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XRN 5'→3' exoribonucleases: Structure, mechanisms and functions

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Abstract

The XRN family of 5'→3' exoribonucleases is critical for ensuring the fidelity of cellular RNA turnover in eukaryotes. Highly conserved across species, the family is typically represented by one cytoplasmic enzyme (XRN1/PACMAN or XRN4) and one or more nuclear enzymes (XRN2/RAT1 and XRN3). Cytoplasmic and/or nuclear XRNs have proven to be essential in all organisms tested, and deficiencies can have severe developmental phenotypes, demonstrating that XRNs are indispensable in fungi, plants and animals. XRNs degrade diverse RNA substrates during general RNA decay and function in specialized processes integral to RNA metabolism, such as nonsense-mediated decay (NMD), gene silencing, rRNA maturation, and transcription termination. Here, we review current knowledge of XRNs, highlighting recent work of high impact and future potential. One example is the breakthrough in our understanding of how XRN1 processively degrades 5' monophosphorylated RNA, revealed by its crystal structure and mutational analysis. The expanding knowledge of XRN substrates and interacting partners is outlined and the functions of XRNs are interpreted at the organismal level using available mutant phenotypes. Finally, three case studies are discussed in more detail to underscore a few of the most exciting areas of research on XRN function: XRN4 involvement in small RNA-associated processes in plants, the roles of XRN1/PACMAN in *Drosophila* development, and the function of human XRN2 in nuclear transcriptional quality control. This article is part of a Special Issue entitled: RNA Decay Mechanisms.

Keywords

Exoribonuclease; XRN; XRN1/PACMAN; XRN4; XRN2/RAT1; RNA decay; Small RNA; Transcriptional surveillance

1.1 Introduction

Messenger RNA turnover is a critical modulator of gene expression. Transcripts are constantly exposed to an array of proteins, small RNAs, and turnover mechanisms primarily

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devoted to regulating their stability. The implementation of these mechanisms also results in the elimination of defective mRNAs. Accordingly, mutations that cause defects in mRNA turnover can have significant consequences.

In the cytoplasm, most eukaryotic mRNAs are degraded by the 5'→3' exoribonuclease, XRN1 (PACMAN), and/or by the exosome complex, which has both endoribonucleolytic and 3'→5' exoribonucleolytic activities [1-3]. In the nucleus, mRNA precursors are degraded by the XRN1 paralog XRN2 (RAT1), or the nuclear exosome complex [2, 4-6]. Additionally, XRNs also participate in diverse aspects of RNA metabolism such as RNA silencing, rRNA maturation and transcription termination [4, 6]. Plants lack an XRN1 ortholog but have an ortholog of XRN2, called XRN4, which resides in the cytoplasm and functions like XRN1 [4, 7]. The XRNs are extremely important at the organismal level as well; loss of nuclear XRN function is lethal in diverse systems and the loss of XRN1 or XRN4 can cause a range of defects such as those affecting growth, development, and responses to hormonal and environmental stimuli [1, 4].

This review discusses the XRN family of exoribonucleases, focusing on their molecular functions and biological impacts. We first indicate the roles of XRNs within fundamental RNA decay pathways, and then describe the structures, locations and contributions of different XRNs to RNA decay in a variety of processes. In addition to reviewing these topics, we highlight three case studies in more detail: small RNA-associated functions of plant XRN4, the role of human XRN2 in 5'→3' decay during pausing and termination by RNAP II, and the role of XRN1 (PACMAN) in *Drosophila* development. Note that although different systems differ in their nomenclature for protein and gene names, primarily with regard to capitalization, this review will use all uppercase for simplicity (with genes italicized and mutant alleles lowercase and italicized).

1.2 Eukaryotic mRNA decay

Much of our understanding of the XRN family and the mechanisms of mRNA decay comes from studies in the yeast *Saccharomyces cerevisiae*. However, studies using other eukaryotic organisms have added to our understanding of the molecular and biological roles of XRNs in multicellular organisms [1, 3-8]. In this section, major mechanisms of both cytoplasmic and nuclear decay are discussed, all of which involve XRN activity.

1.2.1 Cytoplasmic mRNA Decay—In general, the decay of most eukaryotic mRNAs occurs by three major pathways 1) deadenylation-dependent 2) deadenylation-independent and 3) endonucleolytic cleavage-dependent decay (Fig. 1). As its name implies, the first rate-limiting step of deadenylation-dependent mRNA decay involves shortening of the poly(A) tail prior to 5' cap removal (i.e. decapping) and subsequent degradation [2, 8]. One of more deadenylase enzymes, CCR4-CAF1-NOT1 or PARN, progressively trim and nearly remove the 3' poly(A) tail [2, 9]. Following this deadenylation, the mRNA can undergo degradation in either the 5'→3' or 3'→5' direction (Fig. 1A). As deadenylation is completed, in the 5'→3' decay pathway, the LSM1-7 proteins bind to the 3' end of the mRNA and recruit the decapping complex [10-12]. Decapping enzymes such as DCP2, with additional cofactors, hydrolyze the 5' cap, exposing the mRNA to decay that is carried out by XRN1, a processive exoribonuclease that completely hydrolyzes decapped (5' monophosphorylated) RNA in the 5'→3' direction (Fig. 1 A1) [4, 5, 8, 13, 14]. This pathway bears a similarity to 5'→3' RNA decay in prokaryotes which is also specific for 5' monophosphorylated RNA [15, 16]. In eukaryotes, after deadenylation the mRNA can also be degraded in the 3'→5' direction, primarily through the activity of the multi-subunit exosome complex (Fig. 1 A2) [17, 18]. This macromolecular complex has a central core arranged in a ring consisting of six catalytically inactive 3'→5' exoribonucleases [18].

Depending on the subcellular localization, the exosome core associates with catalytically active subunits: a distributive RNase D 3'→5' exoribonuclease, RRP6 (nucleus and nucleolus), and/or a processive RNase II 3'→5' exoribonuclease RRP44/DIS3 (cytoplasm and nucleus) [19-22]. RRP44 also has a highly conserved PiIT N-terminus (PIN) domain with endoribonucleolytic activity [23-26]. Exosome-mediated 3'→5' degradation in the cytoplasm is followed by hydrolysis of the remaining cap-structure by DCPS (DCS1 in yeast), a “scavenger” type decapping enzyme [27-29]. These two directions of mRNA degradation occurring after poly(A) shortening are referred to as deadenylation-dependent RNA decay and represent the major decay mechanisms for RNA turnover in the cytoplasm, at least in yeast. SOV, another component of cytoplasmic 3'→5' RNA decay, was first identified in *Arabidopsis* as a suppressor of *VARICOSE/HEDLS*, a decapping scaffold protein [30]. SOV is a member of the RRP44/DIS3 family that contains a conserved RNaseII domain but SOV lacks the PIN-domain required interacting with the core exosome and falls in a separate cluster within the family [30]. RNA stability data indicate that substrates of SOV overlap with those of the decapping complex [30]. Recently the function of SOV homolog, DIS3L2 has been described in yeast *Schizosaccharomyces pombe* and humans [31, 32]. DIS3L2 preferentially degrades uridylylated substrates in *S. pombe in vitro* and it appears to function independent of the exosome in all three systems [30-32]. This suggests that SOV/DIS3L2 represents a cytoplasmic RNA decay pathway alternative to XRN1- and exosome-mediated degradation (Fig. 1 A2).

Another mRNA degradation mechanism involves the internal cleavage of mRNA to create unprotected 5' and 3' fragments that are substrates for exoribonucleolytic decay (Fig. 1B). Until recently, study of mRNA degradation had focused mainly on exoribonucleolytic decay from the ends. Yet, it is now apparent, that many pathways utilize endoribonucleases (e.g AGO, SMG6, RRP44/DIS3) [33]. One example of this in both plants and animals occurs via small RNAs (20-30nt long) acting as guides in silencing complexes by directing AGO proteins to specific target mRNAs [34-37]. Endonucleolytic cleavage is achieved by an AGO slicer activity if the small RNA is highly complementary to the target, if not, other decay mechanisms that may be linked to translational inhibition can take place [34, 36]. If cleavage by AGO does occur, XRN1 or XRN4 degrade the 3' mRNA fragment while the 5' fragment is degraded by the exosome [36, 38, 39]. In *Drosophila*, transcripts containing premature termination codons (PTCs) are degraded via a SMG6-mediated endonucleolytic mechanism, followed by exoribonucleolytic decay of the cleaved 5' and 3' fragments by the exosome and XRN1, respectively [40-42].

As shown in Fig. 1B2, some mRNAs undergo 5'→3' decay without the removal of poly(A) tail (e.g. *S. cerevisiae* transcripts recognized for Nonsense-Mediated Decay (NMD), and *RPS28B* and *EDC1* mRNAs) [43-45]. As part of the cytoplasmic mRNA surveillance system, aberrant mRNAs, mainly NMD substrates, also predominantly undergo 5'→3' degradation without the need for deadenylation [44, 46]. NMD factors (e.g. UPF and SMG proteins) recognize and facilitate decay of transcripts containing PTCs, and thereby prevent the generation of 3' truncated proteins that could be detrimental to the cell [44, 47]. XRN1 degrades these aberrant RNAs following 5' cap removal by DCP2 initiated by the NMD factors [5, 44]. An alternative pathway contributes to the degradation of PTC-containing mRNAs and relies on rapid deadenylation followed by 3'→5' decay via the exosome [46].

1.2.2 Nuclear mRNA decay—As in the cytoplasm, the nucleus also has 5'→3' and 3'→5' decay mechanisms that are critical for nuclear RNA turnover. Unlike mRNAs stabilized by polyadenylation, several nuclear RNAs, including rRNAs, are *destabilized* via polyadenylation by the TRAMP complex, leading to accelerated exosome-mediated 3'→5' decay [48]. Unspliced and incorrectly polyadenylated RNAP II transcripts undergo degradation by the nuclear exosome [17, 49]. Work in yeast indicates that nuclear-restricted

mRNAs are degraded by XRN2 (RAT1) after they are decapped by machinery recruited by the LSM2-8 proteins [6, 50].

2. Structure and mechanistic functions of XRNs

The XRN family members were first identified in *S. cerevisiae* (XRN1 175 kDa and XRN2, 115 kDa) and have been studied extensively over the past three decades [51-54]. Orthologs of XRN1 and XRN2 have been identified in most key model organisms, and in humans [55-65].

XRN1 and XRN2 (Fig. 2A and B) show extensive conservation within their N-terminal regions and share an active site and mechanism of action. The sequence similarity between the two is greatest around the active site, where several residues are strictly conserved and required for function [4](Fig. 2C). Sequence conservation is much lower outside the active site and the C-terminus of XRN1 extends much further than the C-terminus of XRN2. Additionally, in *S. pombe*, XRN2 is known to associate with another protein, RAI1, which confers stability to XRN2, but is not well conserved in other eukaryotes [66, 67]. Orthologs of XRN1 and XRN2 are also widely conserved, most strongly around the active site. Fig. 2D illustrates the conservation of XRN1 with residues colored by conservation between 195 eukaryotic XRN1s (XRN2s excluded) using ConSurf [68].

The crystal structure of *Drosophila* XRN1 has been determined at high resolution and reveals a well-supported model for the mechanism of action of the enzyme. The mechanism for XRN2 is likely to be very similar, due to the strong conservation of the active sites [69]. The main structural features and how they relate to function are summarized in Table 1. The entrance to the active site is narrow, preventing access to double-stranded RNA and removing secondary structures as the RNA is pulled through the gap. The first three nucleotides of the RNA are held in position by two highly conserved residues, His41 and Trp540. A pocket of basic residues interacts with the 5' phosphate and positions the first nucleotide to expose the phosphate bond to two Mg²⁺ ions, which interact with a strictly conserved group of acidic residues. After cleavage, His41 and the basic pocket cause the next nucleotide to move in to the active site by a Brownian ratchet mechanism (Supplemental Figure S4, panel C in [69]). The structure of the active site limits the type of RNA that can be degraded to RNA with an exposed 5' phosphate, because larger structures such as the m⁷G cap or triphosphorylated RNAs do not fit into the basic pocket. A single-stranded overhang of around 4nt or longer is also necessary for efficient degradation, since a sufficient length of single-stranded RNA is required to reach into the active site of the enzyme [69]. To appreciate the 3D structure of the protein, a visit to the XRN1 crystal structure page at the Protein Data Bank (<http://www.rcsb.org/pdb/explore/jmol.do?structureId=2Y35>) is recommended.

The structure elucidated above does not include all of the C-terminal portion of the protein (from residues 1140 - 1612), presumably because it is rather flexible. This part of the protein is less conserved overall but does include some areas of conservation, which may be important to its activity. Also, it is well established that deletions of this domain are detrimental to the activity of XRN1, suggesting it has a key role in XRN1 function [70]. It is possible that the C-terminal half of the protein may act as a scaffold for other proteins involved in 5'→3' degradation, such as DCP1, in a similar manner to the C-terminal protein of *E. coli* RNaseE [71].

3. Molecular functions of XRNs

3.1 Molecular functions of XRN1

XRN1s have molecular functions in a number of key processes (listed in Table 2). In addition to bulk 5'→3' mRNA turnover (Fig. 1 A1), XRN1 degrades a wide range of cytoplasmic RNAs, including noncoding RNAs and NMD-substrates. Yeast XRN1 degrades a novel class of long noncoding RNAs (lncRNAs) called XRN1-sensitive Unstable-Transcripts (XUTs) after they are decapped, particularly those antisense to open reading frames [72]. XRN1 substrates also include *GAL* lncRNAs, which overlap with *GAL* protein-coding genes involved in galactose utilization and repress their activities [5, 73]. These lncRNAs bypass deadenylation and undergo DCP2-dependent decapping (Fig. 1 B2) [73]. Interestingly, in the nucleus, XRN2 degrades the *GAL* lncRNAs following DCP2-mediated decapping, suggesting the existence of lncRNA degradation pathways in both the nucleus and cytoplasm [73].

Perhaps the most prominent example of deadenylation-independent decay (Fig. 1 B2) is the recruitment of yeast XRN1 by the NMD-complex after 5' cap removal to degrade PTC-containing polyadenylated mRNA that is shown to occur in the polyribosomes (see section 1.2 and 4.1) [44, 46, 47, 74]. In *Drosophila*, the NMD pathway is initiated primarily by an endonucleolytic cleavage mediated by PIN-domain containing SMG6, without initial decapping or deadenylation events [42]. The decay of the 3' cleavage product is XRN1 dependent [42]. This also occurs in human cells, in addition to deadenylation-independent decapping of PTC-containing transcripts as discussed above [40]. In plants, it has been proposed that XRN4 is not essential for NMD but may participate in a minor NMD pathway [75].

A major type of mRNA cleavage event leading to 5'→3' decay by XRN4 in plants is that catalyzed by the slicer activity of a miRNA-guided AGO protein (see section 7.1) [36, 39]. In animals, such cleavage occurs only rarely (e.g. *miR-196* guides cleavage of *HOXB8* mRNA) because most miRNAs lack the degree of complementarity required [76]. However, using a genomic approach called PARE (Parallel Analysis of RNA Ends) or RNA degradome analysis [77-79], additional experimental evidence for these rare instances of target cleavage that generate XRN1 substrates in mammalian cells has been obtained [80-82]. More often, miRNA function in animal cells is associated with translational inhibition and the corresponding targets undergo deadenylation-dependent decay by XRN1 [34, 36, 83, 84]. Models based on prominent cases in multiple systems indicate this decay is largely subsequent to, and potentially directly coupled to, translational inhibition [35].

Recently, the PARE approach was used to search for endonucleolytic cleavage sites in yeast [85]. Few were found suggesting that endonuclease-initiated mRNA decay in yeast is not prevalent. However, the *dcp2Δxm1Δ* mutant used for this study was found to have increased accumulation of debranched polyadenylated lariat intermediates, suggesting that XRN1 is involved in a discard mechanism for some intron-containing endogenous mRNAs that exit splicing prematurely [85]. It will be exciting to test if this pathway is conserved in multicellular eukaryotes and contributes to differential regulation.

3.2 Molecular functions of XRN2

3.2.1 Role of XRN2 in transcription termination—XRN2 is of critical importance during termination of RNA Polymerase (RNAP) II transcription to prevent the formation of long aberrant mRNAs that may be deleterious to the cell [86]. XRN2 is required after cleavage and polyadenylation of the nascent pre-mRNA, to degrade the downstream cleavage product (the cleavage product downstream of the cleavage site) in the 5'→3'

direction [6, 87]. Loss of XRN2 (or its interacting partner RAI1), results in defective termination of RNAP II [88]. To explain this, the “torpedo model” proposes that XRN2 chases and rapidly degrades the unprotected downstream cleavage product of nascent RNA, and in the process collides with and displaces RNAP II to cause termination [89]. More recent findings indicate additional cofactors interacting with XRN2 might be required to displace RNAP II [90]. Although the role of XRN2 in transcription termination was originally deduced from yeast work, it parallels the situation in human cells with a seemingly more complex set of cofactors (see section 7.2).

3.2.2 Role of XRN2 in rRNA processing—In eukaryotes, 18S, 5.8S and 25S rRNAs are transcribed by RNAP I as a single long precursor molecule, which is then processed by the actions of site specific endoribonucleases, and exoribonucleases that trim in the 5'→3' and 3'→5' directions [91]. The pre-rRNA is comprised of a 5' and a 3' External Transcribed Spacer (ETS), and two Internal Transcribed Spacers (ITS1 and ITS2) flanking the 5.8S sequence [91]. XRN2 trims the 5' ends of precursor rRNA molecules by degrading ITS1 [92, 93]. rRNA processing is a general role of XRN2 because its orthologs in *Arabidopsis thaliana* (XRN2) and mouse cells also process 5' ends of precursor rRNAs [94, 95].

3.2.3 Other functions of XRN2—Regulation of telomere length is a critical determinant for organisms and has wide implications for cellular aging and senescence [96]. In yeast, XRN2 promotes telomere elongation by degrading Telomeric Repeat-containing RNA (TERRA), a lncRNA that represses telomerase activity [97, 98]. The prevalence of TERRA in diverse eukaryotes indicates that the function of XRN2 in telomere maintenance is highly conserved [97]. Additional roles of XRN2 in transcriptional nuclear quality control in human cells are discussed in detail in section 7.2.

3.3 Overlapping functions of XRN1 and XRN2

Both XRN1 and XRN2 degrade and process a wide range of other RNA substrates and do not exclusively function in mRNA decay and rRNA processing. Reports from several eukaryotic systems point towards an overlap of substrates between the XRNs. Yeast XRN1 and XRN2 participate in accelerated degradation of mature tRNA that are under-modified and defective with reduced stabilities of acceptor and T-stems [99, 100]. In yeast, both XRN1 and XRN2 are involved in processing of nuclear structural RNA (e.g. snoRNA, and pre-rRNA) [101-103]. These effects either suggest that XRN1 can be located in the nuclear or nucleolar compartment or that it plays an indirect role by targeting an ‘unknown’ RNA that in turn affects nuclear processing.

Two groups have provided evidence that XRNs are involved in degrading small RNA. One study followed miRNA decay in human cells after inhibiting transcription of the miRNA precursors (pri-miRNA) in knockdowns of the exosome and XRN1. The conclusions from the data were that the exosome, and to a lesser extent XRN1, degrade selected mature miRNAs [104]. Two studies in *Caenorhabditis elegans* by a second group used *in vitro* decay and *in vivo* miRNA accumulation to conclude that XRN2 degraded selected miRNAs [63]. The second study also found evidence for XRN1 in degrading selected miRNA*s (passenger strands) [105]. The second study also found evidence for XRN1 in degrading selected miRNA*s (passenger strands). Whether these different observations with respect to XRN participation in small RNA degradation are due to the differences between human cells and *C. elegans* awaits further study. Recently, in human cells, both XRN1 and XRN2 have also been implicated in the formation of a different class of small RNAs called Transcript Start Site-associated (TSSa) RNAs, which map to promoter-proximal pausing sites of RNAP II [106](see Section 7.2).

Johnson et al. (1997) demonstrated that XRN1 and XRN2 are functionally interchangeable [107]. In yeast, XRN1 has been shown to compensate for the loss of XRN2 by degrading ITS1 [107]. Yeast XRN2 can substitute for the function of XRN1 when localized to the cytoplasm, while XRN1 tagged with a nuclear localization signal (NLS) can rescue the lethal phenotype of temperature-sensitive XRN2 mutant, *rat1-1^{ts}* [107]. In the reference plant *Arabidopsis*, no XRN1 ortholog is present, instead there are three XRN proteins, XRN2, XRN3, and XRN4, which are XRN2 orthologs [64]. Among them XRN4, which lacks an NLS, fails to complement *rat1-1^{ts}*, but complements *xrn1Δ* [39, 64, 108]. Given that XRN4 functions like cytoplasmic XRN1 in yeast, its inability to complement *rat1-1^{ts}* can be explained by its lack of nuclear localization. The results with XRN4 indicate that the functional interchangeability of XRN1 and XRN2 extends across kingdoms [64, 109].

4. Co-factor interactions of XRN1 and XRN2

The diversity of molecular functions of XRN1 and XRN2 predict that multiple interacting factors are involved. This is indeed the case, as highlighted in Table 3. This table and the sections below are not intended to provide an exhaustive description of interacting partners, but instead focus on key interactions of XRN1 and XRN2 with cofactors from specific decay pathways.

4.1 Interactions of XRN1

Yeast XRN1 associates with DCP1/DCP2, decapping enhancers, and LSM1-7 proteins [10-12]. LSM1-7 is a RNA-binding heptameric ring-complex that interacts with the 3'-region of oligo-adenylated or deadenylated mRNA to promote and enhance decapping, probably after the mRNA has exited translation [11, 110-113]. LSM1-7 forms a complex with XRN1 and decapping enhancers such as PAT1 that binds and stabilizes 3'-regions of oligo-adenylated RNA and prevents 3'→5' decay [110, 113]. This binding also stimulates DCP2 activity and consequently results in translational repression [11]. PAT1 recruits LSM1 to P-bodies, and directly interacts with XRN1, thereby acting as a scaffolding protein for decapping factors and XRN1-mediated decay [11]. In yeast, XRN1 also interacts with DCS1, a scavenger-decapping enzyme that degrades m7GpppN cap to m7Gp, catalyzing the last step of mRNA decay [27-29]. Yeast DCS1 interacts with XRN1 and stimulates its activity *in vitro* [114]. Their interaction is required for production of proteins essential for mitochondrial function and respiration under environmental stress conditions [114].

XRN1 associates with UPF proteins, which are core components of the NMD pathway that help discriminate PTC-containing transcripts from normal transcripts [44, 46, 115, 116]. Unlike yeast, where decay of PTC-containing mRNAs proceeds primarily from the 5' end, in human cells it depends on 5'→3', 3'→5', and endonucleolytic decay pathways (Section 1.4) [46, 117, 118]. NMD factors UPF1/UPF2/UPF3A coimmunoprecipitate with DCP2, XRN1 and 3'→5' decay factors [116]. The ATP-dependent RNA helicase activity of UPF1 removes UPF1, UPF2, UPF3, SMG6 and SMG7 from the 3' cleavage product generated by SMG6-endonucleolytic activity [119]. This is crucial for the disassembly of the NMD complex allowing access to DCP2 and XRN1-mediated degradation of PTC-containing mRNAs.

Among the most well-studied mRNA-stability determinants are the AU-rich elements (ARE) that are generally found in the 3' UTR of many unstable mRNAs in eukaryotic organisms [120, 121]. Several studies indicate that ARE-containing mRNAs are rapidly degraded in the 5'→3' or 3'→5' direction by XRN1 and the exosome, respectively [122-125]. In human cells, Tristetraprolin (TTP) and its paralog BRF1, are Zn finger (CCC-H type) proteins that bind ARE-containing mRNA, resulting in the destabilization and degradation of the mRNA in the cytoplasm [123, 126]. These proteins localize to processing

bodies (P-bodies) and to stress-granules under certain stress conditions [127] (see Section 5). TTP is found in a complex with XRN1, DCP2, and exosome components, and recruits the decapping complex to ARE-containing transcripts [123]. In support of these results, deletion of XRN1 stabilizes TTP-regulated ARE-containing mRNA in P-bodies in the cytoplasm [125]. These studies point to the important role of TTP in XRN1-mediated degradation of ARE-containing transcripts.

4.2 Interactions of XRN2

In yeast, RAI1 forms a stable complex with XRN2 and stimulates its activity [66, 67]. RAI1 has pyrophospho-hydrolase activity that removes the 5' end of aberrantly capped mRNA (mRNA with an unmethylated cap), and is thought to be involved in the surveillance of mRNA capping in the nucleus especially during environmental stress conditions [128]. The XRN2-RAI1 interactions are not conserved or observed in *Drosophila* and human cells, despite the presence of RAI1 homologs in these systems [4, 66].

XRN2 interacts with a host of 3'-processing factors for its recruitment to the RNAP II elongation complex to facilitate transcription termination. In yeast, RTT103, a protein that binds the Ser2 phosphorylated CTD of RNAP II, interacts with XRN2-RAI1. In the torpedo model (see Section 3.2.1), RTT103 is one of the cofactors that recruits XRN2 complex to 3'-regions of pre-mRNA during RNAP II transcription [88-90]. Similarly, in human cells, XRN2 interacts with P54NRB/PSF for 3'-processing during transcription [129]. These proteins are involved in numerous aspects of RNA processing (e.g splicing and polyadenylation) [130]. The P54NRB/PSF recruit XRN2 to the elongation complex by binding to its C-terminal domain and aid in termination of RNAP II transcription [129, 130]. Human XRN2 also interacts with DCP2 and TTF2 to remove aberrantly capped pre-mRNA from paused RNAP II and cause transcription termination [131]. Details of the nuclear interaction between DCP2, TTF2 and XRN2 are described in section 7.2.

5. Location of XRN family proteins within eukaryotic cells

The location of XRN enzymes in the cell has provided information on their function and probable targets. XRN2 and related enzymes (e.g. *Arabidopsis* XRN2/3 and *Trypanosoma brucei* XRND) are known to be located in the nucleus and nucleolus, where they are involved in the maturation (5' trimming) of rRNAs and snoRNAs, as well as contributing to transcription termination by nuclear RNAP I and II [64, 65]. The larger XRN1s and functionally-related proteins (e.g. *Arabidopsis* XRN4, *Drosophila* PACMAN, *T. brucei* XRN/C) are localized in the cytoplasm where they participate in the degradation of mRNAs, miRNAs and aberrant RNAs (see details above) [4, 64, 65, 104, 132, 133].

The specific locations of cytoplasmic XRN1s and their interactions with other proteins have provided interesting insights into their role in translational repression as well as in degradation pathways. In the cytoplasm, XRN1 is predominantly localized to P-bodies along with other proteins required in the 5'→3' degradation pathway such as the decapping enzyme DCP1, the helicase DDX6 (also termed DHH1/RCK/ME31B), the deadenylase CCR4-CAF1-NOT complex and PAT1 [134-136]. P-bodies are known to be highly dynamic and increase in number and size in response to stress and when mRNA decay is inhibited (e.g. in an XRN1 mutant or knockdown) [137]. Although 5'→3' degradation is generally thought to take place in P-bodies, aggregation of mRNPs into microscopically visible P-bodies is not a requirement for removal of transcripts targeted for decay by NMD or by AU-rich elements [138]. Further, mRNAs can re-emerge from P-bodies to resume translation. A current model is that cytoplasmic mRNAs present in polysomes undergo repeated rounds of translation and then in response to an unknown signal(s), are deadenylated and then bound by sequence/structure-specific proteins (and possibly miRNAs), leading to translational

repression. These repressed transcripts are then bound by non-specific RNA-binding proteins such as DDX6 to produce masked mRNAs. mRNAs are then transported to P-bodies where they can either be returned to a translationally competent state, or recruited to the degradation machinery and degraded by XRN1 [132, 134, 139]. The localization of proteins in the 5'→3' degradation pathway into P-bodies is thought to improve the efficiency of degradation and/or to sequester these proteins away from signaling proteins such as MAP kinases.

In addition to P-bodies, XRN1 and its homologues are known to be recruited to other cytoplasmic foci such as stress granules, neuronal granules, or germ cell granules (nuage) [132, 134, 136, 140]. Like P-bodies, stress granules are dynamic complexes whose assembly is dependent upon the available pool of non-translating RNAs. However, in contrast to P-bodies, stress granules contain translation initiation factors as well as a number of other RNA-binding proteins [135]. Granules containing XRN1 have also been detected in motor neurons and germ cells [140-143]. The function of XRN1 in these granules is not yet known but it may be required as a “scaffold” to co-ordinate mRNP interactions and/or to be available to initiate mRNA degradation of specific mRNAs upon receipt of the relevant signal. This idea is supported by data showing that XRN1/PACMAN is likely to be involved in the degradation of full-length transcripts and decay intermediates targeted by piRNAs and produced by retroelements [144]. Since mutations in *XRN1/PACMAN* have reduced male and female fertility and affect dendritic arborization the presence of XRN1/PACMAN would appear to be critical in these complex cell types [141-143].

6. Biological functions of XRNs

Although the molecular functions of XRNs have been elucidated (see section 3), the biological functions of XRNs responsible for observed phenotypes in mutants, are less well known (Table 4). Experiments to determine the biological consequences upon mutation or knockdown of XRN1 or XRN2 homologues are essential in order to determine the true biological targets of XRNs.

6.1 XRN1

In unicellular eukaryotes, it seems that XRN1 is particularly involved in control of growth rates. Mutations in *S. cerevisiae* *XRN1* show slower growth rates than isogenic controls but their viability was not affected [52, 145]. Other phenotypes include reduced rates of diploid formation, decreased sporulation, increased sensitivity to a microtubule-destabilizing drug (benomyl) [145], and deficiencies in meiotic homologous pairing of the DNA [146, 147]. In the yeast *Candida albicans* and in the protozoan parasite *T. brucei*, reduced growth rates have also been observed in *XRN1* mutants [65, 148]. In these unicellular organisms, it would therefore appear that XRN1 targets particular mRNAs involved in growth and meiosis and that degradation of these target mRNAs by the exosome cannot compensate. However, it is at present unclear whether these phenotypes are due to direct effects of XRN1 on particular targets or downstream “indirect” effects.

In plants, XRN1 homologues appear to have more complex biological functions. Mutations in *Arabidopsis* *XRN4*, result in insensitivity to ethylene, a key plant hormone influencing developmental and stress responses [149-153]. Although null mutants of *xrn4* up-regulate the *EBF1* and *EBF2* genes, the XRN4 effect is proposed to be indirect because these mRNAs are not stabilized in the mutants, suggesting that XRN4 targets another mRNA encoding a protein which in turn regulates *EBF1* and *EBF2* [39, 150, 151]. Mutations in *XRN4* also result in derepression of post-transcriptional gene silencing (PTGS) of transgenes and probably endogenous genes, a phenomenon discussed in more detail in the first case study (section 7.1) [154-156]. Abnormal developmental phenotypes have been

reported for *xrn4* mutants such as serrated leaves, larger rosettes, and late flowering [150, 151, 153, 155, 157]. Although there are possible explanations of each of these phenotypes, the flowering time delay is likely caused by increased levels of *FLC* mRNA, that encodes a protein regulating flowering time [157]. It has also been suggested, on the basis of Affymetrix array and PARE analysis, that *XRN4* may influence stamen development by an indirect mechanism [158]. As more growth conditions and analysis tools are applied to study specific tissues of *XRN4*-deficient plants, additional phenotypes may be identified to provide further insights into its biological roles.

In invertebrates such as *C. elegans* and *D. melanogaster*, complete loss of *XRN1* is lethal suggesting that *XRN1* targets particular mRNAs required for development and that the 3' → 5' degradation pathway cannot rescue viability [61] and unpublished observations]. A developmental role for *XRN1* is also suggested in the nematode worm, *C. elegans*. In this organism, RNAi-mediated knockdown of *xrn-1* results in defects in ventral enclosure, where the edges of the epithelial sheet move together and seal to enclose the embryo. Failure of this process, termed epithelial sheet sealing, causes lethality [61]. In *D. melanogaster*, *PACMAN* (*XRN1*) mutants also show developmental defects such as defective epithelial sheet closure (a process analogous to the ventral sealing in *C. elegans*) and reduced fecundity [1]. Further details of the morphological defects of *PACMAN* mutations are discussed in section 7.3. These data suggest that *XRN1* is required at specific points in development to target a specific set of RNAs involved in cell shape change, cell movement or cell proliferation.

In humans, mutations in *XRN1* can result in osteosarcoma, which is the eighth most common form of childhood cancer, and arises from cells of mesenchymal origin that fail to differentiate appropriately to produce an unmineralised portion of the bone matrix termed an osteoid [159, 160]. In a study examining primary osteogenic sarcoma cell lines and patient biopsy specimens, it was found that the majority of cell lines and biopsy specimens showed a substantial reduction in *XRN1* mRNA expression. Analysis of the *XRN1* sequence in one cell line showed a homozygous mis-sense mutation leading to a change in a conserved amino acid (Asp1137 → Asn1137) [160]. Therefore *XRN1* could be essential for correct bone formation in humans.

The above information suggests that *XRNs* in multicellular eukaryotes are often required during periods where the organism is responding to changes in the developmental program. Presumably this is because the cell needs to “switch off” a suite of target mRNAs so that the encoded protein products do not interfere with the subsequent developmental program. The nature of the cell signaling pathways controlling *XRN* activity or protein levels is not yet clear. However, in *S. cerevisiae* and *A. thaliana*, it has been shown that 3'-phosphoadenosine 5' phosphate (pAp) can inhibit both *XRN1* and *XRN2* *in vivo*, perhaps by binding inside the catalytic site of *XRN* [156, 161-164]. Since the protein controlling pAp conversion to 5' AMP + Pi (HAL2 in yeast, FIERY1/FRY1/SAL1 in *A. thaliana*) is functionally conserved in humans, *C. elegans* and *D. melanogaster*, this would appear to be a conserved signaling pathway.

6.2 *XRN2*

The nuclear *XRNs* such as *XRN2* has been shown to be an essential gene for viability in *S. cerevisiae*, *S. pombe*, *D. melanogaster*, *C. elegans*, and *A. thaliana* (*XRN3*) [51, 57, 63, 156, 165-167]. Since the *XRN2* proteins are involved in many essential functions (see section 3.2) this is not surprising. In *Arabidopsis*, the nuclear *XRNs* (*XRN2* and *XRN3*), like *XRN4*, are also independently involved in the suppression of PTGS (Section 7.1) [64, 156]. Another biological function of the nuclear *XRNs* revealed by the analysis of an *xrn2/xrn3* double mutant in *Arabidopsis* relates to drought, one of the most important environmental

challenges in agriculture [163, 164]. Not only is the expression of several drought-responsive genes elevated in the double mutant, but the plants are more drought tolerant than the wild-type [163, 164]. Although the *xrn4* mutant is not considered drought-tolerant, it enhances the effect of the *xrn2/xrn3* mutant; the triple XRN mutant plants survive long-term water deprivation better than *xrn2/xrn3* and also outlive the wild-type [164]. The drought-tolerant phenotype of the triple XRN mutant mimics that of FIERY1/FRY1/ SAL1 mutants, which are thought to reduce activity of all XRNs [164]. These observations support a role of the XRN family in the negative regulation of drought-tolerance responses in plants.

7. Case Studies

7.1 Small RNA-associated functions of plant XRN4

The *Arabidopsis* system has been the source of most information about plant XRNs and their roles in interesting small RNA-associated RNA decay mechanisms. The earliest report of an XRN enzyme playing a role in an RNA decay pathway initiated by small RNA-mediated cleavage came from *Arabidopsis* with the demonstration that XRN4's substrates include miRNA targets. Specifically, XRN4 degrades the downstream cleavage product produced when a miRNA guides an AGO-protein to cleave the target in the middle of miRNA/target RNA complementary region (Fig. 1B) [39].

To examine miRNA targets and their cleavage on a genome-wide scale, techniques called PARE or RNA degradome analysis were developed using *Arabidopsis* [77-79]. These analogous approaches involve cloning and deep sequencing of the 5' ends of miRNA-guided downstream cleavage products. Because mutants of *xrn4* were known to stabilize such mRNA decay intermediates, PARE was carried out on an *xrn4* mutant as well as wild-type control plants to increase sensitivity. The PARE data contained sequences corresponding to the vast majority of experimentally validated miRNAs targets; most exhibited a high abundance PARE sequence corresponding to the 5' end of the downstream cleavage product which was often elevated in *xrn4* [78] (Fig. 3). Nevertheless, the effect of *xrn4* on the accumulation of the downstream product is known to be selective despite the fact that all have a 5' monophosphate and should be substrates [39]. For *ARF10*, transgenic plant experiments showed that a 150nt region downstream of the miRNA cleavage site was sufficient to make the downstream cleavage product accumulate in *xrn4* [158]. This region contains several hexamer sequences, particularly near the 5' end, that were enriched in other downstream cleavage products of miRNA targets that preferentially accumulated in *xrn4*. Taken together, these data suggest that sequence elements influence the susceptibility of uncapped 5' ends to XRN4 and potentially other XRNs [158].

miRNA-dependent translational inhibition of target mRNAs has also been reported in plants. The process requires VARICOSE, a component of the decapping complex, and could involve XRN4-mediated 5'→3' decay [168]. However, 3'→5' decay of the target transcript cannot be ruled out.

Similar to miRNA-mediated decay in plants, PTGS is a gene silencing process that involves RNA decay, small interfering RNA (siRNAs) and XRN activity. One of the roles of PTGS (a counterpart of RNA-interference in animals) is to protect plants from invading RNAs such as RNA viruses, and is often studied when a transgene becomes silenced in transgenic plants [37]. The first mutant demonstrating that XRN could suppress PTGS was found to have a lesion in the *XRN4* gene [154]. This mutant was found to accumulate decapped transgene RNA and small RNAs. The former was proposed to be converted into double stranded RNA and subsequently into siRNAs that silenced the transgene in the mutant [154]. It has been known for many years that PTGS arising from an overexpression of an endogenous gene can also silence the native gene in a sequence-specific manner [169].

Recently, this “cosuppression” of both genes was shown to be sensitive to *XRN4* mutations as well [170].

Mutations in either *Arabidopsis XRN2* or *XRN3* can also cause PTGS of transgenes, presumably due to deficient degradation of aberrant transgene RNA in the nucleus (leading to siRNA production), but the effect is weaker than that of an *xrn4* mutant [156]. Another mutant that suppresses PTGS is the pAp overaccumulator *fry1* (*fiery1*), which is thought to act by simultaneously down-regulating all three *Arabidopsis* XRNs [156]. Both *fry1* and *xrn4*, but not *xrn2* or *xrn3*, mutants are hyper-resistant to Cauliflower Mosaic Virus (CMV), a cytoplasmic virus used to study PTGS. The model is that CMV produces aberrant viral RNA that leads to siRNA production and viral silencing; the aberrant RNA is a substrate of *XRN4* but not of the nuclear XRNs [156].

A deep-sequencing analysis of the small RNA population in *xrn4* indicated that *Arabidopsis* *XRN4* functions to negatively regulate the levels of small RNAs processed from endogenous decapped transcripts [155]. In this study, *XRN4* affected a class of small RNAs (21nt) that were processed from both sense and antisense strands of ~130 endogenous mRNAs. An enhancement in the abundance of *XRN4*-affected small RNAs was observed in the absence of both *XRN4* and ABH1 (CBP80), the latter a subunit of nuclear Cap-Binding Complex (CBC) [155]. This suggests that the stability and/or processing of these small RNAs are dependent on the activities of these proteins. The results from this study indicate that the 5' cap structure of mRNA acts as a deterrent against siRNA production, probably by denying access to a RNA-dependent RNA polymerase (RDR) needed to make dsRNA. *XRN4* suppresses RNA silencing by removing uncapped RNA that could serve as template for RDR-mediated dsRNA production.

Arabidopsis *XRN4* is also involved in degrading mRNA targets of a novel class of long siRNA (lsiRNA) that does not require a prior small RNA-mediated endonucleolytic cleavage of the target mRNA. These unique lsiRNAs range between 30 – 40nt in length [171]. *lsiRNA-1* is induced in response to *Pseudomonas syringae* infection, and processed from an overlapping pair of Natural Antisense Transcripts (NAT) by components of a distinct siRNA biogenesis pathway involving RDR6, DCL1, DCL4, HEN1, HYL1 and AGO7 [171]. This lsiRNA specifically deregulates the expression of *RAP* mRNA, which encodes an RNA-binding domain-containing protein. Unlike known small RNA-mediated mRNA decay, *lsiRNA-1* employs decapping to create an entry site for *XRN4*-mediated degradation of *RAP* mRNA [171]. Biotic stress also induces additional lsiRNAs that originate from overlapping NAT pairs at other loci [171]. Small RNA profiling of *Arabidopsis* and rice genomes identified siRNAs (on both strands) arising from overlapping regions for 16 and 34 NAT pairs, respectively [172]. Although the exact mode of action of these siRNAs in gene regulation is unclear, it could be hypothesized that they mediate degradation of their NAT pair, probably via an *XRN4*-dependent mechanism. Overall, these studies on small RNA-associated functions of *XRN4* highlight the crucial roles played by plant *XRN4* in eliminating decay intermediates and aberrant RNA, thereby acting as a safeguard for cells against RNA silencing occurring at protein-coding genes.

7.2 Role of mammalian *XRN2* in 5'→3' decay during RNAP II pausing and termination

As in yeast, *XRN2* in human cells plays a role in degrading the 3' fragment of pre-mRNA after an endonucleolytic cleavage, and is thought to cause transcription termination by releasing the RNAP II paused downstream of the poly(A) site [62, 86, 88, 173]. Recent studies of human cells have revealed novel roles of *XRN2* in degrading nascent pre-mRNA while RNAP II is stalled near promoters, adding further complexity to transcriptional quality control in the nucleus.

Promoter-proximal pausing of RNAP II is a regulatory mechanism of transcriptional control necessary for rapid gene expression in response to development and environmental cues [173]. It has also been shown to coincide with 5' capping of the nascent transcript [174]. Although this phenomenon is widespread, the factors and mechanisms controlling promoter-proximal pausing are not yet understood.

Using ChIP experiments, Glover-Cutter et al. (2008), showed that human genes have increased densities of RNAP II occupancy primarily at promoter regions and downstream of the poly(A) site, while the density is decreased for the rest of the gene body [175]. In a recent study, using genome-wide ChIP-Seq analysis, Brannan et al. (2012), demonstrated that *XRN2* associates with decapping proteins DCP1a/DCP2 and termination factor TTF2 and localizes near transcription start sites (TSS) coinciding with paused RNAP II [131]. The knockdown of *XRN2* or *TTF2* resulted in repositioning the RNAP II density away from the TSS for genes where RNAP II accumulates near the 5' region. However, simultaneous depletion of *XRN2* and *TTF2* resulted in a much stronger effect, with increased relative RNAP II occupancy downstream of the TSS. Knocking down *DCP2* also results in shifting RNAP II occupancy away from the TSS and the effect was as strong as depletion of both *XRN2* and *TTF2* together [131]. Based on these results, it was hypothesized that at promoter-proximal pause sites, co-transcriptional decapping creates a 5' monophosphate serving as an entry point for *XRN2*. This results in a 'torpedo-like' function of transcription termination with the assistance of *TTF2* and eviction of RNAP II. Therefore, *XRN2*-mediated degradation of decapped RNA and associated co-transcriptional termination at promoter-proximal pause sites, could regulate RNAP II elongation.

Another recent study showed that RNAP II pausing and termination are mediated by both endo- and exo-ribonucleolytic activities as a regulatory mechanism of transcriptional elongation in HeLa cells [176]. *XRN2*, along with RRP6 (3'→5' exoribonuclease) and SETX (helicase), are recruited to the promoter-proximal region of *HIV-1*. Premature RNAP II pausing and termination at the *HIV-1* promoter are initiated by *XRN2* and termination factors, followed by the cleavage of a stem-loop structure in the nascent transcript by a double-strand-specific endoribonuclease called DROSHA. The stem-loop is further processed by RRP6 to generate small RNA, which represses transcription via chromatin modeling at the *HIV-1* promoter [176]. These results suggest that the DROSHA complex, *XRN2* and RRP6 cooperate to mediate RNA-dependent transcriptional gene silencing at the *HIV-1* promoter in HeLa cells.

Paused RNAP II also appears to overlap with the presence of small RNAs associated with transcription start-sites in mammalian cells [177, 178]. These so called TSSa RNAs (uncapped RNAs between 18-24nt) are found in sense and antisense orientations downstream (approximately +40) of the TSS [179]. Simultaneous siRNA-mediated knockdown of *XRN1* and *XRN2* in HeLa cells resulted in longer TSSa RNA fragments with 5' extensions mapping to the TSS [106]. This suggests that the *XRN* activities could play a role in the formation of these small RNAs and aid in the removal of paused RNAP II on an aberrantly processed nascent transcript.

7.3 Role of PACMAN in *Drosophila* development

Studies using human or *Drosophila* tissue culture cells have provided extensive information on the molecular functions of *XRN1* in animal cells. However the elucidation of the function of *XRN1* in development and differentiation requires use of a model organism such as *D. melanogaster*. By analysis of mutant phenotypes in *Drosophila*, it has been shown that *XRN1*/PACMAN is required for a number of developmental processes that provide insights into its biological targets and tissue-specific regulation (Table 4).

Loss-of-function mutations in *XRN1/PACMAN* in *D. melanogaster* show that *PACMAN* is required for male and female fertility [142, 143]. Males carrying the hypomorphic *PACMAN* allele *pcm⁵*, which causes a reduction in the level of *PACMAN* RNA and protein, develop testes of normal length that are around 25% thinner than those of wild-type males [1]. The number of mature sperm present in these testes is reduced to almost half the wild-type number and consequently only half the number of offspring is produced by *pcm⁵* males compared to wild-type. A less severe phenotype also occurs in males carrying weaker *PACMAN* alleles such as *pcm³*. *PACMAN* in the testes is expressed most strongly in the stem cells and spermatogonia at the tip of the testes and localizes to P-bodies within these cells [143]. The observed phenotypes suggest that *PACMAN* plays a role in the self-renewal or maintenance of germline stem cells. Defects in stem cell renewal would lead to fewer stem cells and thus less spermatogonia and sperm. Females of homozygous *pcm* mutants show reduced egg production compared to the wild-type, with the egg chambers degenerating around stage 8 and a dramatic reduction in the number of offspring [142]. *PACMAN* is also required for female fertility [142].

PACMAN also appears to be involved in the development of the wings and thorax of adult flies, as *pcm* mutants often have defects of the wings and thorax. These adult structures are formed from the wing imaginal discs, which grow and differentiate during late larval and pupal development [180]. Wings of *pcm³* or *pcm⁵* flies frequently appear to be dull, lacking their natural iridescence and are sometimes crumpled along the posterior wing margin. Phenotypes on the thorax range from disrupted bristle formation, such as missing bent or duplicate macrochaetae, to a more severe cleft thorax phenotype (Fig. 4) [180]. The epithelial sheet movement involved in dorsal/thorax closure is a conserved morphogenetic process which is similar to that of hind brain closure in vertebrates and wound healing in humans [181]. The cleft thorax phenotypes observed closely resemble those observed in flies mutant mutations in the JNK (c-Jun N-terminal kinase) signaling pathway such as *hemipterous (JNKK)* or *kayak (Dfos)* [182, 183]. A similar epithelial sheet sealing defect termed ventral enclosure is also apparent in *C. elegans* embryos upon *xrn-1* knockdown [61]. These phenotypes suggest that *PACMAN* targets a subset of mRNAs involved in morphogenesis. One possibility, which is consistent with other thorax phenotypes and with the phenotypes seen in testes, is that *PACMAN* is normally required to prevent apoptosis of specific cells during development.

It has also been shown that mutations in *PACMAN* result in defects in wound healing. Flies homozygous for the *pcm⁵* and *pcm³* mutant alleles severe defects in wound healing such that the survival of *pcm⁵* females was half that of wild-type controls [180]. Since the JNK signaling pathway is known to be activated in wound healing, it is possible that *PACMAN* targets mRNA(s) which suppresses this pathway [184, 185]. The phenotypes described above emphasize the importance of *XRN1* in developmental processes and highlight the usefulness of examining its role in multicellular organisms.

8. Conclusions and future prospects

The 5'→3' exoribonucleases *XRN1*, *XRN2* and related *XRNs* have proved to be fascinating enzymes, with key functions in RNA decay and roles in a number of other important cellular processes including NMD, PTGS and small RNA-mediated decay. During the past several years, especially exciting discoveries have been made. Solving the *XRN1* crystal structure made it easy to understand the enzymatic mechanism of the *XRN* family in general. New substrates and interacting partners have been identified, and the phenotypes of *XRN* knockdowns have led to both mechanistic and biological insights about the roles of both nuclear and cytoplasmic members of the *XRN* family.

When evaluated on a global scale, the molecular phenotypes of XRN mutants have led to significant advances, particularly in *Arabidopsis*. Small- and large-scale array-based approaches were important initiators of this trend leading to the identification of novel XRN4 substrates, most notably cleaved miRNA targets. This has naturally led into deep sequencing approaches to quantify gene expression genome-wide, and as a result, we will soon see RNA-seq data emerge from XRN mutants from several plant and animal species. The expected outcomes will allow a better picture of the genes affected by XRN deficiency, including which functional groups and potential *cis*-regulatory sequences are overrepresented among them. Although RNA-Seq experiments are not designed to separate partially degraded from intact RNA, the PARE technique is a potent way to characterize the former. Further application of PARE and related technologies to the study of the RNA degradome in XRN mutants will detect XRN substrates on the basis of their decay patterns, and thus help identify direct targets of specific enzymes. As these transcriptome and RNA degradome data are integrated into multi-network models that also include various types of interaction data, new hypotheses about the roles of individual XRNs should emerge.

In order to further understand the biological functions of XRNs it is also necessary to examine the role of XRN1 in model organisms. Research using the invertebrates *C. elegans* and *Drosophila* has shown that XRNs have specific functions during development, suggesting that particular sets of transcripts are targeted. In *Drosophila*, our unpublished results indicate that XRN1 may target different sets of transcripts in different tissues, indicating that 5'→3' degradation can be dependent upon tissue-specific factors. Remarkably, there are not yet any reports on the specific biological functions of XRNs in the mouse or in zebrafish, even though the *Drosophila* data would suggest that this gene is likely to be important in the development of these organisms. Further work on the biological role of this enzyme in model organisms is likely to shed light on its role in human disease and development.

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HIGHLIGHTS

- XRN1s degrade diverse RNAs in the nucleus and cytoplasm of eukaryotes
- Crystal structure and interaction studies enhance mechanistic understanding
- Mutant phenotypes illustrate roles of XRN1s in development, stress and more
- Cases highlight exciting *Drosophila* XRN1, human XRN2, and plant XRN4 functions

Fig 1. Mechanisms of mRNA degradation in eukaryotes

A) Most mRNAs undergo decay by a deadenylation-dependent pathway. Deadenylation activity of CCR4-CAF1-NOT1 or PARN removes nearly all of the poly(A) tail. Following deadenylation, mRNAs can be degraded by either 5'→3' or 3'→5' decay pathways. A1) In 5'→3' decay, a decapping complex, typically containing DCP2, hydrolyzes the 5' cap exposing the mRNA to exoribonuclease XRN1. A2) Alternatively, the deadenylated mRNA is degraded by the exosome complex in the 3'→5' direction, and the 5' cap structure is hydrolyzed by the scavenger-decapping enzyme DCP5. SOV/DIS3L2 preferentially degrades uridylated transcripts in the 3'→5' direction and its substrates overlap those of the deadenylation-dependent 5'→3' pathway. B) mRNA degradation can occur independent of deadenylation as in endonucleolytic cleavage (scissors) mediated decay and Nonsense-Mediated Decay (NMD) pathways. B1) Internal cleavage due to endonucleolytic activity results in 5' and 3' mRNA fragments with unprotected ends that are degraded by XRN1 and the exosome complex, respectively. B2) NMD targets (and certain long noncoding RNAs) bypass deadenylation and undergo 5' cap removal by the decapping complex followed by 5'→3' degradation by XRN1.

Fig 2. Structural comparisons between XRN1 and XRN2

A) Crystal structures of *D. melanogaster* XRN1 (PACMAN, PDB ID: 2Y35, residues 1-1141 of 1612) [69]. The catalytic domain (N-terminal) of PACMAN is shown in blue with three nucleotides of decapped RNA (red) bound in the active site. The C-terminal is shown in grey. B) *S. pombe* XRN2 (RAT1, PDB ID: 3FQD, residues 1-885 of 991) with its binding partner RAI1 (all of 352 residues) [66]. XRN2 is shown in blue with the active site marked by a red asterisk. RAI1 is shown in light brown. C) Similarity of residues between XRN1 and XRN2. Residues of PACMAN are colored by similarity to residues in *D. melanogaster* XRN2. Residues are most strictly conserved around the active site and less so towards the C-terminal, much of which is not present in XRN2. D) Conservation of residues across 195 eukaryotic XRN1s. Conservation is greatest around the active site, but there is also good conservation in parts of the C-terminal. Multiple alignment and conservation scores were calculated using ConSurf [68], which takes into account the phylogenetic distance between species. XRN2 alignments were excluded from the calculation. All images (and the alignment of PACMAN and XRN2 sequences) were produced in UCSF Chimera [186].

Fig 3. Example of decay plots (D-plots) showing accumulation of the downstream decay intermediate following miRNA-directed cleavage is elevated in an *xrn4* mutant of *Arabidopsis* The abundance of PARE sequences are plotted versus their position within the *ARF10* (*AT2G28350.1*) mRNA for the wild-type (WT) and loss-of-function mutant, *xrn4*. The arrows indicate the PARE sequence that corresponds to the position of *miR160*-directed cleavage. Accumulation of the downstream cleavage intermediate represented by this sequence is enhanced due to the absence of XRN4, which normally degrades this product. TP10M is transcripts per 10 million reads mapped.

Cleft thorax phenotype of *pcm* mutants **Fig 4. Phenotypes of *Drosophila xrn1/pacman* mutant**

A) Thorax of wild-type *D. melanogaster* B) Thorax of a *pcm*⁵ mutant (*w*¹¹¹⁸ *pcm*⁵/*Df*(1)JA27) exhibiting mild cleft thorax (red arrow) and cleft scutellum (blue arrow) phenotypes.

Table 1

Structures, functions and interactions of important residues identified within *Drosophila* XRN1 (PACMAN) [69]

Structure	Characteristics	Residues	Interactions/functions
Catalytic domain		1-674	
	Mg ²⁺ binding/active site	Asp35, Asp86, Glu177, Asp205, Asp207, Asp288	Co-ordination of two Mg ²⁺ ions and water molecules for RNA hydrolysis
	Tower domain	533-542	Allows only exonuclease activity in the active site and interacts with the Winged helix domain
	Stacking residues	His41, Trp540	Organizes first three nucleotides into a π - π stack; His41 required for processive degradation
	Basic pocket	Lys93, Gln97, Arg100, Arg101	5'-phosphate binding excludes capped RNA and maintains 5'-nucleotide position for cleavage
	α 1-helix and Trp540 loop	4-13, 533-542	Steric barrier at entrance to active site to exclude double stranded RNA and facilitate duplex unwinding
β -barrel domain	Similarity to PAZ/Tudor domains	674-779	Interacts with catalytic domain for stability
β -barrel domain	Similarity to KOW/SH3 domains	800-850, 1031-1045	
α -helical domain	Winged helix domain	851-1026	Interacts with Tower domain
SH3-like domain	Lacks canonical SH3 residues for proline rich binding	1046-1140	Interacts with N-terminal for stability

Table 2

Major molecular functions of XRNs

	Molecular function ^a	Substrate	System(s) ^b	References
XRN1				
	mRNA degradation	Decapped mRNA	Sc; At (XRN4 ^c); Hs	[116, 154, 158, 187, 188]
	mRNA degradation	3' intermediate from endonucleolytic cleavage	Sc; At (XRN4 ^c); Dm; Hs	[38-42, 189]
	mRNA degradation	Splice-defective debranched intronic lariat	Sc	[85, 190]
	Noncoding RNA degradation	Long noncoding RNAs ^d (lncRNAs, e.g. XRN1-sensitive Unstable Transcripts (XUTs))	Sc	[72], [73]
XRN2				
	Pre-mRNA degradation	3' fragment of co-transcriptionally cleaved nascent pre-mRNA	Sc; Hs; At	[62, 88]
	Pre-rRNA processing	5' end of 5.8S and 25S rRNA precursor	Sc; At (XRN2)	[92, 93, 95, 103, 191]
	Noncoding RNA processing	Excised loops of miRNA precursor	At	[156]
	Noncoding RNA degradation	Telomeric repeat-containing RNA (TERRA)	Sc	[98]
XRN1 and XRN2				
	Noncoding RNA formation	Transcription Start Site-associated (TSSa) small RNA	Hs	[106]
	Noncoding RNA degradation	Mature miRNA	Ce; Hs (XRN1)	[63, 104, 105]
	Noncoding RNA degradation	Hypomodified mature tRNA	Sc	[99]
	Pre-mRNA degradation	Uncapped aberrant pre-mRNA	Sc; Hs (XRN2)	[50, 131, 192]
	Pre-mRNA degradation	Unspliced nascent pre-mRNA and lariat introns	Sc; Hs (XRN2)	[193]
	Noncoding RNA processing	Small nucleolar RNA (snoRNA)	Sc	[101, 102, 194, 195]

^aMolecular function(s) reported for XRN1 and/or XRN2; some functions have not been tested for both^bSc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*^c*Arabidopsis* XRN4 is a cytoplasmic homolog of XRN1^dAt least one type of lncRNA (*GAL* lncRNAs) is a substrate of both XRN1 and XRN2 [73]

Table 3

Key co-factors/interacting partners of XRN1 and their proposed functions

	Interacting Protein	Method ^a /System ^b	Proposed function of interacting protein	References
XRN1				
	LSM1-7	Y2H,TAP/Sc	RNA-chaperones that affect pre-mRNA splicing and degradation, and snRNA/tRNA/rRNA processing; Enhances decapping by facilitating interaction of DCP2 with mRNA	[10, 12]
	DCS1	FW/Sc	Scavenger decapping enzyme with His triad pyrophosphatase activity; Increases affinity of XRN1 for RNA	[114]
	PAT1	TAP/Sc	Topoisomerase II-associated protein that interacts with translation initiation factors, LSM1 and XRN1; Activates mRNA decapping <i>in vitro</i>	[11]
	UPF1/2/3A	CoIP/Hs	UP-FRAMESHIFT proteins involved in PTC-recognition during NMD pathway	[116, 119]
	TTP/BRF-1	CoIP/Hs	Tristetraprolin (TTP), Zn-finger protein; Binds AU-rich Elements (ARE) and activates RNA decay by associating with DCP1a, DCP2 and XRN1	[123]
XRN2				
	RAI1	CoIP/Sc	Preference for demethylated cap and stimulates XRN2 activity	[67]
	RTT103	TAP/Sc	RNAPII interacting protein and involved in transcription termination; Interacts with XRN2-RAI1	[88]
	DCP1a/2	CoIP/Hs	Hydrolyzes caps of misprocessed nuclear mRNA precursors	[131]
	TTF2	CoIP/Hs	A DNA-dependant ATPase that releases RNAP II and nascent RNA from DNA templates; Involved in terminating transcription and nuclear decapping	[131]
	P54nrb/PSF	CoIP,FW/Hs	Associated with transcription, splicing, and polyadenylation. Recruits XRN2 to nascent RNA for 3' processing and transcription termination	[129]

^aCoIP, Coimmunoprecipitation; FW, Far Western; TAP, Tandem Affinity Purification; Y2H, Yeast-2-Hybrid^bHs, Homo sapiens; Sc, Saccharomyces cerevisiae

Table 4

XRNs affecting key biological processes/functions

XRN^a	Mutant phenotype^b	Other name(s)	References
XRN1			
ScXRN1	Reduced growth rate Microtubule destabilization Reduced sporulation Defects in filamentous growth	SEP1 KEM1 KEM1	[52] [196] [146] [145]
CaXRN1	Defects in filamentous growth	KEM1	[148]
CeXRN1	Defective ventral enclosure during embryogenesis	XRN-1	[61]
AtXRN4	Insensitivity to ethylene Suppression of post-transcriptional gene silencing (PTGS) Enhances drought tolerance of <i>xm2/xm3</i> mutant ^c	EIN5/AIN1	[150-153] [154, 156] [97, 164]
TbXRN1	Reduced growth rate	XRNA	[65]
DmXRN1	Reduced male fertility Reduced female fertility Defects in epithelial sheet sealing	PACMAN	[143] [142] [180]
XRN2			
ScXRN2	Cell lethality Reduced sporulation	RAT1	[51] [166]
SpXRN2	Cell lethality Defects in chromosomal segregation during mitosis	DHP1	[167] [57]
AtXRN3	Embryo lethality		[156]
AtXRN2/3	Suppression of PTGS Reduced fertility Activation of high-light responses Activation of drought responses; drought tolerant		[156] [156] [163] [163, 164]

^aAt, *Arabidopsis thaliana*; Ca, *Candida albicans*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tb, *Trypanosoma brucei*

^bPhenotype(s) reported for indicated *xm* mutant

^cTriple mutant (*xm2/xm3/xm4*) is more drought tolerant than double mutant