Structural basis and function of XRN2 binding by XTB domains

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The RNase XRN2 is essential in RNA metabolism. In Caenorhabditis elegans, XRN2 functions with PAXT-1, which shares a putative XRN2-binding domain (XTBD) with otherwise unrelated mammalian proteins. Here, we characterize the structure and function of an XTBD–XRN2 complex. Although XTBD stably interconnects two XRN2 domains through numerous interacting residues, mutation of a single critical residue suffices to disrupt XTBD–XRN2 complexes in vitro and to recapitulate paxt-1-null mutant phenotypes in vivo. Demonstrating conservation of function, vertebrate XTBD-containing proteins bind XRN2 in vitro, and human CDKN2AIPNL (HsC2AIL) can substitute for PAXT-1 in vivo. In vertebrates, which express three distinct XTBD-containing proteins, XRN2 may partition into distinct stable heterodimeric complexes, which probably differ in subcellular localization or function. In C. elegans, complex formation with PAXT-1, the sole XTBD protein, serves to preserve the stability of XRN2 in the absence of substrate.

RNases are key enzymes in RNA metabolism and are central to both RNA processing and degradation. The nuclear 5′→3′ exorNase XRN2 functions in processing of ribosomal and small RNAs1–6; transcriptional termination7,8; clearance of aberrant precursor mRNA9 and tRNA10; degradation of microRNAs11; and other pathways (reviewed in refs. 12,13). Accordingly, XRN2 is conserved across eukaryotes instead forms a complex with the protein Partner of XRN2 (PAXT-1)23. Previous, we have shown that the N-terminal amino acids 1–121 of C. elegans PAXT-1, which contain the XTBD domain, are sufficient for XRN2 binding in complex with XRN2 and identify a single XTBD amino acid that makes crucial and conserved contributions to complex formation in vitro and in vivo. We demonstrate that XTBDs are general XRN2 binders in vitro and that, despite its limited sequence similarity, HsC2AIL can substitute for C. elegans PAXT-1 in vivo. Finally, we provide evidence that PAXT-1 buffers cellular RNase activity by preserving XRN2 stability in the absence of substrate.

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Conserved binding interface of an XTBD–XRN2 complex

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We sought to elucidate XTBD structure and function. Here, we identify a core XTBD that binds XRN2, solve its crystal structure with XRN2 and identify a single XTBD amino acid that makes crucial and conserved contributions to complex formation in vitro and in vivo. We demonstrate that XTBDs are general XRN2 binders in vitro and that, despite its limited sequence similarity, HsC2AIL can substitute for C. elegans PAXT-1 in vivo. Finally, we provide evidence that PAXT-1 buffers cellular RNase activity by preserving XRN2 stability in the absence of substrate.

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Although PAXT-1 is 375 amino acids long, its N-terminal 121 amino acids are sufficient for XRN2 coimmunoprecipitation, XRN2 accumulation and animal viability23. This portion of PAXT-1 contains a predicted domain of unknown function, DUF3469, and although PAXT-1 is not well conserved outside nematodes, DUF3469 also occurs in unrelated vertebrate and ciliate but not yeast proteins23. Although it is not known whether these DUF3469-containing proteins bind XRN2, three of them, mammalian NKRF (NRF) and CDKN2AIP (CARF) and ciliate Tan1 have been observed in XRN2 complexes3,24,25. Hence, DUF3469 may function as an XTBD23.

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The crystal structure confirms that PAXT-1_{1-75} is the core XTBD. It binds to a large groove on XRN2, which is formed by NCS2 and PBS. XRN2 residues 645–681 form a long loop protruding from the globular core of the PBS and fold around the base of a large α-helix (residues 108–139) known as the tower domain\(^\text{21}\) (Fig. 1b and Supplementary Fig. 1b). XTBD folds into a globular four-helix bundle (H1–H4) connected by three loops (L1–L3) (Fig. 1c). H1–H3 form an antiparallel helical array, and H4 folds back on top of H2 and H3 at a 90° angle. H1 is short, comprising only six residues, whereas helices H2–H4 are much longer (10–15 residues). The four-helical bundle is mainly stabilized by hydrophobic helix-helix interactions together with additional polar interactions between side chains located on neighboring helices. One peculiarity is that L3, which connects H3 and H4, is in a completely linear conformation (Supplementary Fig. 1c). Although a Dali search\(^\text{30}\) against the Protein Data Bank (PDB) identified many structures with a topologically similar arrangement of three to four α-helices, either as single units or as part of a larger helical array; these differed substantially in their helix-to-helix angles and therefore represent only distant hits with low Z-scores (Z < 4.5) and rather large r.m.s.d. values (>3.0 Å). Hence, it seems that the four-helix bundle of XTBD represents a structurally unique arrangement for XRN2 binding with no closely related protein structure present in the PDB.

### Table 1 Data collection and refinement statistics

| XTBD–XRN2\(^{\text{ZLC}}\) |
|-----------------------------|
| **Data collection** |
| Space group | P2\(_1\)2\(_1\)2\(_1\) |
| Cell dimensions |
| a, b, c (Å) | 170.21, 200.77, 202.99 |
| α, β, γ (°) | 90, 90, 90 |
| Resolution (Å) | 50.00–2.84 (2.91–2.84)\(^a\) |
| \(R_{\text{sym}}\) | 0.126 (1.261) |
| \(R\) / \(R_{\text{free}}\) | 0.185 / 0.238 |
| No. atoms |
| Protein | 33,362 |
| Ligand/ion | 135 |
| Water | 53 |
| B factors |
| Protein | 85.9 |
| Ligand/ion | 122.9 |
| Water | 56.4 |
| r.m.s. deviations |
| Bond lengths (Å) | 0.011 |
| Bond angles (°) | 1.18 |

*One crystal was used for data collection and refinement.

\(^a\) Values in parentheses are for highest-resolution shell.

### XTBD Tyr56 is critical for complex formation

Crystal packing analysis suggested that the XTBD–XRN2\(^{\text{ZLC}}\) complex exists as a single heterodimer, and size-exclusion chromatography coupled to multiangle light scattering (SEC MALS) experiments confirmed this: the XTBD–XRN2\(^{\text{ZLC}}\) complex eluted as a monodisperse heterodimer with a measured mass of 80.9 kDa (calculated mass of 83.0 kDa) (Supporting Information Fig. 2a). Analysis of the protein–protein interface by Proteins, Interfaces, Structures and Assemblies (PISA)\(^\text{31}\) and Evolutionary Protein-Protein Interface Classifier (EPPIC)\(^\text{32}\) revealed a solvent-accessible area on either protein of ~1,000 Å\(^2\) upon complex formation. Twenty-four residues of XTBD interact with 36 residues of XRN2\(^{\text{ZLC}}\) in a hydrophobic and polar manner, and these interface residues are highly conserved, as shown by ConSurf analysis\(^\text{33,34}\) of both proteins (Fig. 2a). The hydrophobic character of the interface is mainly due to XTBD residues on L1, H3 and L3 (Trp14, Ile37, Cys41, Leu52, Cys54, Tyr56 and Leu60), and it contributes to interactions with XRN2\(^{\text{ZLC}}\) residues Val553, Phe560, Pro591, Pro650, Ile652, Asp653, Pro656, Pro677 and Phe678 (Fig. 2b and Supplementary Fig. 2b). The formation of 13 hydrogen bonds and three salt bridges provides additional binding energy (Fig. 2c,d and Supplementary Fig. 2c). Although the XTBD–XRN2 interface is relatively small at ~1,000 Å\(^2\), the complex is very stable and resists disruption by high-salt washes (data not shown) and high dilution (Supplementary Fig. 2a).

To further dissect the XTBD–XRN2\(^{\text{ZLC}}\) interaction, we leveraged the structural information to design PAXT-1–interface point-mutant proteins, which we coexpressed with XRN2. We examined complex formation by purifying the PAXT-1 proteins through their hexahistidine (His\(_6\)) tags by immobilized metal affinity chromatography (IMAC). The PAXT-1 point mutations W14A and C41G did not compromise XRN2 copurification (Fig. 2e and Supplementary Data Set 1). Less XRN2 copurified with PAXT-1 C54G, but this mutation also decreased the amount of PAXT-1 purified (Fig. 2e and Supplementary Fig. 2e). C54G may thus disrupt protein integrity, although we note that the level of PAXT-1 C54G in bacterial lysates was comparable to that of wild-type PAXT-1 (Supplementary Fig. 2f). In contrast, the Y56A mutation completely abrogated

(residues 788–975) of XRN2, thus yielding XRNX2\(^{\text{ZLC}}\). After performing bacterial coexpression, purification and crystallization of XRNX2\(^{\text{ZLC}}\) and PAXT-1_{1-75}, we determined the structure of the 83-kDa complex at 2.85-Å resolution (Table 1). The macromolecular complex had six XTBD–XRN2\(^{\text{ZLC}}\) heterodimers in the crystallographic asymmetric unit and a solvent content of ~64%. We performed molecular replacement and model building with phased anomalous difference Fourier electron density maps obtained from selenomethionine-derivative crystals. The final XTBD–XRN2\(^{\text{ZLC}}\) structure consists of residues 4–25, 35–148, 153–409 and 534–787 for XRNX2\(^{\text{ZLC}}\) (chain A) and 1–73 for XTBD (chain B), which show clear electron density.

Previous work has used sequence conservation to assign domains to XRN family proteins, namely conserved region (CR) 1 (residues 1–409) and CR2 (residues 543–713), which are connected by a nonconserved disordered region and extended by a poorly conserved C terminus, respectively\(^\text{21}\) (Fig. 1a). To facilitate description and functional dissection of XRNX2, we will use an alternative annotation system including nuclease core segment 1 (NCS1; residues 1–310), nuclease core segment 2 (NCS2; 311–612), PAXT-1–binding segment (PBS; 613–705), C-terminal segment 1 (CTS1; 706–787) and C-terminal segment 2 (CTS2; 788–975) (Fig. 1a,b).

The structure of XRNX2\(^{\text{ZLC}}\) is highly similar to that of *Schizosaccharomyces pombe* Rat1 (PDB 3FQD\(^\text{23}\)); these proteins superimpose with an r.m.s. deviation (r.m.s.d.) of 1.46 Å over 580 aligned ßα atoms (53% sequence identity). XRNX2\(^{\text{ZLC}}\) also superimposes very well onto the exonuclease cores of *Drosophila melanogaster* and *Kluyveromyces lactis* XRN1s, with r.m.s. values of 1.51 Å (503 aligned ßα atoms, 44% sequence identity, PDB 2Y35 (ref. 20)) and 1.36 Å (475 atoms, 42% identity, PDB 3P1E\(^\text{29}\)), respectively.

### Footnotes

\(^{a}\) Values in parentheses are for highest-resolution shell.
interaction of XTBD with XRN2 without affecting PAXT-1 levels or purification (Fig. 2e and Supplementary Fig. 2f), thus highlighting Tyr56 as a key interface residue.

Because numerous side chains contribute to the total binding energy of the interface, loss of binding through a single point mutation appears surprising. However, it may be explained by the crucial role of Tyr56 in shaping the unique linear conformation of loop L3, with all seven residues of XTBD 51–57 engaged in XRN2 binding (Supplementary Fig. 2b). Overall structure of the XTBD–XRN2ΔZLC complex, shown as a cartoon model with transparent surface for XRN2ΔZLC. Colors for XRN2ΔZLC and for XTBD correspond to those in a. (c) Cartoon representation of XRN2 (blue, orange, cyan and magenta) in complex with the XTBD (yellow). Selenomethionine residues are shown as sticks with corresponding anomalous difference Fourier peaks in magenta (5σ).

To validate the importance of XTBD Tyr56 for XRN2 binding under physiological conditions, we used genome editing35,36 to create the Y56A point mutation in endogenous PAXT-1. Strikingly, when we grew the resulting paxt-1(xe29) worms at 26 °C, they arrested as L1-stage larvae, as did paxt1(0) animals (Fig. 2f). In addition, as previously observed in paxt-1(0) animals23, accumulation of XRN2 was reduced, and PAXT-1 was undetectable by western blotting (Fig. 2g, Supplementary Fig. 2d and Supplementary Data Set 1). Formally, we cannot exclude that destabilization of PAXT-1 through the Y56A mutation causes these effects. However, the protein was stable in vitro (Fig. 2e), as was a human XTBD protein with an analogous mutation in human cells (discussed below). Moreover, loss of XRN2 destabilizes PAXT-1 in vivo23. Hence, we propose that PAXT-1 depletion in vivo is a secondary consequence of its inability to bind XRN2. At any rate, our data identify Tyr56 as a critical residue for PAXT-1–mediated stabilization of XRN2 in vitro and in vivo and explain the structural basis of this importance. They also explain the high degree of conservation of this residue: In XTBDs, this position is always held by tryptophan; residues with aromatic side chains (tyrosine, phenylalanine and tryptophan; Fig. 1a and Supplementary Fig. 2b), which can occupy the XRN2 Pro656 pocket via a stacking interaction.

**XTBD is a general XRN2 adaptor**

Although different XTBD-containing proteins occur in complexes with XRN2 (refs. 3,23–25), these interactions have generally been examined through coimmunoprecipitation, thus leaving it unclear whether they are direct and are mediated by XTBDs. The high degree of conservation of the interaction surfaces of both XRN2 and XTBD (Fig. 2a and Supplementary Fig. 3b) supports a conserved and direct interaction. To test this notion experimentally, we produced recombinant XRN2 and the XTBD-containing proteins CDKN2AIP and...
CDKN2AIPNL (C2AIL in the following) from *Danio rerio* (zebrafish) in *Escherichia coli* and determined their interactions. Whereas CDKN2AIP is a tumor suppressor that can activate p53 (refs. 37–39), HsC2AIL can substitute for *C. elegans* XRN2 (zebrafish) and is as yet uncharacterized. When purifying the N-terminally point-mutant variants coexpressed with XRN2. M, marker; mol. mass, molecular mass. (Fig. 3a) Gel showing results of Ni-NTA–mediated pulldown of His6-PAXT-1 and its position-specific conservation scores computed by ConSurf33,34 are displayed from white (weak to average conservation, ConSurf levels 1–6) to red (most conserved, level 9). The dashed-dotted line on XRN2 projection of bound XTBD. Dashed lines on XTBD indicate residues analyzed in detail. (Fig. 3b) Representation of XTBD residues Cys54 and Tyr56 and zoomed-in views showing (c) interactions of the critical Tyr56 and (d) side chain arrangement of Cys54 and Tyr56. Hydrogen bonds are indicated by gray dotted lines. (e) Gel showing results of Ni-NTA–mediated pulldown of His6-PAXT-1 and its point-mutant variants coexpressed with XRN2. M, marker; mol. mass, molecular mass. (f) Stereomicroscopic images of *C. elegans* grown as indicated at top. The paxt-1(xe29) allele encodes PAXT-1 1–121 and was obtained by genome editing of endogenous *paxt-1*. (g) Western blot of 100 μg lysate from worms of the indicated genotype grown at 26 °C. Antibodies used were specific to the indicated endogenous proteins. Actin, ACT-1 loading control.

**XTBD binding or substrate binding stabilizes XRN2**

Previously, we have examined the influence of PAXT-1 on the enzymatic activity of XRN2 (ref. 23) and have found that the PAXT-1–XRN2 complex has greater activity than that of XRN2 alone over extended periods of time. However, initial velocities remain unchanged in the presence or absence of PAXT-1 (ref. 23), and examination of Michaelis-Menten enzyme kinetics also failed to provide evidence for modulation of XRN2 activity by PAXT-1 (Supplementary Fig. 4a). Because XRN2 protein but not mRNA levels are reduced in *C. elegans* lacking PAXT-1 (ref. 23), PAXT-1 thus appears to have a stabilizing rather than stimulatory effect on XRN2. To test this hypothesis experimentally, we monitored unfolding of recombinant XRN2 and its complex in *vitro* and determined the melting temperature, Tm (Fig. 4a). We found a striking shift in Tm of 24 °C, from Tm = 31 °C for XRN2 to Tm = 55 °C for the PAXT-1–XRN2 complex, thus demonstrating a pronounced stabilizing activity of PAXT-1 toward XRN2. The activity largely resides in the XTBD, because the Tm values of PAXT-1–XRN2, PAXT-1–XRN2ΔZLC and XTBD–XRN2ΔZLC complexes differed little at 55 °C, 54 °C and 53 °C, respectively.

Although PAXT-1 is dispensable at lower temperatures, *paxt-1(0)* animals arrest as L1-stage larvae when grown at 26 °C, a phenotype that is rescued by expression of PAXT-111–121 (ref. 23). Strikingly, the HsC2AIL–transgenic worms similarly developed into adults and were able to produce F2 progeny (Fig. 3e). Hence, not only the molecular but also the developmental function of PAXT-1 can be compensated for by HsC2AIL, despite only ~35% sequence identity between their XTBDs. This result is consistent with the good correlation of the C2AIL homology model with the XTBD structure (Supplementary Fig. 3a).

**HsC2AIL can substitute for *C. elegans* PAXT-1 in vivo**

To validate our insights into XTBD conservation from *in vitro* and cell-based studies *in vivo*, we expressed a codon-optimized HsC2AIL single-copy transgene in *paxt-1(0)* mutant bearing a FLAG sequence in *C. elegans*. Endogenous *C. elegans* XRN2 coimmunoprecipitated with FLAG-HsC2AIL from worm lysates, as confirmed by western blotting (Fig. 3d and Supplementary Data Set 1) and mass spectrometry (data not shown). This confirms the formation of a chimeric complex *in vivo*.
At 31 °C, the $T_m$ of XRN2 alone was close to the temperature at which paxt-1(0) larvae arrest development (26 °C), thus suggesting that XRN2 destabilization in vivo may occur through its unfolding and subsequent degradation. To examine the consequences of temperature increases on XRN2 activity in vitro, we assessed XRN2 destabilization at two temperatures within, or close to, the physiological range of C. elegans: 25 °C, which is the default temperature of previous activity assays, and 30 °C. From the melting curve, we estimated that 45% of XRN2 would be unfolded at 30 °C. Nonetheless, and contrary to our expectations, we found that XRN2 activity was consistently, and over extended times, higher at 30 °C than at 25 °C (Fig. 4b).

Although an increase in temperature accelerates enzymatic reactions, according to Arrhenius's equation we expected at most a modest effect with the narrow temperature window that we used. Hence, we considered that XRN2 might be unexpectedly stable under the conditions of the assay, possibly because of substrate binding. To test this, we examined XRN2 thermal stability in the absence and conditions of the assay, possibly because of substrate binding. To understand the interaction of XRN2 with substrate, we superimposed the XRN1–RNA substrate complex (PDB 2Y35 (ref. 20)) onto the XTB–XRN2 structure. This revealed a crucial role of the PBS-prolonging loop in substrate binding, in which its Trp670 base-stacks with three unpaired 5′-terminal nucleotides and His60 of α-helix 2 (Fig. 4e). Moreover, Trp670 in XRN2, like Trp540 in XRN1, which was previously crystallized in the absence of both substrate and additional proteins (PDB 3P1T)24. Strikingly, whereas the PBS is fully structured in XRN2–XTBD and Drosophila XRN1–RNA complexes, it is heavily disordered in the structure of K. lactis XRN1, and the domain could not be entirely built in the crystallographic model (Fig. 4f). Hence, although they do not constitute proof, these structures are consistent...
with the notion that stabilization of XRN2 is achieved through maintenance of the integrity of the PBS, which can be accomplished by both XTBD binding and substrate binding (Fig. 4f).

**DISCUSSION**

On the basis of the recurring identification of XTBD (DUF3469)-containing proteins in complexes that also contain XRN2 (refs. 3,23–25), we have previously hypothesized that XTBD might mediate XRN2 binding. Here, we tested this notion and demonstrated that a core XTBD, substantially smaller than initially predicted, mediates binding to XRN2. Chimeric interactions between XTBD-containing proteins and XRN2 from different species in vivo and in vitro confirmed the generality of this finding. Although XRN2 and its paralog XRN1 are highly similar, notably in the PAXT-1–binding groove (Supplementary Fig. 1e), binding of PAXT-1 to XRN1 is disfavored: the NCS2 of XRN1 extends further into the groove than that of XRN2, thus suggesting a steric clash with XTBD (Supplementary Fig. 1f) XRN2 in the presence of an RNA substrate or tRNA. Results for XRN2 alone and XRN2–PAXT-1 from a are included for comparison. (a,c) Data points of the sigmoidal part of each melting curve and their fits to the Boltzmann equation, normalized to 1 and scaled, are indicated. Squares represent melting points ($T_m$), A.u., arbitrary units. (b) Plot of activity over time of XRN2 and its complex at 25 °C and 30 °C. Mock, negative control (no enzyme) at 30 °C. (d) Plot of activity of XRN2 alone and its complex with or without preincubation at 30 °C. (e) Representation of XRN2 substrate binding, inferred by superposition of XRN2$Δ^{ZLC}$ (gray, red and cyan) in complex with XTBD (yellow) on D. melanogaster (Dm) XRN1 in complex with substrate (PDB 2Y35 (ref. 20)). For the latter, only substrate is shown, in stick model. An $m_F$–$D_F$ map of the XTBD–XRN2$Δ^{ZLC}$ complex (calculated in the absence of $SO_4^{2–}$) is overlaid and displayed at the $SO_4^{2–}$ position only (5.5σ). (f) Comparison of the PBSs of C. elegans XRN2 (cyan) in complex with XTBD (yellow, surface representation), DmXRN1 (purple; PDB 2Y35 (ref. 20)) in complex with a trinucleotide substrate (stick model) and K. lactis (Kl) XRN1 (orange; PDB 3PIF (ref. 39)) by superposition (top) or individual representation (bottom). Disordered regions within the K. lactis PBS are marked in orange dashed lines.

**Figure 4** XTBD or RNA substrate stabilize XRN2 by promoting PBS folding. (a,c) Graphs showing unfolding of (a) XRN2 alone or its indicated complexes and (c) XRN2 in the presence of an RNA substrate or tRNA. Results for XRN2 alone and XRN2–PAXT-1 from a are included for comparison. (a,c) Data points of the sigmoidal part of each melting curve and their fits to the Boltzmann equation, normalized to 1 and scaled, are indicated. Squares represent melting points ($T_m$), A.u., arbitrary units. (b) Plot of activity over time of XRN2 and its complex at 25 °C and 30 °C. Mock, negative control (no enzyme) at 30 °C. (d) Plot of activity of XRN2 alone and its complex with or without preincubation at 30 °C. (e) Representation of XRN2 substrate binding, inferred by superposition of XRN2$Δ^{ZLC}$ (gray, red and cyan) in complex with XTBD (yellow) on D. melanogaster (Dm) XRN1 in complex with substrate (PDB 2Y35 (ref. 20)). For the latter, only substrate is shown, in stick model. An $m_F$–$D_F$ map of the XTBD–XRN2$Δ^{ZLC}$ complex (calculated in the absence of $SO_4^{2–}$) is overlaid and displayed at the $SO_4^{2–}$ position only (5.5σ). (f) Comparison of the PBSs of C. elegans XRN2 (cyan) in complex with XTBD (yellow, surface representation), DmXRN1 (purple; PDB 2Y35 (ref. 20)) in complex with a trinucleotide substrate (stick model) and K. lactis (Kl) XRN1 (orange; PDB 3PIF (ref. 39)) by superposition (top) or individual representation (bottom). Disordered regions within the K. lactis PBS are marked in orange dashed lines.

Note: The text is not fully transcribed due to the image format limitations. The full text is available in PDF format.
immediately in front of the XRN2 nuclease cleft, and because analysis by PSIPRED and DisEMBL suggests that the C terminus is structured (data not shown). Because we have thus far been unable to observe regulation of XRN2 activity by PAXT-1 in vitro, such functions might require specific substrates or conditions that remain to be identified. Alternatively, the PAXT-1 C terminus might modulate PAXT-1 stability or binding to XRN2.

In support of a more specialized role of the PAXT-1 C terminus, this region is dispensable for C. elegans viability. Together with the ability of heterologous HsC2AIL to substitute for PAXT-1 in vivo, this finding implies functions of XTBD beyond that of a passive XRN2 adaptor. We propose that one such function might be stabilization of XRN2 by promoting folding of its PBS segment. The Tm is a measure of a protein's thermal stability and depends on numerous molecular parameters such as the amino acid composition, surface hydrophobicity, number of hydrogen bonds and labile secondary structures such as loops. Upon XTBD binding, 13 hydrogen bonds are formed, a hydrophobic patch is covered, and the flexibility of the PBS loop is reduced. Decreased thermal stability of XRN2 in the absence of XTBD in vitro may then reflect decreased half-life in vivo, because even partially unfolded proteins exhibit increased susceptibility to proteolysis. These results then explain how PAXT-1–XTBD binding increases XRN2 levels in vivo.

Although PAXT-1 is expressed throughout C. elegans development, its loss causes defects only at specific stages: when paxt-1(0) mutant animals are shifted to the restrictive temperature of 26 °C during the L4 stage, they continue development through adulthood and produce F1 progeny, which, however, arrest at the L1 stage. This delayed effect does not simply reflect slow depletion of XRN2, because L1-stage arrest of F1 animals also occurs if parental animals are shifted to 26 °C during their L1 stage. Moreover, although animals may conceivably be more dependent on XRN2 activity during some developmental stages than others, developmental arrest can be induced in virtually any stage upon direct inactivation of XRN2 (ref. 15). Hence, we speculate that it is the stability of XRN2 that varies with developmental stage. Specifically, substrate binding may confer some stability to XRN2, and the stabilizing activity of PAXT-1 may be more important when substrate levels are low. Thus, a key function of PAXT-1 may be to preserve, rather than promote, XRN2 activity, thereby guarding enzyme not bound by substrate against decay and causing a constant pool of active protein to be retained.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank under accession code PDB 5FR1.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.R. and H. Großhans designed research. H.R. designed and performed in vitro and in vivo experiments. H. Gut and H.R. performed crystallographic structure determination and analyses. I.K. performed clustered regularly interspaced short palindromic repeats (CRISPR) procedures, injected worms and assisted in crossing worm lines. H.R., H. Großhans and H. Gut wrote the manuscript. All authors edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Experimental replicates and sample sizes. The following numbers of replicate experiments were performed: Figure 2e, n = 3; Figure 2f, n = 3; Figure 2g, n = 3; Figure 3a, n = 2; Figure 3b, n = 2; Figure 3d, n = 2; Figure 3e, n = 3; Figure 4a, n = 3; Figure 4b, n = 2; Figure 4c, n = 2; Figure 4d, n = 3. For enzymatic and unfolding assays in Figure 4 and Supplementary Figure 4, replicate experiments were carried out on separate occasions but used the same sample preparations. All other experiments used biological replicates. For C. elegans experiments, animals were cultured independently, on separate occasions, for replicate experiments.

Animal experiments. Experiments were not blind or randomized; no statistical calculations were performed to predetermine sample size. Animal procedures were approved by the Veterinary Department of the Canton Basel-Stadt (Switzerland).

Protein expression and purification. Expression constructs were cloned into a modified bicsistrionic pCOLADuet vector from Novagen. XTBD-containing constructs were cloned into the first multiple cloning site (MSC) and thus N-terminally extended by a hexahistidine (6xHis) tag followed by a tobacco etch virus protease (TEV)–cleavage site, whereas the XR2N constructs in the second MSC remain untagged. All constructs were expressed in E. coli BL21 (DE3). Cells were lysed by sonication, and the lysate was cleared by centrifugation. Immobilized metal affinity chromatography (IMAC) slurry (Qiagen) was used to bind the His6 tag of the target protein. Next, proteins were eluted through cleavage by TEV (homemade)50. IMAC was repeated, and the unbound fraction was collected, diluted and purified on a Mono Q 5/50 GL (GE Healthcare) anion-exchange column. Positive fractions were pooled, filtered with a Milllex-GV 0.22-µm filter and loaded onto a Superdex 200 HiLoad 16/600 (GE Healthcare) column. Finally, the target fractions were collected and concentrated with Millipore Amicon Ultra centrifugal filter concentrators with suitable pore size.

Incorporation of selenomethionine was performed as described by Molecular Dimensions’ SelenoMethionine media kit, with E. coli BL834 (DE3).

Crystallization, data collection, and structure solution. Protein crystallization was carried out at 20 °C with the sitting-drop vapor-diffusion method with a Phoenix robot (Art Robbins) dispensing 300-nl drops (100 nl crystallization buffer and 200 nl protein solution). XR2NΔZLC–XTBD crystals, obtained in 2 M ammonium sulfate, 0.1 M Bis-Tris, pH 6.5, or 0.1 M Tris, pH 8.5, were harvested and flashed and cooled in liquid nitrogen after cryoprotection with 3.2 M ammonium sulfate, 0.1 M Bis-Tris, pH 6.5, or 0.1 M Tris, pH 8.5. The highest-resolution data were collected at the SLS PX-II beamline (Villigen, Switzerland) and allowed, 99.6%; outliers, 0.4%) and the wwPDB Validation Server (http://wwpdb-validation.wwpdb.org/validservice/). Atomic coordinates and structure factors for the XR2NΔZLC–XTBD complex have been deposited in the PDB under accession code 5FIR. Structural images for figures were prepared with PyMOL version 1.3.1 (http://www.pymol.org/).

Sequence logo. The profile hidden Markov model (HMM) of DUF3469 from Pfam was exported and uploaded to http://skyline.gr/49. For concise representation, the following parameters were set: (i) alignment Processing to ‘Create HMM - remove mostly-empty columns’, (ii) fragment handling to ‘Alignment sequences are full length’ and (iii) letter height to ‘Information Content - Above Background’. The resulting logo was exported without showing the gap parameters.

Thermal shift assay. In a 20-µl reaction, 2 µg of assayed protein was mixed with Protein Thermal Shift Dye and Buffer from Applied Biosystems to a final concentration of 1×. In the presence of RNA substrate, however, 1 µg of protein was used instead to reduce the otherwise high concentrations of RNA. Purified yeast tRNA (Ambion) or a 30-mer XR2N substrate identical to that used in the kinetic assays (described below) were used when examining the effect of substrate binding. The reactions were prepared in a 96-well MicroAmp optical microplate at 4 °C, which was sealed with MicroAmp Optical Adhesive Film. After centrifugation for 25 s at 1,000 g, the plate was read in a StepOnePlus real-time PCR system with a melting-curve program from 25–99 °C. No normalizing quencher was used, and reactions were run in triplicate. Raw data were exported and plotted with Microsoft Excel. The sigmoidal part of the curve was fit to the Boltzmann equation (equation (1)) with nonlinear regression by maximizing R² and varying Tm and C, and the melting point, Tm, was calculated as the inflection point. The resulting function was normalized to 1 and plotted (as described below).

Raw data were also normalized to 1 and plotted as dashed lines. In addition, XR2N, XR2N + tRNA, and XR2N + RNA curves and values were rescaled by a factor of 1.9, 1.3, and 1.8, respectively, for concise representation.

where Fpre is baseline fluorescence, Fpost is the maximal fluorescence, T is the temperature in °C, Tm is the melting temperature in °C, and C is enthalpy.

Real-time exonuclease activity assay and kinetic analysis. The kinetic assay was performed as described in ref. 56 with the following modifications. The RNA-DNA duplex substrate, in which the RNA was FAM labeled, and the DNA carried a quencher, was diluted with a four-fold excess of unlabeled 5’-phosphorylated let-7, which increased the dynamic range of the qPCR system. 300 fmol enzyme was assayed with varying concentrations of total substrate (sum of RNA-DNA duplex and let-7) in an excess of 5 to 200 fold. Each kinetic reaction was run in triplicate and yielded one data point. After each data point was normalized to its baseline, the initial linear phase from 0 to 180 s was fitted by linear regression. The slope of this function equals the velocity at the corresponding substrate concentration, [S]. Furthermore the average of three velocities was plotted over [S] and fitted to the Michaelis-Menten model with the least-squares method:

where V is velocity, Vmax is maximal velocity, [S] is substrate concentration, and Km is the Michaelis-Menten constant. A 50% substrate excess was used in Figure 4b. The preincubation time in Figure 4d was 5 min at 30 °C.

Strains. Standard procedures were used to culture the Bristol N2 wild-type, mutant and transgenic strains57: HW1091, paxt-1(xs5) is the null mutation described by ref. 23; HW 1614, paxt-1(xs5); e; sex203(xs1); paxt-1:1::C. elegans_co_Hs C2A11_FLAG_operon_GFP_tf1-2UTC unc-119(+); B; and HW 1644, paxt-1(xs5): [I 8597012–8597014 → TAT → GCT, I; 8597041 A → T7] (expressing PAXT-1 Y56A and containing a silent mutation to facilitate screening for genome editing events) are described in detail below.

Genome editing of C. elegans with CRISPR-Cas9. A suitable site for a 20-nt-long sgRNA was identified by considering the availability of a PAM site to create a double-strand break on the paxt-1 gene. Two complementary oligonucleotides were hybridized and cloned by Gibson assembly58 into pIK111, a derivative of the PU6:: sgRNA backbone59,60. Furthermore, a 100-nt DNA single-stranded repair oligo was designed with the desired point mutation and a silent mutation at the sgRNA-binding site. A CRISPR coconversion mix (analogous to that in
ref. 35), also containing Peft-3::Cas9::tbb-2′-UTR (ref. 36), the sgRNA and oligonucleotide to recreate the sqt-1(sc1) mutation was injected into N2 animals35. Roller progeny were cloned, and their progeny were analyzed for the desired mutations by PCR.

**Rescue experiments with MosSCI.** The paxt-1 promoter, H. sapiens C2AII (codon optimized plus two artificial introns) coding sequence and tbb-2′-UTR sequences were cloned into pCEF header by Gateway cloning. An operon-GFP sequence between C2AII and the tbb-2′-UTR was included to permit visualization of transgene expression without altering the sequence of the encoded protein. The resulting plasmid was inserted into the ttx5605 site by MosSCI (refs. 62,63), thus yielding xehs203[paxt-1::C. elegans co_Hs_C2AIII_FLAG_operon_GFP_tbb-2′_UTR unc-119(+)] II animals. The integrants were outcrossed to wild-type N2 and crossed into the paxt-1(0) mutant. Rescue of the paxt-1(0) mutant phenotype was examined as previously reported23.

**Cell culture and transient transfection of HEK293T cells.** HEK293T cells were available in our laboratory and were tested for mycoplasma contamination and grown in DMEM-F12 (Gibco) supplemented with 10% (v/v) FCS and 25 U/ml penicillin-streptomycin (Gibco) at 37°C. Before transfection (described in ref. 64) with the PEI method, 2 × 10⁶ cells were plated on a 6-cm dish per construct and grown for 1 d. Each transfection was run in two technical duplicates with plasmids derived from pIREsneo (Addgene 10822) to encode N-terminally FLAG-HA–tagged wild-type or Y82A-mutated Hcc2AII. pSD44 containing a GFP sequence was used as a mock control. It was modified from the pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene 12252) backbone, containing the SV40 enhancer/early promoter driving expression of a puromycin-selectable marker. Cells were harvested 72 h after transfection.

**Western blotting and coimmunoprecipitation experiments.** Worm lysates were prepared by Douche homogenization of harvested worms with 150 pestle strokes in extraction buffer (50 mM HEPES, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 0.1% w/v Triton X-100, and 10% w/v glycerol) with 7 mg protease inhibitor (Roche complete EDTA free), 2 µl 1 M DTT and 10 µl 100 mM PMSF added per milliliter of extraction buffer. The lysate was cleared by centrifugation at 16,000×g for 20 min, and protein concentrations of the lysates were measured with Bradford reagent (Bio-Rad Protein Assay). Subsequently, 100 µg of lysate was loaded and separated by a Bis-Tris SDS-PAGE 4–12% gradient gel (NuPAGE). The lysate was cleared by centrifugation at 16,000×g for 10 min. For each IP, 40 µl of bead slurry (described above) was washed twice with cold TBS (50 mM Tris, pH 7.4, and 150 mM NaCl), added to the lysate and filled to 1 ml final volume with TBS. After 2 h of incubation on the rotating wheel, beads were washed three times with 400 µl TBS and boiled at 70°C for 10 min in 40 µl SDS loading buffer for western blot analysis detecting the transgene with anti-HA antibody (1:1,000 dilution; clone 3F10, Roche). Human XRN2 was detected as described23.

**Microscopy.** Stereoscopic images were taken with a M205 A stereo microscope (Leica).

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