms of mRNA decay

2.1 mRNA Decay Pathways

Eukaryotic mRNA decay is primarily exonucleolytic, therefore the structures on the ends of the mRNA are important determinants of transcript stability. Eukaryotic mRNAs have a 5' N7-methyl guanosine cap (^{m7}G) and a 3' end poly(A) tail added to the pre-mRNA in the nucleus (see figure 1) [37]. Upon export of the mRNA to the cytoplasm, the 5'-^{m7}G-cap structure protects the mRNA from 5'-to-3' exonucleolytic degradation and allows activation of translation upon binding of the translational initiation complex [38]. The pathways for general cytoplasmic decay described in this section are depicted in figure 1.

Shortening of the 3' poly(A) tail (deadenylation) is the first and often rate-limiting step in the regulated mRNA decay pathways [39-42]. Two cytoplasmic deadenylase complexes contribute to the reduction in poly(A) tails [43]. The Pan2/Pan3 deadenylase complex may be responsible for the initial stages of deadenylation [44]. However, the most significant effect on mRNA half-life is observed with a large complex of proteins termed the Ccr4-Not complex. The Ccr4-Not complex consists of highly conserved exoribonucleases and adaptor proteins that hydrolyze the poly(A) tail progressively reducing its length from ~60 to ~10 nucleotides in yeast [40]. In this complex, both Pop2 and Ccr4 exhibit deadenylase activity *in vitro* [45,46],but *in vivo* catalytic activity resides with Ccr4 [46,47]. After deadenylation, the unprotected 3'-end of the mRNA can potentially be degraded in a 3'-to-5'

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direction catalyzed by the exosome. The exosome is a macromolecular protein complex consisting of 3'-to-5' exo- and endo-ribonucleases, with its activity attributed to the Rrp44/Dis3 subunit (Reviewed in [48]).

In yeast, however, deadenylation is primarily followed by the removal of the 5'-^{m7}G-cap (decapping) and decay in a 5'-to-3' direction promoted by the cytoplasmic exoribonuclease Xrn1 [39,42]. In the 5'-to-3' general mRNA decay pathway, the removal of the cap is a highly regulated step catalyzed by the decapping enzyme.

Dcp2, the catalytic subunit of the decapping enzyme, is a member of the Nudix (nucleotide diphosphates linked to other moieties, X) superfamily that hydrolyzes a wide range of substrates [49]. In isolation, Dcp2 can recognize and bind to mRNA. However, its activity is low without its binding partner Dcp1, which is required for decapping *in vivo* and stimulation of Dcp2 *in vitro* [50-52]. The mechanism of how Dcp1 enhances decapping was provided by the structure of the Dcp1/2 complex [53-56]. While Dcp1 does not assist in RNA binding, it increases the rate of the catalytic step by changing the conformational state of Dcp2 [54,55].

Decapping is an important step since it is an irreversible reaction except under certain conditions in mammalian cells [57]. After Dcp2 hydrolyzes the phosphate bond in the 5'-cap structure to generate a 5'-monophosphorylated mRNA, the mRNA is rapidly and processively hydrolyzed by the major cytoplasmic 5'-3' exonuclease Xrn1 [58,59]. Decapping of the 5'-^{m7}G-cap structure is a prerequisite for 5'-3' decay since Xrn1 has less than one percent relative activity on capped or 5' triphosphorylated mRNA as compared to monophosphorylated substrates [60,61].

2.2 Alternative decapping enzymes

Dcp1/2 appears to be the only cytoplasmic decapping enzyme in yeast. The situation has recently been found to be more complex in mammals with the identification of Nudt16

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[62]. In cultured mouse cells, it was found to be partially redundant with Dcp2 [63,64]. However the role of this decapping enzyme is not completely resolved as it has previously been reported to be predominantly nucleolar in *Xenopus* and human cells [65,66]. The *Xenopus* homolog (X29) was shown to remove (hyper)methylated guanosine caps *in vitro* and *in vivo* [65], while another study found that the human Nudt16 had higher activity in conversion of inosine diphosphate to inosine monophosphate suggesting its primary function may not be in mRNA degradation [66]. The discrepancies could be attributed to accessory proteins which remain to be identified. Despite these caveats, the possibility of more than one decapping enzyme introduces new possibilities in the regulation of mRNA decapping.

The addition of Nudt16 to the canon of decapping enzymes coincides with the recent discoveries of decapping-analogous proteins in bacteria, which also contain the Nudix motif. The approach used to identify them was similar to the more recent Nudt16 work. All the *E. coli* Nudix proteins were purified and tested *in vitro* to identify an enzyme (RppH) that catalyzed the removal of a triphosphate to generate a monophosphate 5' end [67]. In *Bacillus*, a similar approach yielded the decapping-like Nudix protein BsRppH [68]. These three proteins demonstrate that the decapping activity can evolve independently from a core Nudix domain, since they do not possess homology outside the Nudix domain.

However, undiscovered non-homologous decapping proteins may exist. A surprising recent observation is that the protein Rai1 acts to remove unmethylated mRNA caps in the nucleus as a quality control mechanism [69]. This protein has no homology to Nudix proteins and was not originally thought to be an enzyme [70]. Furthermore, the identification of a unique activating protein for Rai1 hints at another potential level of regulation of mRNA decapping.

2.3 Enhancers of decapping

Despite being responsible for catalysis, Dcp2 requires additional proteins for maximal activity. Activation of decapping *in vivo* has been shown to occur in one of two manners. In

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general, either the accessory protein can directly activate the decapping enzyme, or it can repress translation, which indirectly leads to enhanced mRNA decapping [71]. However, it seems that at least one protein (Pat1) can function in both fashions [71].

Dcp1 is essential for the activation of decapping and is the constitutive binding partner of Dcp2. It not only plays a major role in acceleration of the catalytic step in Dcp2, but also links co-activators and other decay factors to the decapping mRNP, adding another control point in the 5'-to-3' decay pathway. Proline-rich proteins such as Edc1 and Edc2 can recognize and bind the EVH1 motif in Dcp1 to enhance both RNA substrate binding and the catalytic step of decapping [72,73]. These proteins are not required for optimal decapping and only come into play when the decapping enzyme is impaired. However, both have been shown to strongly activate the decapping enzyme *in vitro* [51,74,75].

Another protein responsible for activation of decapping is Edc3. It was identified from genome-wide analyses of interactions with the decapping complex [76]. Similar to the other Edc proteins, Edc3 is not required for efficient mRNA decapping under normal conditions but it can activate decapping *in vitro* [77]. Edc3, in contrast to Edc1 and Edc2, enhances decapping by binding directly to Dcp2 [77]. The significance (if any) of the choice of Dcp1 or Dcp2 as a binding partner is currently unclear [75].

The second manner of decapping activation is via repression of translation initiation. This class is exemplified by Dhh1 [71,78], a DEAD-box helicase that belongs to a family of proteins with RNA-dependent ATPase activity. Dhh1 was identified through different screens linked to mRNA metabolism [79,80]. It plays a significant role in the general mRNA decay pathway but does not affect the decay of non-translating mRNA *in vivo* [78,80,81]. Therefore it is likely to enhance decapping through repression of translation [71]. Dhh1 has been shown to inhibit translation *in vitro* and is one of two proteins necessary for translational repression *in vivo* [78]. Consistent with this proposal, it does not stimulate decapping *in vitro* [71]. However, it should be noted that an earlier *in vitro* study did report stimulation [80].

Pat1 is perhaps the most important decapping activator (Reviewed in [82]). To date, it

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is a unique member of the class of decapping activators that enhance decapping activity via both activation of the decapping enzyme and repression of translation initiation [71]. Pat1 is required for normal rates of mRNA decay *in vivo* [83-86], specifically in mRNA decapping [87]. Pat1 works in concert with Dhh1 as essential factors in translation repression in yeast [78]. Biochemical evidence supports these observations as Pat1 has been shown to repress translation and directly activate decapping enzyme in assays *in vitro* [71,87].

Associated with the Pat1 complex are a ring of seven Sm-like proteins, Lsm1-7 [88]. Similar to Pat1, lesions in the complex result in significant increases in mRNA half-lives in vivo [86]. The Lsm1-7 may serve to direct the Pat1 complex to mRNAs with short half-lives such as mRNAs with shorter mRNA tails and U rich sequences [89].

2.4 Assembly of a decapping complex

The most straightforward manner a decapping complex could assemble on an mRNA would be by simply binding Dcp1/2. Given the wide diversity of proteins that affect decapping and its (essentially) irreversible nature, the regulated assembly of the complex would be necessary.

At least two proteins have been shown to act as platforms to assemble the decapping complex: Edc3 and Pat1 [71,90]. Edc3 has significant interactions with most known decay factors, but does not globally affect mRNA half-lives [76,90-92]. However, Pat1 has the most identified *in vitro* and *in vivo* interactions with components of the yeast 5'-3' decay machinery. More importantly, it has a significant effect on the decay of virtually every mRNA examined. In humans it may also act to directly recruit the Ccr4-Not deadenylation complex [93,94].

Pat1 is integrally associated with the Lsm1-7 complex, which may confer enhanced binding specificity and affinity [84,87]. Both *in vitro* and *in vivo,* this complex preferentially binds the shortened poly(A) tail that is a prerequisite for decapping [89,95].

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2.5 Cis *factors play a role in regulation of mRNA stability*

It has long been a goal of mRNA stability studies to identify specific sequence elements that coordinate and determine the degradation rate. Some of these elements such as presence of a long 3' UTR, AU rich elements in the 3' UTR, and binding sites for specific RNA binding proteins such as the PUF proteins, appear to correlate negatively with mRNA stability in both mammals and yeast [12,21,96,97]. In general, these sequences assist in recruitment of specific factors that target mRNA for rapid degradation.

However, factors can bind to mRNA to enhance stability as well. Paradoxically, the most well known amongst these proteins bind to AU rich elements in mammals: HuR and AUF1/hnRNP, both of which can promote the stability of some mRNAs [98-101]. The mechanism that these proteins employ to enhance stability is not entirely clear, although there is some evidence that they may compete with proteins that promote decay for binding to AU rich elements.

2.6 Specific targeting of mRNA

Thus far, we have described the general mRNA decay pathway. Certain classes of transcripts, mRNA with errors, and specific messages can be targeted for degradation differently. They utilize the basic machinery but possess alternative entry-points. There are two major ways these specific mechanisms can feed into the general pathways. One is by recruitment of deadenylases to promote rapid entry into the pathways, since deadenylation is the rate-limiting step for general eukaryotic mRNA decay. The other is via deadenylation independent pathways. To illustrate these mechanisms for recruitment of proteins to accelerate the mRNA decay pathway, we will briefly discuss a few examples with emphasis on experiments performed in yeast and cite references for more detailed discussions.

First, recruitment of a deadenylation complex promotes more rapid destruction of the poly(A) tail. This specific requirement can accelerate the entry into the general decay

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pathways by making the mRNA vulnerable to either of the exonucleolytic decay processes. The deadenylase complex can be recruited to transcripts in several manners. One way is by specific recruitment by miRNA to promote degradation of their target as demonstrated in mammalian cells [102-104]. More recently, several groups have identified the mechanism of recruitment of deadenylases to transcripts through the miRNA RISC complex in *Drosophila* and humans [105-107]. The details of what deadenylase is recruited differ in these studies, but it seems that in all cases the GW182 protein is responsible.

An example of a protein that targets specific messages for rapid deadenylation is Puf5. Puf5 regulates a class of mRNAs including the mRNA encoding for the HO endonuclease in yeast [108]. mRNA bound Puf5 recruits deadenylase complexes both *in vitro* and *in vivo* and can thus promote decay. This protein is only one of many that can specifically act on deadenylases to recruit or inhibit the enzymes and regulate decay. For a recent detailed discussion see [109].

Second, mRNA decay can be accelerated by bypassing the requirement for deadenylation to initiate decay. This can occur through several mechanisms. Deadenylation independent decapping ultimately leading to 5'-3' decay is one mechanism that functions in this manner. Specific proteins can recruit the decapping complex for circumventing the deadenylation requirement for further decay. One of the several examples of these proteins is the yeast decapping activation protein Edc3. However, it has only been shown to regulate the stability of two mRNAs encoding the ribosomal protein Rps28b and the mRNA export factor Yra1 [91,110]. In both cases, it activates decay by apparently promoting deadenylation independent decapping and subsequent 5'-3' degradation. The existence of only two examples limits the determination of the exact mechanism of its recruitment and the binding sequences.

The specific recruitment of the decapping complex can be utilized in the quality control system of Nonsense Mediated Decay (NMD) is one of the most important and well studied (Reviewed in [111-113]). In NMD, mRNA-binding proteins Upf1, Upf2 and Upf3 recognize

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mRNAs with premature stop codons. In yeast, this results in the recruitment of the decapping machinery, deadenylation-independent decapping, followed by 5'-to-3' degradation [114]. However, it has recently been demonstrated that in NMD in both yeast and mammalian cells, deadenylation is activated along with the 5'-to-3' and 3'-to-5' exonucleolytic processes (Reviewed in [111]).

NMD can also be initiated by endonucleolytic cleavage by the protein Smg6 in metazoans [115-117]. Endonucleolytic decay is also used to initiate decay in certain other classes of messages. One is the miRNA pathway, where perfectly matched target mRNA sequences are cleaved, which is especially prevalent in plants [118]. However, there exists an endonucleolytic quality control system in yeast and other eukaryotes to detect and resolve stalled ribosomes [119,120]. After endonucleolytic cleavage, further degradation is via both 5'-3' degradation by Xrn1 and 3'-5' by the exosome.

Non-Stop Decay (NSD) is another quality control system to release stalled ribosomes in yeast and mammalian cells [121,122]. When a mRNA lacks a stop codon, the ribosome advances to the 3' end and stalls. After resolution of the stall, the poly adenylated mRNA is destroyed even in strains deleted for the major cytoplasmic deadenylase (Ccr4). This implies an exosome mediated deadenylation independent degradation at work. [122].

3. Sub-cellular localization of mRNA degradation

3.1 *Initial steps of mRNA decay*

The question of where mRNA decay occurs in eukaryotes has existed for a considerable amount of time. An early fundamental question was whether mRNA degradation begins in the nucleus or only after export to the cytoplasm. At least three lines of evidence suggest that deadenylation is cytoplasmic. These are based on the co-transcriptional addition of poly(A) to mRNA [123] and rapid export from the nucleus [124]. However, the poly(A) tail must be removed for the general mRNA decay pathways to begin

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[90]. First, evidence supporting poly(A) shortening from early pulse-chase fractionation experiments in HeLa cells. They indicate that the nuclear pool of poly(A) tails remains stable, while the cytoplasmic fraction deadenylates over time [125]. Second, the major mRNA deadenylase Ccr4-Not complex is distributed evenly in the cytoplasm with nuclear exclusion [43,126]. Finally, these points are consistent with evidence that mRNA is hyper-adenylated upon inhibition of mRNA export factors [127,128].

While it is clear that mRNA decay occurs in the cytoplasm, the exact location remains unknown. Several methods to estimate where mRNA decay occurs provide clues to its localization, but none are currently definitive.

First, cellular fractionation can give an indication of the cellular compartment where the enzymatic activity is found. However, its resolution limits its utility. It therefore serves mostly to demonstrate that the mRNA degradation pathway can occur in the cytoplasm, which has been shown in mammalian cells [129]. Second, fractionation can be performed by sucrose gradients, which is customarily used to identify the association of factors with translating ribosomes. Finally, the localization of individual proteins can be examined and co-localized with mRNA. A more definitive experiment is localization of individual decaying transcripts by single molecule analysis. (Co-)Localization of the mRNA and the dynamic association of the factors would provide the clearest answer to where in the cell mRNA decay occurs. However, on a technical level, it is currently unclear when this will be accomplished [130]. This review will examine and summarize the results from the above approaches.

3.2 *Cytoplasmic 3'-5' degradation by the exosome*

Of the two pathways of mRNA decay, 3'-5' degradation is a less significant contributor to general cytoplasmic mRNA decay in yeast and perhaps in metazoans as well. The exosome is the primary mediator of this type of decay. The exosome is a large complex of RNases with the Rrp44 subunit responsible for its catalytic activity. Examining the localization of the core subunits is complicated, because, in addition to their role in cytoplasmic mRNA decay, they are also essential for ribosomal RNA processing in the

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nucleus. Consideration of the localization of the exosome therefore must concentrate on factors that are required only for cytoplasmic mRNA decay such as Ski2 and Ski7. Similar to the deadenylase, in exponentially growing cells these are evenly distributed throughout the cytoplasm [131,132].

3.3 *Cytoplasmic decapping and 5'-3' decay*

The 5'-3' pathway is the major decay pathway in yeast. It requires many proteins to effectively decap and exonucleolytically decay the mRNA body. These have been extensively studied both by microscopy and by sucrose gradient fractionation. Most of these proteins have similar localization, therefore, we will focus on major factors: the decapping enzyme Dcp1/2, the cytoplasmic 5'-3' exonuclease Xrn1, and the decapping activating proteins Lsm1-7, Pat1, Dhh1, and Edc3.

These proteins generally have two features in common: First, they exhibit overall cytoplasmic localization. Second, they are often found to be associated with ribosomes or polysomes. These points are consistent since ribosomal proteins are localized evenly through the cytoplasm [133]. Early in the study of mRNA decay, a subset of the decay factors was localized by GFP or by immunofluorescence. The factors examined initially (Xrn1, Lsm1, Pat1 and Dhh1) were reported to be cytoplasmic in actively growing, unstressed cells [80,86,134]. Foreshadowing subsequent localization experiments, both Xrn1 and Pat1 did show a granular appearance in the cytoplasm [86,134].

Specific sub-cellular localization of decay factors was first observed with the presence of Xrn1 in cytoplasmic foci [135]. The role of these foci was proposed to be sites of mRNA turnover. After this discovery, different decay factors were localized to foci in human cells, the first being Lsm1 [136]. Further investigation of tagged human Lsm proteins localized these to foci and FRET studies further demonstrated their interaction [136]. The Lsm1-7 foci and the decapping complex both co-localized with Xrn1, providing further evidence that these might be sites of mRNA decay [136,137]. However, since no RNA localization in the foci was examined, it left open the possibility for simply being sites of storage of the mRNA

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decay machinery.

Finally, localization of mRNA decay intermediates to foci in yeast provided the first experimental evidence that these could be sites of mRNA degradation [126]. Thus, the foci were termed P-bodies for RNA processing bodies.

To further support this finding, the authors followed this observation with two experiments in a subsequent study [138]. They first observed that a constitutively expressed mRNA, that produces a decay intermediate, formed P-bodies in exponentially growing cells. Second, they generated P-bodies by induction of the decay intermediate, which provided further evidence for P-bodies as sites of mRNA decay. However, an unresolved caveat still remains. Since these mRNAs are roughly ten times more stable than the full length mRNA [41], they may persist as non-translating mRNAs and therefore accumulate in P-bodies [139].

3.4 The role of P-bodies as sites of cytoplasmic decay

The identification of a specific site of mRNA decay in the cell generated the possibility of a more localized regulation of mRNA degradation. Given the strong evidence that mRNA decay intermediates accumulate in P-bodies, several groups examined the effect of P-bodies on many types of mRNA decay. The results from these initial studies seemed to indicate that they are not important for most pathways of mRNA degradation under the conditions examined.

Several groups have studied the role of microscopically visible P-bodies in mRNA decay. These involved reduction or elimination of P-bodies by knockdowns or deletions of core proteins. However, most of these experiments could arguably have residual P-bodies, which may be sufficient for activity. Another difficulty in comparing these results is that the knockdowns were often performed against different proteins and with different cell types or organisms.

In yeast, the effect on the general mRNA pathway was examined through mutations that eliminated all microscopically visible P-bodies [90]. These mutations did not produce

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any effect on the half-lives of either a stable or an unstable transcript.

In addition to the general mRNA decay pathway, several additional classes of mRNA decay have been described. Three of the most significant are the AU-rich element (ARE) Mediated Decay (AMD), Nonsense Mediated Decay (NMD) and the miRNA-mediated decay pathway. None of these required P-bodies for their function under the conditions examined.

One of the most common sequence motifs affecting mRNA stability in mammalian cells is the AU-rich elements (ARE) [140]. The role of P-bodies in ARE-mediated decay (AMD) has been studied in mammalian cells by knockdown of the Lsm1 and GW182 P-body proteins, which reduce the number of P-bodies [141]. Depletion of GW182 and reduction of P-bodies did not affect half-life of any ARE containing mRNA suggesting that P-bodies are not a prerequisite for AMD. Similarly, the Izaurralde group found that knocking down of core P-body proteins (Lsm1 and Lsm4) in *Drosophila* S2 cells eliminated P-bodies, but had no effect on NMD [142]. Similar results were also obtained in mammalian cells [143].

Several components of the miRNA machinery has been shown to be concentrated in P-bodies in mammalian cells [144]. The role of P-bodies in miRNA-mediated decay was examined by a similar methodology. In human cells, knockdown of the RCK/p54 (the Dhh1 homolog) and Lsm1 eliminated visible P-bodies [145]. The depletion of RCK resulted in effective siRNA cleavage and the Lsm1 knockdown still allowed *let7* miRNA mediated cleavage to occur. Similar studies from the Izaurralde group showed that while formation of P-bodies is linked to an intact silencing pathway, depletion of microscopically visible P-bodies has no effect on miRNA silencing of gene expression or RNAi [142].

However, more recent experiments indicate that perhaps the effect is more subtle and that P-bodies may affect decay under certain conditions or for specific (classes of) transcripts and simply may not be important in exponentially growing cells [111,146]. There have been some clues that mRNA decay may be altered by P-bodies or the sub-microscopic P-body aggregates. Moreover, human and *Drosophila* Dcp1 can form trimers, which enhance their accumulation in P-bodies [147]. In *Drosophila* cells, the deletion of the same trimerization domain results in a three fold increase in half-life, perhaps suggesting a role for

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P-bodies in mRNA decay [147].

The strongest evidence for the role of P-bodies in mRNA decay comes from studies in mammalian cells [148]. Knockdown of the Pan2/3 complex of deadenylases to reduce P-bodies affected the half-lives of several classes of mRNA decay. Pan3 knockdown was observed to decrease the decay rate of the NMD mRNA, but increased the degradation rate of a stable and an AMD mRNA. These results suggest that P-bodies can have differing effects on classes of mRNA decay. However, the interpretation of these results is complicated by the reduction in mRNA deadenylation rate in the Pan3 knockdown, most significantly with the NMD substrate mRNA.

Apart from the presence of decay intermediates, P-bodies as sites of mRNA decay is supported by experiments demonstrating that they enlarge when translation is inhibited and are characterized by the absence of ribosomes [138,139]. These observations are consistent with conditions that enhance degradation. For example, mRNA decay rate is inversely proportional to translation initiation [149,150]. Furthermore, the mRNA decapping enzyme competes for the cap with the translational factor eIF4E [149]. This resulted in the proposal that mRNA would first be repressed in translation and then degraded. When P-bodies were discovered, their response to translational repression suggested that they could function within the existing paradigm. One unexplained result linking translation and mRNA decay is that cells treated with cycloheximide stall ribosomes, but are inhibited in mRNA decapping [151].

Conversely, the decay of messenger RNA has also been linked to active translation. For example, the decapping enzyme is associated with purified salt-washed ribosomes [152]. Subsequently, several groups have observed the mRNA decapping enzyme and the Xrn1 exonuclease to be associated with polysomes [153,154].

Consistent with these data, recent evidence suggests that the ribosome-free state of a P-body is not necessary for decapping and degradation. Based on the observation that decapped mRNA in an exonuclease defective strain was associated with polysomes, the Coller lab examined if mRNA decay could indeed occur during translation and therefore not

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require entry into P-bodies [155]. They demonstrated that polysome associated decapped mRNAs are in translation and that degradation can occur co-translationally using several techniques. This is not limited to general bulk decay, as NMD was also shown to be possible co-translationally [156]. It is interesting to note that the decaying mRNA observed in translation might be analogous to the co-translational 5'-3' decay observed in bacteria [155,157].

4. Concluding Remarks

mRNA degradation is clearly an important contributor to gene expression. The global importance has been revealed by the advent of genome-wide studies of mRNA decay. This is especially true in the case of modulating gene expression in response to altering conditions. However, how it is orchestrated is not well understood. What we do know is that many proteins are involved in mRNA decay and they coordinate this highly regulated process by associating with mRNA and forming mRNPs with diverse properties.

The formation of such mRNPs, most likely by binding to the 3' UTR, could provide instructions to the mRNA on where to go for degradation and set its rate of decay. These decisions can be driven through protein-RNA interactions via specific binding sites on the mRNA such as ARE or PUF binding sites or through interactions with a core scaffold protein such as Pat1 that recruits additional factors through protein-protein interactions. Genome-wide analyses using recent advances in mRNP purification, such as CLIP, might be very useful in resolving this question. The formation of different mRNP states determining decay could be thought of as an "mRNA decay code" and analogous to the combinatorial control of translation through binding of proteins to the 3' UTR of mRNA [158].

One of the mRNPs that has been widely studied is the P-body aggregate state. The experiments of the last ten years taken together presents a complex picture for the role of P-bodies as sites of degradation. In general, it is clear that lack of microscopically visible P-bodies do not substantially affect mRNA stability. Additionally, the semantic discussion of what is and is not visible P-bodies remains a caveat for all these experiments. This may be

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reflected in the results from genome-wide studies, in which the regulation of mRNA decay rates do not seem to have a significant impact on the expression of most genes under normal conditions. However, it does matter for a small subset and in kinetics of adaptation. Perhaps the conditions where P-body mutants are tested will be a significant determinant as well. A subtler role for P-bodies as sites of decay is certainly possible and is suggested by many of these experiments. The actual role of P-bodies in mRNA decay is not yet clear. Do they prevent or enhance mRNA degradation? Can they enforce commitment of decay to specific mRNAs? Can P-bodies only affect decay under certain (as yet) untested conditions? The current sets of experiments certainly leave many questions to be explored.

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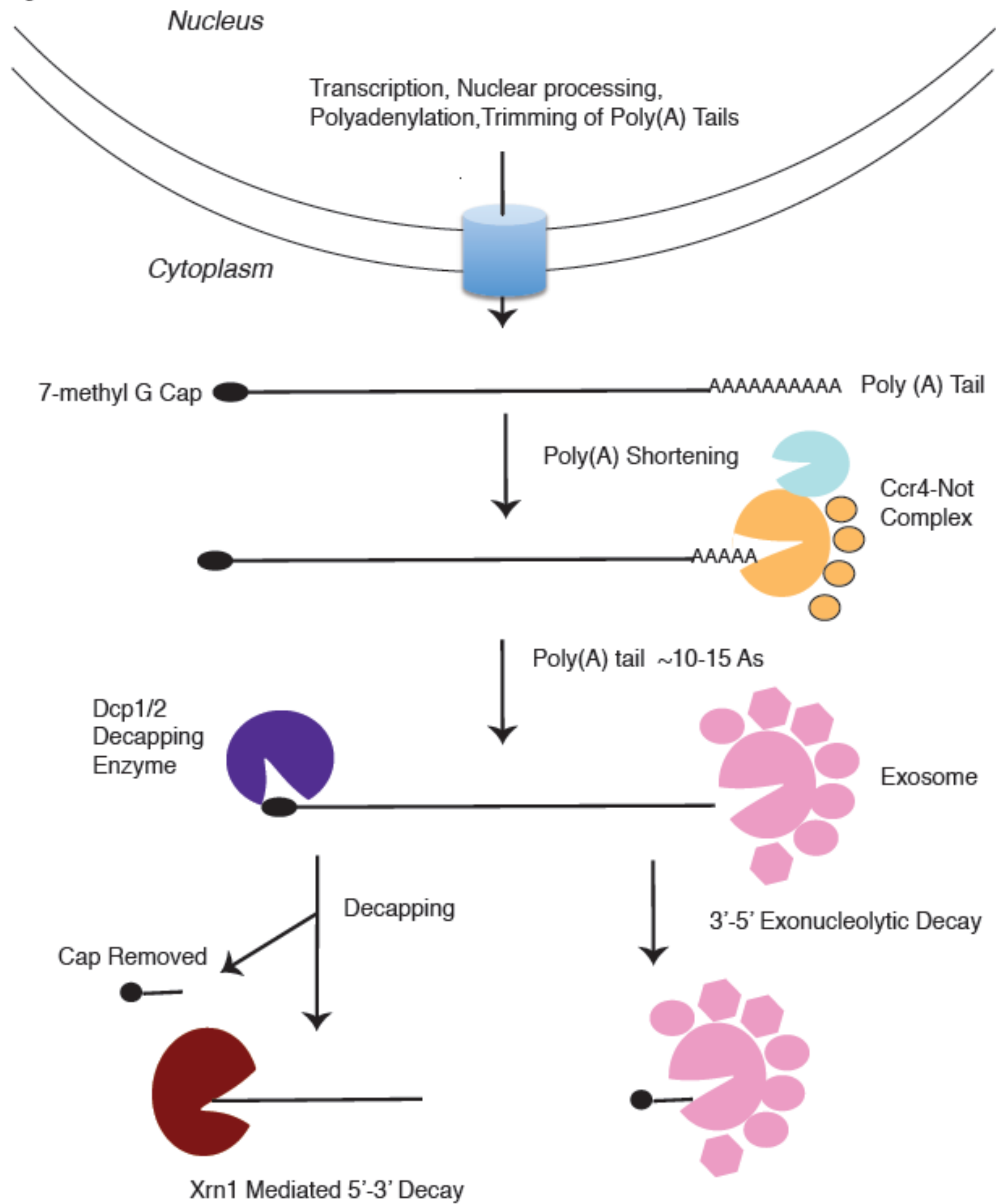

Figure 1

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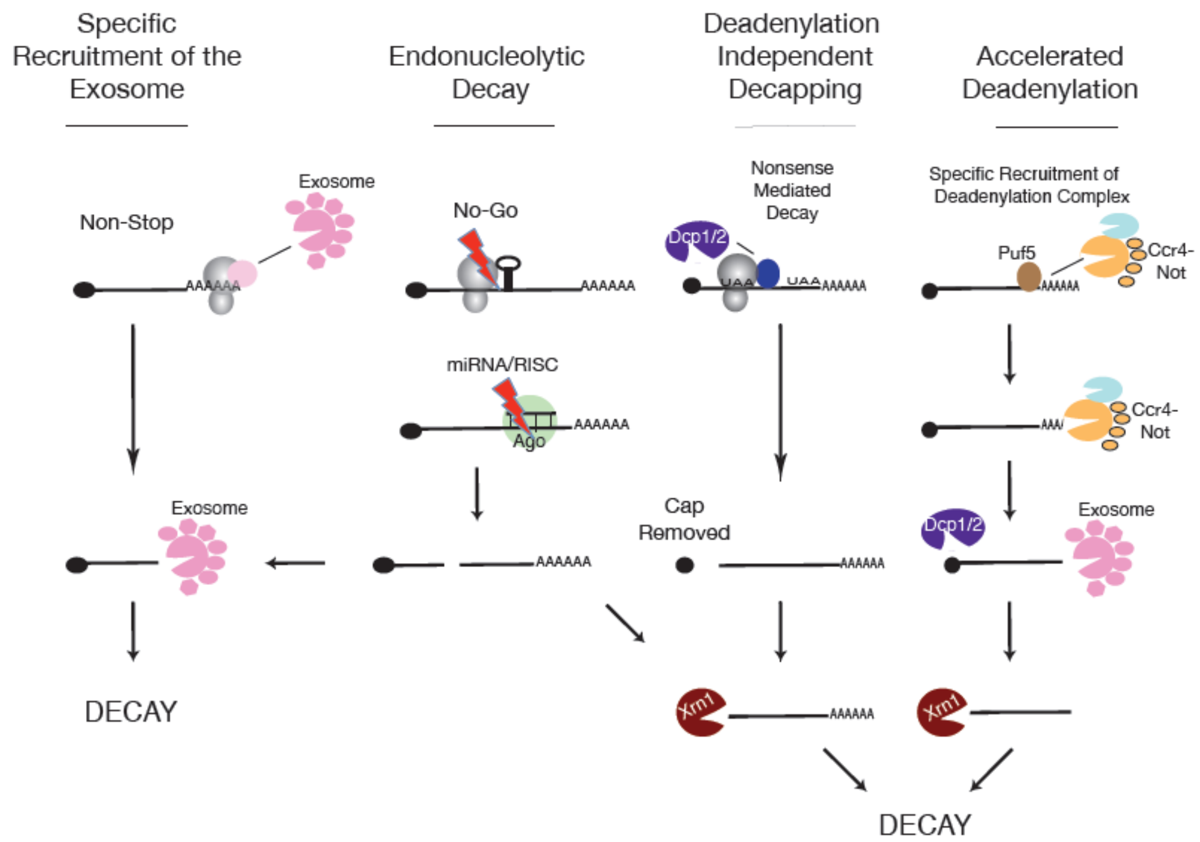

Figure 2

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Figure 1. **General cytoplasmic mRNA decay pathways.** mRNA is spliced and exported from the nucleus with 5' cap and a poly(A) tail of defined length. The general degradation pathways are initiated with poly(A) shortening by the Ccr4-Not deadenylase complex. Once the poly(A) length is reduced to 10-15 adenosines, mRNA can decay from either the 5' end (decapping followed by Xrn1 mediated) or 3' end (exosome mediated) exonucleolytic decay.

Figure 2. **Specialized mechanisms of mRNA decay.** Four mechanisms are depicted: 1) Non-stop decay occurs when there is no stop codon. The ribosome advances to the 3' end of the mRNA, the exosome is recruited and 3'-5' decay ensues. 2) Endonucleolytic pathways depicted are miRNA and No-Go decay. Micro RNA cleavage is directed by miRNA that is perfectly complementary to its target (this pathway does not exist in budding yeast). No-Go decay detects stalled ribosomes on the mRNA which leads to endonucleolytic cleavage of the message. The mRNA is then degraded in both the 5'-3' and 3'-5' directions at the site of cleavage. Nonsense Mediated Decay (NMD) can also occur by endonucleolytic cleavage in metazoans. 3) The NMD pathway detects premature stops codons in mRNA and promotes decapping prior to poly(A) shortening. 4) Accelerated deadenylation can occur by recruitments of deadenylases by specific proteins such as Puf5 or by the miRNA RISC complex (does not occur in budding yeast). After deadenylation, mRNA can be subject to decapping followed by 5'-3' degradation or 3'-5' decay by the exosome complex.

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Highlights

> mRNA decay is an important determinant of gene expression

> Discuss insights into gene expression gained from global mRNA decay studies

> Review the mRNA decay pathways and mechanisms emphasizing studies in yeast

> Detail recent developments in the mechanism and control of mRNA decapping

> Discuss the role of mRNPs and higher order aggregates in mRNA degradation

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