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Information available at cut rates: structure and mechanism of ribonucleases

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Ribonucleases are counterweights in the balance of gene expression and are also involved in the maturation of functional RNA. Recent structural data reveal how ribonucleases recognize and cleave targets, in most cases with the catalytic assistance of metal cofactors. Many of these enzymes are 'processive', in that they make multiple scissions following the binding of substrates; crystallographic data can account for this solution behaviour. These data not only explain how ribonucleases turn over transcripts, but also provide hints about how they often play dual roles in quality control checks on structured RNA.

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Introduction

Only a few decades ago, when the genetic code was still in its infancy of abstraction, Crick, Brenner, Jacob and Monod reasoned that the information encoded by DNA must be converted into protein through an intermediary that is short-lived and consequently does not accumulate in the cell. This message was subsequently shown to be composed of RNA and its short life is a well-known curse of experimentalists who try to extract it from cells. The instability is caused principally by the activity of ribonucleases, which are abundant in cells and seem to lie eagerly in wait of vulnerable substrates. Seemingly nuisances, these enzymes in fact play important roles in the regulation of gene expression, for example, in the course of development or in response to environmental change [1]. In prokaryotes, nucleases affect the differential processing and rate of degradation of polycistronic transcripts, and thus may contribute to the coordinated stoichiometric synthesis of the subunits of multicomponent complexes [2].

The apparent wanton destruction of RNA by ribonucleases poses several questions: how do cells maintain folded RNA structures, such as tRNA and ribosomes, in the presence of all these keen ribonucleases? Conversely, how are unstructured or damaged RNAs identified and turned over? Lastly, many different types of structured RNAs are synthesized as precursors that must be trimmed to form the mature species. Are there dedicated, highly specific nucleases that do this controlled trimming? Perhaps the answer to all these questions stems from the observation that the turnover of mRNA and the processing of structural RNA share common steps; for instance, in *Escherichia coli*, the processing of rRNA and the degradation of mRNA both start with endonucleolytic cleavage followed by exonucleolytic attack on the fragments [3••]. Many of the ribonucleases that turn over transcripts play dual roles in the degradation of stable RNA and the processing or fidelity checking and stress-triggered decay of structured RNA. As proposed by Deutscher [3••], the structure of the RNA itself may be the signal that steers it to a course of maturation or to a fate of destruction; we shall explore how these implicit signals are recognized. As is often the case, eukaryotes are more complicated and might have dedicated complex machinery for the different tasks of degradation and quality control [1]. Here, the choice of processing versus turnover may be affected by the time lag of processing, in analogy to kinetic proofreading of translational fidelity [4••].

We summarize some recent structural and functional data that provide insight into these processes, as represented by key bacterial ribonucleases and a few from archaea and eukaryotes. For convenience and perspective, we group these ribonucleases into the context of enzymes from *E. coli*, an organism for which we are rapidly approaching comprehensive coverage (Table 1). Not all of our questions about ribonucleases are answered by these structures, but some good hints are provided. The role of RNA structural determinants is a unifying theme, but the related catalytic chemistries also provide another connection between these new structures.

Structure and function of RNase E

In *E. coli*, the endoribonuclease RNase E plays a key role in initiating transcript turnover [5]. RNase E is an essential enzyme that affects the balance and composition of the mRNA population [6,7]. RNase E targets specific transcripts for destruction, in conjunction with the destruction of small regulatory RNAs [8]. In addition to its role in destroying transcripts, RNase E also plays a creative role in generating certain RNA species by maturing larger precursors. These include rRNA (9S

Table 1

Summary of recent structural studies of ribonucleases, grouped according to the closest homologue from *E. coli*[a].

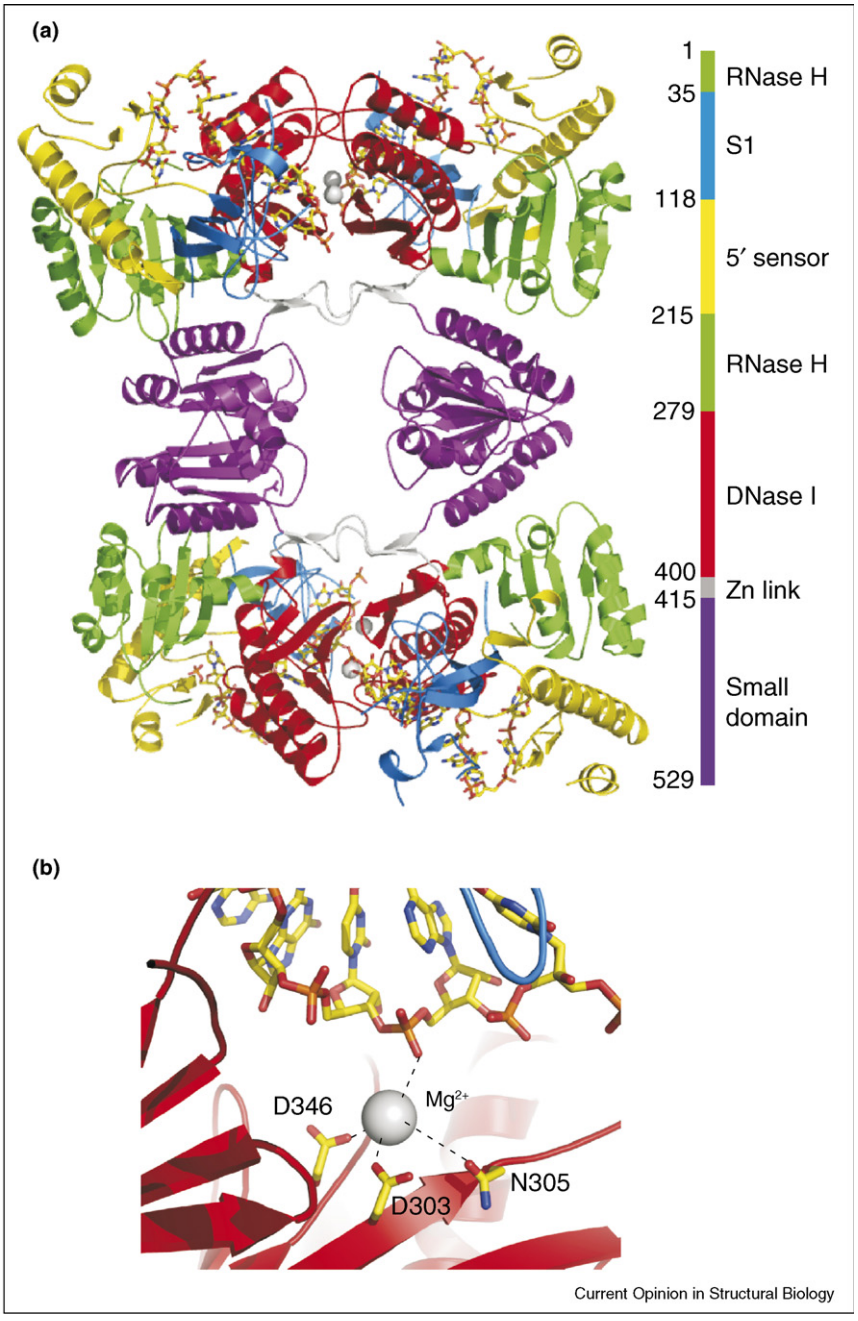
Family representative from *E. coli*	Function	Mechanism	Architecture	Distribution	References
RNase E	Initiates transcript turnover; processes structured RNA precursors.	Hydrolytic ssRNA endoribonuclease. Activated by 5′ monophosphate. Mg^{2+} dependent.	Modular: DNase I like active site, RNase H domain and an S1 domain.	Paralogue RNase G found in most bacteria (*B. subtilis* is a notable exception).	[14••]
RNase II	mRNA degradation	Hydrolytic, processive 3′ to 5′ ssRNA exoribonuclease. Possibly two Mg^{2+} ion dependent.	Modular: S1 domain and two CSDs.	Related to subunits of the exosome (see also PNPase below). Homologue RNase R can 'drive through' secondary structure in substrates, such as rRNA. Homologues of RNase R are components of the exosome.	[21••,22••]
RNase III	Processes rRNA, mRNA, small non-coding RNAs and (in eukaryotic homologues) small nuclear RNAs.	Hydrolytic endoribonuclease specific for dsRNA, leaves two-base 3′ overhangs.	Modular: endonuclease domain (endoND) and dsRNA-binding domain (dsRBD).	All domains of life. Homologues include Dicer from RNAi machinery. Bacterial enzymes have one endoND and dsRBD. Eukaryotic Dicer has two endoNDs and one dsRBD.	[25,26,48]
RNase H	Specific for DNA–RNA hybrids	Hydrolytic endoribonuclease. Two Mg^{2+} ion dependent.	Mixed α/β with αβα Rossmann-like fold. Found in non-enzymatic context as a structural domain.	All domains of life. Nucleotidyl-transferase superfamily, including DNA transposases, retroviral integrase, Holliday junction resolvase and Argonaute nuclease of the eukaryotic RISC.	[25,27•,28–31,49]
RNase BN	Involved in tRNA processing	Hydrolytic endoribonuclease. Zn^{2+} dependent (an exoribonuclease *in vitro*).	β-lactamase fold	Found in archaea, eukaryotes and most eubacteria. The β-lactamase fold is also likely to occur in *B. subtilis* RNase J1 and J2, which are functional (not sequence) homologues of RNase E.	[32•–34•,50]
RNase PH	RNA maturation, rRNA processing, mRNA degradation.	3′ to 5′ ssRNA exoribonuclease using a phosphorolytic cleavage mechanism. Gene duplication of RNase PH fold found in PNPase, a processive ssRNA exoribonuclease.	Compact α/β: the RNase PH fold.	Homologues of RNase PH found in the archaeal and eukaryotic exosome, with a similar protomer arrangement to that seen in PNPase, which itself has a modular organisation of S1 and KH domains and an internal duplication of the RNase PH fold.	[36•,37••,38•,51–53]
No known homologue	Processing U-rich segments of snoRNAs	Hydrolytic ssRNA endoribonuclease. Probably Mn^{2+} dependent.	New architectural class	XendoU from the amphibian *Xenopus*. Homologues found in mammalian viruses (SARS, mouse leukaemia); these form the NendoU subclass.	[43,44•,45,46•,47]
RNase D	tRNA processing	3′ to 5′ exoribonuclease characterised by conserved acidic residues. Two divalent metal ions (Zn^{2+}, Mn^{2+}) activate a water molecule for hydrolysis of the terminal phosphodiester.	Modular: two HRDC-like domains, which determine substrate specificity, and a catalytic DEDD domain (Figure 2).	Homologues include the nuclear exosome auxiliary factor Rrp6p from yeast, the proofreading subunit of DNA polymerase I and the poly(A)-specific ribonuclease.	[39•,40•,41]
RNase P	Matures the 5′ end of tRNA precursors	Hydrolytic Mg^{2+} ion dependent	Ancient ribozyme consisting of an RNA chain and a polypeptide chain.	Found in all domains of life.	[54–57]

[a] *E. coli* has eight 3′ to 5′ exoribonucleases, which can be arranged in four family groups according to structure or mechanism: PNPase and RNase PH; oligoribonuclease, RNase D and RNase T; RNase II and RNase R; and RNase BN. There are three endoribonuclease families: RNase E and RNase G; RNase III; and RNase I/M. Many of the enzymes have overlapping roles [3••]. RISC, RNA-induced silencing complex.

RNA), tRNA, tmRNA, which is required to rescue stalled ribosomes, and RNase P, a conserved ribozyme involved in tRNA processing [9–13].

The crystal structure of the RNase E catalytic domain has been solved in complex with RNA substrate at 2.85 Å (Figure 1a) [14••]. The ribonuclease is a composite of

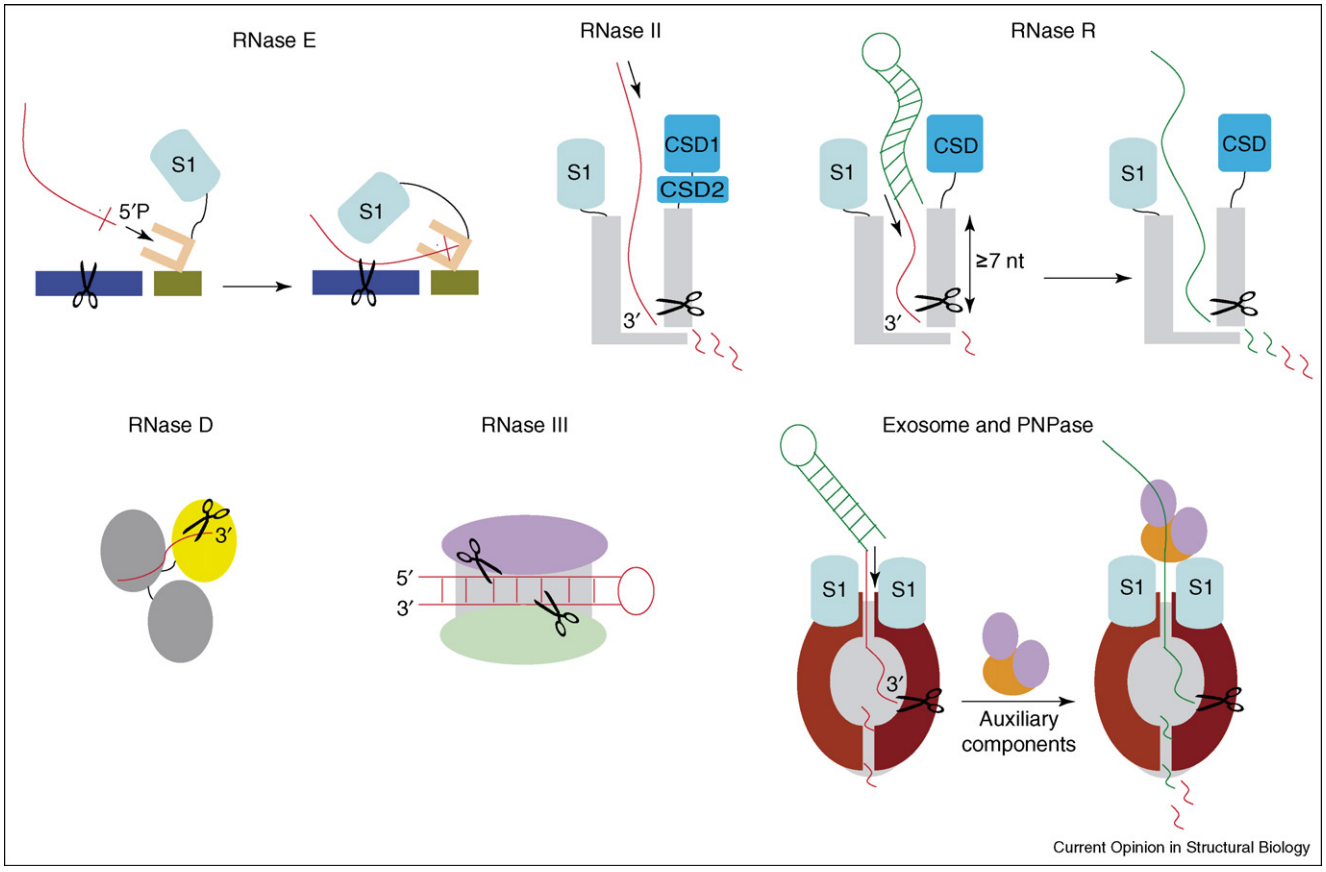
Figure 1



Escherichia coli RNase E. **(a)** Ribbon representation of the homotetramer of the catalytic domain of RNase E from *E. coli* (PDB code 2c0b). The different subdomains of the four protomers revealed by the structure are shown as indicated in the colour key, with a bound 13-mer ssRNA shown as sticks and magnesium ions as spheres. **(b)** Close-up view of the catalytic active site of an RNase E protomer. The magnesium ion is required to activate a water molecule for nucleophilic attack on the scissile phosphate of the RNA. Hydrogen-bond interactions between the magnesium ion, amino acid sidechains and the phosphate backbone of the ssRNA are represented as dashed lines.

recurrent folds, although these were not detected by sequence similarity. The catalytic domain of RNase E contains structural subdomains, such as DNase I and RNase H (Figure 1a), and an S1 subdomain, which occurs in many different RNA-binding proteins. In RNase E, the S1 subdomain clamps down on the RNA substrate and appears to be flexibly tethered to the body of the nuclease (see the summary in Figure 2). The catalytic site is situated in the DNase I subdomain, where a single metal-binding site is found (Figure 1b); however, it is possible that there is a second metal-binding site in the transition state. (The use of two-metal clusters as catalytic

Figure 2



Schematic summary of the modes of RNA processing of some of the endoribonucleases (RNase E) and exoribonucleases discussed in the text and in Table 1. Common protein domains are indicated and scissors represent the positions of the active sites. For the exosome and PNPase schematic, only one active site is represented for clarity. RNase D and RNase III share no common structural features with the other ribonucleases. The two-colour scheme for the RNA substrates of RNase R, the exosome and PNPase highlights how the duplex regions, indicated in green, become melted as the single-stranded regions, shown in red, are cleaved.

centres is a recurrent theme in other ribonucleases; see Table 1.) Cleavage is by nucleophilic attack on the phosphate backbone within a single-stranded A/U-rich region, generating fragments with a free 3′-OH (on the 5′ side of cleavage) and a 5′-monophosphate (on the 3′ side). The RNase E structure suggests that recognition of the 5′ end of the substrate induces a conformational switch that results in the phosphate backbone being oriented for attack by an OH group activated by a coordinated magnesium ion.

In *E. coli* and probably many other related Gram-negative bacteria, RNase E is part of a multienzyme assembly known as the RNA degradosome [15,16]. The other components of the degradosome are the glycolytic enzyme enolase, an ATP-dependent RNA helicase (RhlB) and the phosphorolytic exoribonuclease polynucleotide phosphorylase (PNPase). The physical association of these proteins within the degradosome coordinates their enzymatic activities. PNPase is a processive phosphorolytic exonuclease that may work together with RNase E to ensure the cooperative destruction of substrates once the first cleavage is made; it is also involved in checking the fidelity of rRNA precursors as a quality control mechanism [17]. RhlB is required to unwind structured RNA substrates so that they become suitable substrates for the nucleases of the degradosome. The role of enolase is unclear, but the available evidence indicates that its recruitment into the degradosome assembly is required to control levels of transcripts for the glucose transporter [18,19]. An enolase recognition motif occurs in RNase E homologues from other Gram-negative bacteria, including pathogens such as *Salmonella*, and it seems likely that, in these organisms, the enolase interacts with RNase E [20].

RNase II and RNase R

The exoribonuclease RNase II is representative of an extensive enzyme family found in all three domains of life, whose members play roles in the maturation, turn-

over and quality control of certain species of structured RNA [3••]. RNase II cuts single-stranded (ss)RNA processively in a 3′ to 5′ direction, using a hydrolytic mechanism, and releases nucleotide monophosphates as it gallops along (summarized in Figure 2). Crystal structures of the apo form of *E. coli* RNase II and of an inactive mutant in complex with ssRNA have been reported [21••,22••] (Figure 3a). This revealed another multidomain molecular montage, composed of two cold-shock domains (CSDs) at the N-terminal end, followed by a catalytic domain and, finally, an S1 domain. The X-ray data reveal that the single-stranded substrate lies deep in a channel at the bottom of which is the catalytic site [21••] (Figure 3a). One magnesium ion is present in the active site, coordinated by aspartates. The inactivating mutation in RNase II lies at a putative metal-coordinating residue, D209N, and Frazão *et al.* [21••] propose that two metals may be involved in the activation of a water molecule for hydrolysis of the terminal phosphodiester, analogous to the active sites of polymerases and perhaps some ribozymes [23]. This hypothesis is corroborated by the mutagenesis studies of Zuo *et al* [22••]. The organization of the active site of RNase II has striking parallels with the active site of the endoribonuclease RNase H; thus, even though the folds differ, the enzymes are likely to share similar catalytic chemistries.

RNase R is a homologue of RNase II, but with an intriguing and distinctive property: it appears to have a built-in ability to unwind the secondary structure of RNA substrates, even if they contain many strong G•C base pairs. In a striking parallel with certain types of RNA helicases, RNase R can sense the polarity of the phosphodiester backbone of the substrate. Thus, the ribonuclease greatly prefers substrates with single-stranded regions at the 3′ end. The ribonuclease can processively degrade structured substrates, provided they have a 3′ single-stranded overhang that is seven or more nucleotides in length [24•]. Cleavage occurs at the single-stranded 3′ overhang and proceeds processively in the 5′ direction, ploughing straight through the secondary structure (Figure 2). Whereas DEAD-box helicases use the free energy of ATP binding and hydrolysis to disrupt secondary structure, RNase R transduces the favourable free energy of RNA backbone hydrolysis into mechanical work that translates the single-stranded substrate further into the catalytic pocket, much like a ratchet. The linkage between RNA cleavage and unwinding of secondary structure is most likely indirect, and may involve changes in the energy of RNA binding to the catalytic pocket and to the S1 subdomain. One hypothesis is that the energy-dependent ratcheting mechanism pulls the duplex RNA against the apex of the tunnel, into which it cannot fit, thus causing it to unwind. It is not presently clear why RNase R manages to perform this operation, whereas homologous RNase II does not. Nevertheless, RNase R seems to have evolved a very clever mechanism to convert the energy of hydrolysis into work of unwinding, and thus seems to be a model of energy conservation. In eukaryotes, the RNase R homologue is a component of the exosome assembly, which we will discuss below.

RNase H and RNase III

Both RNase H and RNase III are representative of components of the RNA interference (RNAi) machinery, which is described elsewhere in this issue [25]. We mention them here briefly to emphasize the role of metals in catalysis, one common theme of the ribonucleases presented here (Table 1). RNase H enzymes cleave duplex RNA or RNA–DNA hybrids, and are representative of a large family whose members include transposases and the Argonaute ribonuclease, which is involved in RNA silencing. RNase III cleaves double-stranded (ds)RNA and the *E. coli* enzyme is a model system for the entire family, which includes eukaryotic enzymes such as Dicer (involved in the RNAi mechanism). The protein uses induced fit to recognize RNA substrates [26] (Figure 2). As seen in the other ribonucleases, divalent metals are again the key components of the catalytic site.

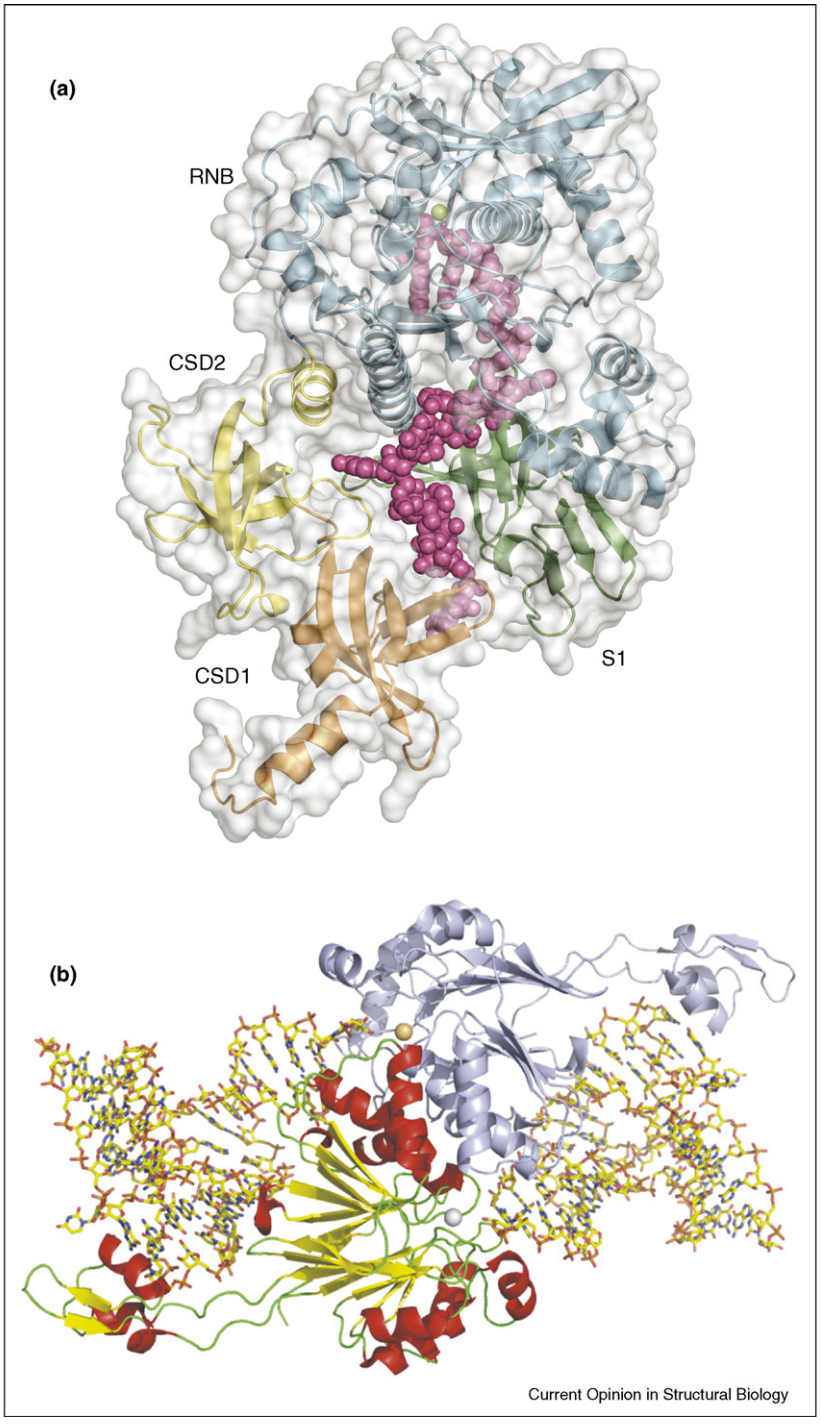
The structure of RNase H from mouse leukaemia virus has been solved at high resolution [27•] and corroborates the presence of a magnesium-binding site in the catalytic site, consistent with the two-magnesium mechanism proposed earlier by Yang *et al.* [28] for *E. coli* RNase H. A recent crystallographic analysis reveals the stepwise participation of the two magnesium metals in the RNase H mechanism, with the first participating in nucleophilic activation of water and the second metal stabilizing the transition state [29,30]. Reflecting the distinct roles of the two metals, they are coordinated in non-equivalent ways. By contrast, metal coordination is symmetrical in the RNase-H-like transposases; this is required because the metals have equivalent roles in the successive steps of nucleophilic activation of water during strand cleavage and 3′-OH activation during strand transfer [31].

RNase Z and the metallo-β-lactamase fold

RNase Z is a conserved endonuclease that cleaves tRNA precursors at the 3′ end in preparation for the addition of a CCA aminoacylation motif. The fold belongs to the β-lactamase structural family and the active site contains two coordinated zinc ions that participate in the hydrolysis of the RNA backbone. Structures are available for the apo and tRNA-bound forms from *Bacillus subtilis* (Figure 3b), revealing that substrate binding causes conformational moulding of both macromolecules to organize the catalytic site [32•,33•]. This induced fit appears to result in recognition of the shape and contour of the tRNA, as well as direct recognition of two conserved guanine bases.

B. subtilis has two other endoribonucleases that are proposed to have a similar metallo-β-lactmase fold: RNase J1

Figure 3



RNA recognition by RNase II and RNase Z. **(a)** Structure of the RNase II active site variant D209N from *E. coli* in complex with RNA (PDB code 2ix1). The individual domains are labelled and the 13-mer ssRNA, which was bound to the 'as isolated' variant, is shown as pink spheres. The magnesium ion found in the active site is represented as a green sphere. **(b)** Structure of RNase Z from *B. subtilis* bound to $tRNA^{Thr}$ (PDB code 2fk6). The monomer of the asymmetric unit is shown in red (for the Zn β-lactamase subdomain), yellow (strands) and green (coils). The functional dimer created by a symmetry-related molecule is shown in blue. The 52-nucleotide $tRNA^{Thr}$ is shown in stick representation, with the zinc ions involved in catalysis indicated as spheres.

and RNase J2 [34•]. These two enzymes appear to be RNase E homologues in function, but emphatically not in sequence or fold.

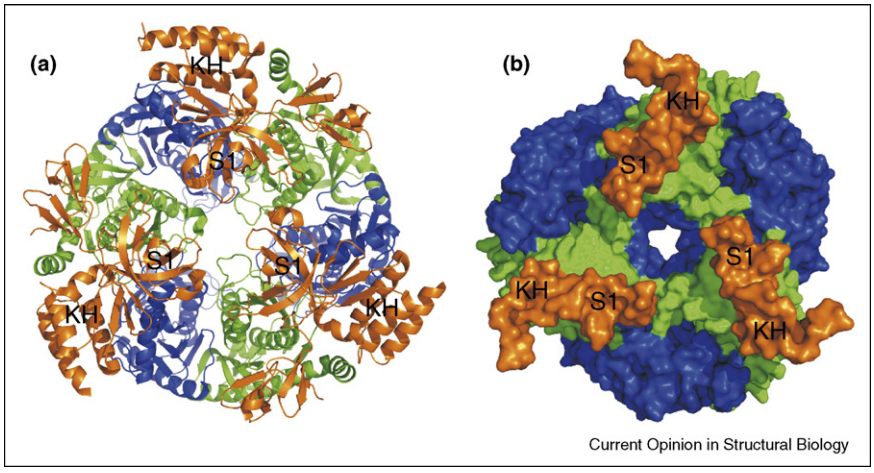
The exosome

In eukaryotes and archaea, the multienzyme exosome plays many key roles in RNA processing and turnover, RNAi and quality control surveillance. The central component of the exosome is a phosphorolytic ribonuclease that is structurally homologous to the bacterial PNPase of the degradosome. The subunits of the exosome and the subdomains of PNPase closely resemble the ancient fold found in the phosphorolytic exoribonuclease RNase PH [35]. Crystal structures of the core of the exosomes from the archaea *Sulfolobus solfataricus* and *Archaeoglobus fulgidus* have recently become available, revealing a hexameric ring comprising two types of RNase-PH-like subunits, known as Rrp41 and Rrp42 (rRNA-processing proteins 41 and 42; see Figure 4a) [36•,37••,38•]. Three of the exosome subunits are likely to have catalytic activity for phosphorolysis, whereas the other three might not. Instead, these non-catalytic subunits might provide a surface for the recruitment of the other exosome components. In the phosphorolytic reaction, the last 3′ phosphodiester bond of the substrate is attacked by phosphate, to leave a 3′-OH and nucleotide diphosphates.

In addition to the hexameric core, the archaeal exosome contains the subunits known as Rrp4, which have the S1 fold (also present in RNase E and RNase II) and the KH fold, another ancient and ubiquitous RNA-binding motif. The RNase PH, S1 and KH domains are organized into a quaternary structure that is remarkably similar to bacterial PNPase (Figure 4b). Although not yet elucidated, it is likely that this structure is also conserved in the eukaryotic exosome. The active site lies within the central channel of the hexameric ring and is 50–60 Å from the exterior surface that engages the Rrp4 subunit, suggesting that a product of 10–20 nucleotides is the limit of digestion; thus, auxiliary components might be needed to complete the processing of undigested residual fragments (Figure 2).

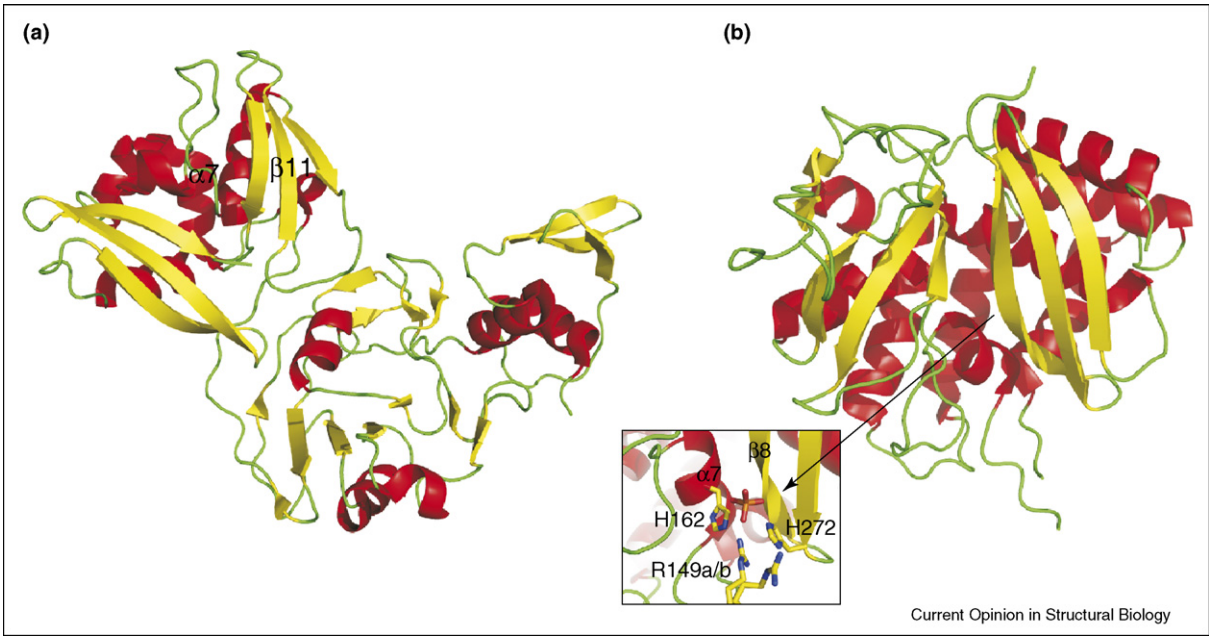
The auxiliary components of the eukaryotic exosome include ATP-dependent helicases and hydrolytic ribonucleases, such as homologues of RNase R (described earlier) and the metal-dependent exoribonuclease RNase D (described below) (Figure 2). In eukaryotes, exosomes are found in the cytoplasm and nucleus, where they have different auxiliary components and specialized function. The cytoplasmic exosome is involved in the turnover of both normal and defective transcripts [4••]. The nuclear exosome is involved in processing precursors of structured RNA, such as 5.8S rRNA, and in quality control surveillance of many different types of RNA [4••]. The structure of the nuclear auxiliary factor Rrp6p has been solved [39•], confirming that this enzyme is related to the RNase D family of exonucleases; this family includes the proofreading subdomain of DNA polymerase I [40•] and the poly(A)-specific ribonuclease [41]. In these enzymes, characterised by conserved acidic residues, two divalent metal ions activate a water molecule for hydrolysis of the terminal phosphodiester. The interaction of a C-terminal domain of Rrp6p with the exosome core might modulate its function [39•]. In eukaryotes, additional hydrolytic exoribonucleases function to hydrolyze substrates in

Figure 4



Ribbon or surface representation of **(a)** the archaeal core exosome from *A. fulgidus* (PDB code 2ba0) and **(b)** PNPase from *Streptomyces antibioticus* (PDB code 1e3p) viewed along the threefold rotation axis. In the exosome, a ring of alternating Rrp41 and Rrp42 subunits (green and blue) forms a hexameric core structure, with three Rrp4 subunits (orange) binding to the top face. The core domains of the exosome share the same RNase PH fold as the bacterial PNPase. The S1 and KH domains, common to both the exosome and PNPase structures, are indicated. The S1 domains of the Rrp4 subunits are ideally situated to guide the 3′ end of ssRNA into the pore (10 Å diameter) for processing. In the PNPase structure, the S1 domains are not well defined, suggesting high mobility in the absence of bound RNA.

Figure 5



Overall fold of two representatives of a new architectural class of endoribonuclease — the EndoU family. **(a)** NendoU, the nsp15 protein from the SARS coronavirus (PDB code 2h85). **(b)** XendoU from *X. laevis* (PDB code 2c1w). A close-up view of a region of the active site of XendoU is shown (inset). The active site is situated in a groove formed by β-strand 8 and α-helix 7, where a phosphate ion, shown in stick form, was found in the crystal. Active site residues His162 and His272 are required for substrate cleavage; Arg149 (found in one of two conformations) is proposed to form a stabilizing interaction with the negatively charged substrate. In NendoU, a similar groove-like feature housing the catalytic residues is found at the C-terminal end of the protein, between α-helix 7 and β-strand 11.

the 5′ to 3′ direction (Rat1 and Xrn1 and their homologues) [1].

EndoU: a new endoribonuclease structural class

In vertebrates, small nucleolar RNAs (snoRNAs) function in ribosome processing in the nucleolus; some have a role in the modification of bases or cutting of rRNA precursors [42]. Some snoRNAs are processed by endonucleolytic cleavage, through the action of enzymes such as the manganese-dependent endoribonuclease XendoU from the amphibian *Xenopus laevis* [43]. Sequence alignment reveals similar proteins in other eukaryotes (including human, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Arabidopsis thaliana*), nidoviruses and, remarkably, the cyanobacterium *Nostoc punctiforme* [44•]. This might indicate the versatile use of this fold in RNA processing. A homologous enzyme, known as NendoU, is a component of the genomic replication apparatus of the SARS virus and other members of the coronavirus family [45]. These enzymes, which we refer to as belonging to the EndoU family, are specific for uracil and the product has a 2′-3′ cyclic phosphate; this is usually an indication that the ribose sugar 2′-OH is oriented and activated to act as a nucleophile. Crystal structures have become available for the NendoU enzymes from SARS virus [46•] and mouse hepatitis virus [47], and for XendoU from *X. laevis* [44•]. These data reveal a common, unique fold that distinguishes the EndoU family from any other ribonuclease family identified thus far (Figure 5). Remarkably, the position of the key catalytic residues in the active sites of XendoU and NendoU mimics the position of corresponding residues in the ribonuclease RNase A (two histidines, which serve as general acid/general base, and a lysine) (Figure 5). This congruence is achieved with different folds and serves as a striking example of convergent evolution. The re-invention of a catalytic site usually indicates an important function, but the precise role of the viral protein is currently not well understood.

Conclusions

The use of metals to activate a nucleophile is a common theme for both endonucleases and exonucleases of the hydrolytic class. These hydrolytic reactions are highly exergonic; by contrast, phosphorolysis, in which inorganic phosphate attacks the backbone to drive bond cleavage, corresponds to a much smaller free energy change. It seems reasonable to expect that selective pressure would affect the balance of one reaction over the other. In the course of the evolution of eukaryotes, the associated genomic expansion and the tremendous amount of non-coding RNA necessitates significant investment of resources and energy. Perhaps the exosome represents an energy-efficient means of coping with the recycling of

oligonucleotides, compared with the costs associated with hydrolytic decay. Some hydrolytic enzymes, such as RNase R, can use the free energy of hydrolysis to 'drive through' secondary structure, so that this does not require the assistance of an energy-consuming RNA helicase. Most RNAs are spared this expensive mode of destruction unless they are specified by a single-stranded 3′ tail of minimal length or an exposed 5′ monophosphate together with a single-stranded region. As the structure rather than the sequence itself is recognized, the fold must be under considerable evolutionary scrutiny and might be optimised to fine-tune the levels of populations of transcripts, so that the enzymes they encode have an extra layer of regulatory connections. Therefore, one speculation is that the targeting of selected transcript classes for turnover by ribonucleases might also permit the coordinated regulation of different genes and thus might deepen connections between metabolic processes [16]. This hypothesis awaits testing.

Acknowledgements

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