RNase E and the high fidelity orchestration of RNA metabolism

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Summary

The bacterial endoribonuclease RNase E occupies a pivotal position in the control of gene expression, as its actions either commit transcripts to an irreversible fate of rapid destruction or unveil their hidden functions through specific processing. Moreover, the enzyme contributes to quality control of ribosomal RNAs. The activity of RNase E can be directed and modulated by signals provided through regulatory RNAs that guide the enzyme to specific transcripts that are to be silenced. At a comparatively early stage of its evolutionary history, RNase E acquired a natively unfolded appendage that recruits accessory proteins and RNA. These accessory factors facilitate the activity of RNase E and include helicases that remodel RNA and RNA-protein complexes, and polynucleotide phosphorylase, a relative of the archaeal and eukaryotic exosomes. RNase E also associates with

enzymes from central metabolism, such as enolase and aconitase. RNase E-based complexes are diverse in composition, but generally bear mechanistic parallels with eukaryotic machinery involved in RNA-induced gene regulation and transcript quality control. That these similar processes arose independently underscores the universality of RNA-based regulation in life. A synopsis and perspective is provided of the contributions made by RNase E to sustain robust gene regulation with speed and accuracy.

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Introduction

It may seem surprising that in almost all known lifeforms, information-encoding transcripts are actively annihilated. Although at first glance this seems to be a potential waste of resource and loss of information, the anticipated advantages of restricting transcript lifetimes include fast response rates and a capacity to rapidly re-direct gene expression pathways. In this way, destroying individual transcripts in a modulated manner might effectively enhance the collective information capacity of the living system. *Escherichia coli* has proven to be a useful model system to study such processes, and nearly forty-five years ago, a hypothetical endoribonuclease was proposed by Apirion as the key missing factor that might account for the observed degradation patterns of mRNA in that bacterium. At the time this hypothesis was formulated, transcript decay in *E. coli* was best described as a series of endonucleolytic cleavages and subsequent fragment scavenging by 3′-exonucleases [1]. A few years later, Apirion and colleagues reported the discovery of the endoribonuclease RNase E and showed it to be involved in processing of rRNA precursors [2–4], and the enzyme was subsequently discovered to also cleave an mRNA from T4 phage into a stable

intermediate [5]. Thus, RNase E seemed to be an ideal candidate for the proposed endonuclease factor to initiate RNA decay in bacteria. What made these findings surprising was that it had previously been thought that ribonucleases might be specialized, with one set presumed responsible for mRNA decay and another set dedicated to stable RNA processing, whereas RNase E could perform both these distinct tasks [6]. This broad functionality has been found to be a recurrent feature of other ribonucleases in *E. coli* and evolutionarily distant bacteria [7].

In the ensuing decades following the discovery of RNase E, more evidence and deeper insights have been gained into the function and importance of the enzyme in RNA metabolism. The data corroborate the numerous roles played by the ribonuclease, including the initiation of turnover for many mRNA species [8–12] and the maturation of precursors of tRNA [13, 14] and rRNA [15, 16]. The roles for RNase E have been expanded to include processing and degradation of regulatory RNAs [17, 18] and rRNA quality control [19].

It is important to note that RNase E is not the sole ribonuclease that can initiate turnover in *E. coli*, as others can catalyze the initial cleavage of mRNAs, including RNase G, RNase P, the double-strand specific RNase III, and RNases from the toxin/antitoxin families [20]

(see also ''Enzymes involved in post-transcriptional RNA metabolism''). Whether any particular RNA will be engaged by RNase E or another ribonuclease is determined by enzyme specificity, substrate accessibility, and which arrives first at the scene. RNase E appears to have privileged access to substrates, and despite the apparent functional overlap with other ribonucleases for all RNA metabolic processes, the enzyme is an essential gene under most growth conditions [21, 22], implicating a unique and dominating role.

The access of RNase E and other ribonucleases to substrates can be modulated by RNA-binding proteins [23]. For instance, the ribosome protein S1 can shield RNase E recognition sites and protect mRNAs against cleavage [24]. Ribosomes can protect mRNAs from enzyme attack during translation, but speculatively these might become accessible in a process of co-translational decay. RNase E recognition sites can either become buried or exposed in locally formed RNA structures [25]. These local structures can be induced or remodeled by base-pairing interactions formed in *cis* or *trans*, or by other binding proteins and the unwinding/remodeling activity of helicases. The actions of all these factors modulate substrate access.

In the degradation pathway of mRNA for *E. coli*, the initial cleavage of a transcript by RNase E is followed closely by

exonucleolytic degradation of the products by PNPase (polynucleotide phosphorylase), RNase II, or RNase R [26] (Figure 1, left panel). Depending on the organism, RNase E forms a complex with some of these exoribonucleases as part of a cooperative system referred to as the RNA degradosome [27]. These assemblies have some mechanistic parallels and, for one component in particular – namely, PNPase - evolutionary relationship to the exosome of archaea and eukaryotes [28]. The exosome complex, like RNase E, recruits RNA helicases and accessory ribonucleases that help to achieve efficient and complete substrate degradation [29, 30] (Table 1).

Figure 1. Ribonuclease dependent processes in bacteria. Ribonucleases play crucial roles in efficient removal of defective or unnecessary RNAs, regulation of gene expression by sRNAs and processing of various types of RNAs. RNA degradation (left panel) is initiated by endoribonucleolytic cleavage that can be preceded by pyrophosphate removal from the primary transcript. The majority of degradation initiation events are RNase E dependent. The initial cleavage generates monophosphorylated RNA fragments which can either boost subsequent RNase E cleavage, or become substrates for cellular exoribonucleases. Fragments resulting from exoribonucleolytic degradation are further converted to nucleotides by oligoribonuclease. When RNA degradation is mediated by sRNA (middle panel), sRNA-chaperones complexes (such as sRNA-Hfq) can recognise a complementary sequence near to the translation initiation region (TIR) and prevent ribosome association on the transcript (left branch). Naked mRNA is rapidly scavenged by endo- and exoribonucleases. The sRNA-Hfq complex can also bind within the coding region of mRNA recruiting RNase E and promoting transcript decay (right branch). In the case of substrates for processing (right panel), the order of RNA processing can be defined by the structure of

precursors and the specificity of the RNases. The processing can form a cascade of interdependent events where some target sites are being revealed only upon specific initial cleavage. RNA – dark blue, endoribonucleases – purple, exoribonucleases – light blue, sRNA – red, ribosomes – grey eclipses, Hfq – orange.

In *E. coli* and numerous other bacteria that are phylogenetically diverse, RNase E is membrane-associated [31–33], and this compartmentalization is expected to give an intrinsic temporal delay between transcription and the onset of decay [34]. mRNAs encoding membrane bound proteins were found to have lower half-lives due to co-translational migration of the mRNA to the membrane [33], where they might potentially become more accessible substrates for the membrane-associated degradosome.

RNase E can work in conjunction with small regulatory RNAs (sRNAs) and RNA chaperones like the Hfq protein to identify specific transcripts [35] (Figure 1, middle panel). The recognition specificity is achieved through base-pairing of an element in the sRNA, referred to as the seed, to the complementary region of the target. This mode of recognition resembles the use of seed pairing for target recognition by eukaryotic microRNAs and siRNAs, and with the bacterial CRISPR/Cas9 anti-phage system [36]. The silencing process also bears some mechanistic analogy to the eukaryotic regulatory RNA-induced silencing complex (RISC) formed by proteins of the Argonaute family

which uses ~22 nt guide RNAs. However, bacterial small RNAs are much larger than RNAi guide RNAs, and include structural and sequence elements used for recognition by Hfq and other facilitator proteins, such as ProQ and cold-shock proteins [37, 38]. The question arises how the seed-target pairings are guiding RNase E and the degradosome machinery to recognize transcripts tagged for degradation.

RNase E is also implicated in RNA processing, releasing mRNA from polycistronic transcripts and participating in sRNA, tRNA and rRNA maturation (Figure 1, right panel). In these processes RNase E is often assisted by other endo- and exoribonucleases, in tandem with which it leads to release of ready-made RNA (Figure 1, right panel).

An overview is provided here of the salient structural, functional and mechanistic features of RNase E from the perspective of accounting for the many *in vivo* functions of the enzyme. The accumulating data provide insights into how RNase E might operate in a cellular context as an intricate machine and interconnected hub of regulatory networks that finds and acts upon general substrates as well as specific targets with optimal speed and accuracy.

A Brief Evolutionary History of RNase E

At 1061 amino acids, RNase E is one of the largest proteins encoded by the *E. coli* genome (Figure 2). The enzyme has a conserved N-terminal domain of roughly 510 residues that encompasses the endonucleolytic active site. The remaining C-terminal portion is predicted to be predominantly natively unstructured, and consequently this region is anticipated to lack a compact and globular character [39]. Again, this gives RNase E the distinction of containing one of the largest encoded segments with this predicted disordered property within *E. coli* [40] (Figure 2). *E. coli* and many other species also express the non-essential RNase G, a paralogue of RNase E. In *E. coli* RNase G is 489 amino acids in length and has roughly 30% identity and close to 50% similarity in amino acid sequence over their common 430 residues, with maintenance of the key residues involved in substrate recognition and catalysis. RNase G and E are likely to have diverged after a duplication within a chromosome early in the evolution of the gamma-proteobacteria, but the enzymes still share some similar activities, including rRNA processing and mRNA turnover [34]. Orthologues of RNase E and RNase G are found in roughly 80% of all bacterial genomes sequenced to date [40]. Although

some Gram-positive bacterial species such as *Bacillus subtilis* encode neither RNase E nor RNase G, they nonetheless express functionally equivalent enzymes such as RNase J1/J2 or RNase Y [41–43] (see also ''RNases and helicases in Gram-positive bacteria'').

In the chloroplasts of plants, an RNase E homologue is implicated in polycistronic RNA cleavage [44] and counterintuitively deficiency of this enzyme appears to have a destabilizing effect on some transcripts [45]. Perhaps RNase E and its plant homologue could have similar roles with respect to non-coding RNAs. Some *E. coli* non-coding RNAs are also destabilized in the absence of RNase E, which may be due to direct interactions of RNase E and sRNA or result from changes in other mRNAs or unidentified interactors [46].

Domains, micro-domains and pseudo-domains of RNase E

X-ray crystallography analysis reveals a quilt work of domains for the N-terminal catalytic region of RNase E. There is an RNase H-like subdomain at the N-terminus probably fulfilling a structural function (1–35, 215-278), an RNA binding S1 domain (36–118), 5' sensor responsible for the enzyme preference of mono-phosphorylated substrates (119-214), DNase I domain (279-400), a

Zn-binding domain (401–414) that stabilizes dimer formation, and a small domain (415–510) that mediates tetramer formation at the dimer-dimer interface [47] (Figure 2).

The crystal structure of the catalytic domain of RNase E identifies two aspartic acid residues involved in metal ion coordination at the active site: D303 and D346 (Figure 2). The metal bound at this site was proposed to be magnesium, and/or manganese, which also supports RNA cleavage [48]. These metals are likely to help activate water for nucleophilic attack of the phosphate backbone. From the crystallographic data and simulations it is inferred that the scissile phosphate must approach the nucleophile in a defined geometry that is incompatible with the A-form conformation found in duplex regions [12, 47]. This requirement accounts for the observation that RNase E prefers single-stranded substrates. The sites recognised by RNase E can be accompanied by stem-loop structures, situated either upstream or downstream of the cleavage site.

The C-terminal domain of RNase E (CTD; 511–1061) forms a scaffold region that serves as a platform for the degradosome complex (Figure 2). Because it is predicted to be predominantly unstructured, it is not a conventional domain with compacted, folded character bearing defined secondary structural elements like strands and

helices. However, the CTD is punctuated by small microdomains that have structural propensity and which recruit protein partners [39]. Biophysical analyses indicate that recruitment of the partner proteins may help to partially compact the natively unstructured portion of the CTD into a conformation that may facilitate RNA interactions [49].

Figure 2. RNase E catalytic domain and a model of the organization of the *E. coli* RNA degradosome. Top panel: RNase E is a tetramer (purple – with a single protomer highlighted in dark purple), and the quaternary organisation is secured through zinc coordination (black spot) linking N-terminal domains. The C-terminal domain is predicted to be predominantly unstructured and provides binding sites for the other degradosome components: RhlB (green), enolase (yellow) and PNPase (blue). The C-terminus also harbours two RNA binding sites (red) and a membrane anchor (dark grey). Bottom panel: structure of the RNase E catalytic domain, with the subdomains of one protomer color-coded. Close view of the phosphate binding pocket (left) and the active site (right) with the main amino acids of functional importance labeled [47].

The interaction partners of RNase E

As the scaffolding core of the RNA degradosome, RNase E interacts with several types of proteins, and its repertoire of partners can vary depending on growth conditions [50–53]. RNase E homologues in divergent bacterial lineages have different interaction modules and partners, making the degradosome a variable machinery with capacity to perform specialized tasks depending on biological context [27, 54, 55] (Table 1).

The *E. coli* RNase E forms a degradosome assembly in which the canonical components associated with the C-terminal domain are a DEAD-box RNA helicase (RhlB), the glycolytic enzyme enolase, and the exoribonuclease polynucleotide phosphorylase (PNPase) (Figure 2

and 3, Table 1). The *Caulobacter crescentus* RNase E forms an RNA degradosome complex together with RhlB, the metabolic enzyme aconitase, PNPase, and the exoribonuclease RNase D [56, 57] (Table 1). As with the *E. coli* degradosome, the proteins of the *C. crescentus* degradosome bind to RNase E mainly through its unstructured C-terminal domain with the exception of RhlB, which binds to a partially helical insert in the S1 domain within the globular N-terminal part of RNase E. We describe the degradosomal interactions and their functional consequences in the following subsections.

The C-terminal domain of *E. coli* RNase E has two RNA-binding domains flanking the helicase binding site (Figure 2). Accumulating evidence indicates that this C-terminal portion of RNase E plays an important role in sRNA mediated regulation. *In vivo*, truncation of RNase E to disrupt the degradosome assembly impacts on the kinetics of substrate cleavage, as shown from results of single molecule studies of the action of sRNA SgrS on the *ptsG* transcript [58]. Removing the RNase E C-terminal domain diminishes the efficiency of co-degradation of the sRNA-mRNA pair, RyhB-*sodB*, implying that the degradosome might be important for presenting the RNA duplex to the catalytic domain of RNase E [17, 59]. The CTD also has a membrane-

association motif that compartmentalizes the assembly to the cytoplasmic membrane [27, 31, 32, 34] (Figure 2 and 3).

i. The exosome-like PNPase

The very C-terminus of RNase E encodes a small segment that recruits PNPase, a 3' to 5' exoribonuclease [60] (Figure 2). PNPase uses phosphate to attack the backbone of the RNA substrate, generating nucleoside diphosphates as products. This mechanism requires magnesium as a cofactor, and it is likely that the metal plays a role in stabilising the charge in the transition state [60]. PNPase contributes to RNA quality control and can degrade improperly folded tRNA and rRNA [61]. As PNPase requires a 3' single stranded region to bind to its substrate, polyadenylation of RNAs may increase their degradation rate by this enzyme [62]. In mammalian mitochondria mRNA is also polyadenylated, and the degradation rate by the Suv3-PNPase complex depends on the length of the poly(A) tail [63].

The first experimental structure of PNPase, from *Streptomyces antibioticus*, was used to propose a model for the archaeal and eukaryotic exosome [28, 64]. The eukaryotic exosome is not a phosphoryltic enzyme like PNPase, but acts as a scaffold to recruit exo- and endo-nucleases on the periphery of the central, PNPase-like

channel, through which some substrates are threaded as single-strands for delivery to the active sites [65]. Analogous cooperation of the RhlB helicase with RNase E and PNPase is described in the following subsection. Crystal structures have been obtained for PNPase from numerous other species, and the structures of the *Caulobacter crescentus* homologue revealed the full domain architecture of this enzyme, and illuminated the path for the RNA to thread into the central channel to the active sites and how the KH RNA binding domains engage single stranded RNA substrate [66].

PNPase may play a chaperone role for some sRNAs [67–70]. The chaperone property opens the possibility that regulatory RNA recruitment by PNPase could modulate the activity of the degradosome.

ii. *RNA helicase partners of RNase E and their role in substrate channeling*

The degradome's RhlB bears the conserved "DExD/H box" sequence motif found in RNA helicases from bacteria, archaea and eukaryotes. Like other RNA helicases, RhlB can harness the energy of nucleoside triphosphate binding and hydrolysis to dynamically remodel RNA structures and protein-RNA complexes. The interaction

site for RhlB has been mapped on RNase E by limited proteolysis and includes a highly conserved motif [40, 49] (Figure 2). The physical interaction of RhlB with its binding site on RNase E boosts the helicase ATPase and unwinding activities. In turn, association of the helicase with RNase E results in efficient unfolding of structured RNAs, and can facilitate the action of PNPase and RNase E to degrade such substrates [71, 72] (Figure 3B).

RNA helicases are associated with phylogenetically diverse RNase E homologs, with some notable variations. In stationary phase or in response to cold stress, other RNA helicases are recruited to the *E. coli* degradosome to replace RhlB [73–76]. A helicase expressed in response to cold shock is also associated with the degradosome in *C. crescentus* [77].

The association of the RhlB helicase with the degradosome has some interesting mechanistic and structural parallels with the complex formed by RNA helicase Mtr4 and the nuclear exosome of the yeast *Saccharomyces cerevisiae* [29] (Table 1). There are also similarities to the eukaryotic Ski complex, which is a conserved multiprotein assembly required for the cytoplasmic functions of the exosome, including RNA turnover, surveillance, and interference [30] (Table 1). The crystal structure of *S. cerevisiae* core complex shows

that Ski3 N-terminal arm and a Ski2 insertion domain allosterically modulate the ATPase and helicase activities of the complex. Interactions with the C-terminal RecA domain of the ATPase may have some functional analogy to the interaction of RNase E with RhlB, and the helicase-mediated operation of threading substrate into the exosome is analogous to substrate channeling by RhlB to PNPase or catalytic domain of RNase E.

Another analogy can be drawn with the yeast mitochondrial exoribonuclease complex (mtEXO), in which the helicase Suv3 acts as a motor that feeds the 3′ end of the RNA into the catalytic channel of the 3'->5' exoribonuclease Dss1 for efficient processive degradation [78] (Table 1). This is particularly important for structured RNAs that cannot be degraded by the nuclease on its own and helicase unwinding activity is required. In higher eukaryotes, including humans, the functional equivalent of the mitochondrial exosome is a complex of human SUV3 and PNPase [79, 80]. All of the described exosome and helicase-exoribonuclease complexes suggest there might be similar cooperation between RhlB and PNPase, which is not capable of degradation of structured RNAs on its own, but can efficiently hydrolyse RNA unwound by RhlB (Figure 3B).

Figure 3. RNase E and interactions with RNA substrates. A. RNase E activation by 5' monophosphorylated substrate binding (5'P depicted as a yellow star). RNase E dimer (purple) is shown for clarity. S1 domain together with 5' sensor (red bar) is capturing the substrate (dark blue) and aligning it in the active site by structural changes induced by RNA binding. B. Substrate (dark blue) channeling by the ATP helicase (green) to the active site of PNPase (blue). Its action may thread substrate down the channel into the active site, as occurs for the exosome and the mitochondrial exoribonuclease-helicase complex of yeast. It is also likely to provide the same threading function for RNase E (purple).

iii. Metabolic enzymes

A longstanding puzzle is why metabolic enzymes are associated with the RNA degradosome. In numerous γ-proteobacteria the glycolytic enzyme enolase is a conserved, canonical component of the RNA degradosome assembly [27], whereas in *Caulobacter crescentus*, RNase E is associated with the Krebs cycle enzyme aconitase [56] (Table 1). Intriguingly, the Gram-positive bacterium, *Bacillus subtilis*, lacks an RNase E homologue, but does have an analogous ribonuclease that interacts with glycolytic enzymes under certain growth conditions [81]. The evolutionary convergence of stable interactions between ribonucleases and metabolic enzymes indicates an important biological function. Moreover, enolase has also been identified as a component of the RNA interactome in human cells [82], suggesting that it might have a secondary role in supporting RNA metabolism.

An earlier study probing enolase function in the degradosome showed that under conditions of phospho-sugar stress in *E. coli*, the depletion of enolase suppresses the sRNA-induced degradation of the *ptsG* transcript by RNase E [83], indicating that enolase may play a role in riboregulation. Another study observed that the deletion of enolase is associated with increased levels of mRNAs encoding proteins that participate in the uptake and utilisation of multiple carbon sources [84]. However, as the proportion of enolase associated with the degradosome is relatively small (~5-10%) [53], removing this key enzyme entirely from the cell could have strong secondary effects unrelated to its function in degradosome assembly. Such perturbations can be circumvented by deleting the enolase binding site in the RNase E gene on the bacterial chromosome. In such a mutated strain of *E. coli*, the growth phenotype is surprisingly little affected under aerobic conditions [85]. However, under anaerobic conditions the mutant shows abnormal cell morphology that is likely to arise from destabilisation of the sRNA DicF and higher levels of its target mRNA *ftsZ* encoding a critical cell-division protein [85]. Biophysical studies indicate that enolase helps to compact an adjacent RNA binding motif in RNase E CTD, known as AR2 [49] (Figure 2). Degradosome-associated enolase could therefore be indirectly

involved in efficient RNA binding by inducing a conformational change in the AR2 RNA binding domain of the RNase E C-terminus. Such structural change could favour RNase E interaction with DicF and confer a certain degree of protection to the sRNA.

Interaction of RNase E with regulatory RNAs and chaperones

Ever since the discovery of the sRNA MicF, which represses the expression of the major outer membrane porin of *E. coli*, *ompF* [86], hundreds of small regulatory RNAs have been discovered that help to regulate gene expression in bacteria [87]. Prokaryotic sRNAs vary tremendously in size and are involved in various aspects of gene regulation. These sRNAs are generally synthesized in response to stress or metabolic conditions and act by pairing to target mRNAs and regulating their translation and stability with the assistance of Hfq, a conserved RNA-binding protein from the extensive Lsm/Sm protein family. Many sRNAs studied so far lead to translational repression by binding to the translation initiation region of target mRNAs and occluding the ribosome-binding site (RBS) [88, 89] (Figure 1, middle panel). sRNA mediated degradation of target mRNAs often involves

RNase E [17, 90, 91], and sRNAs paired with target mRNAs can be degraded together in an RNase E-dependent manner [17]. Hfq, along with Hfq-binding sRNAs, associates with RNase E, resulting in the formation of a ribonucleoprotein effector complex that allows for the tethering of RNase E near the base-pairing region of target mRNAs [92].

Bacterial sRNAs that bind to their target within the coding region can alter transcript stability by creating a ribonuclease cleavage site [93]. Additionally, sRNAs can differentially alter transcript accumulation within an operon. An example is RyhB, which downregulates the *iscSUA* genes within the *iscRSUA* operon to allow independent accumulation of *iscR* [94]. sRNAs can be degraded by RNase E and PNPase, or by RNase III if the sRNA has long stretches of complementarity [95]. sRNA can act *in trans* to allosterically activate RNase E, which we will describe further in the next section [96].

The question arises where and how the RNase E-dependent degradation of the mRNA–sRNA hybrid is initiated [97]. The key challenge faced in answering this question is that the complexes are formed transiently and are therefore difficult to capture. One experimental approach to overcome the challenge is to crosslink the RNase E-RNA complexes *in vivo*, in their cellular context, and then

identify the components. Such an approach was employed with a variant of RNA sequencing, the CLASH methodology (UV-crosslinking, ligation and sequencing of hybrids) [35]. The data obtained using this method support an interaction/displacement model whereby RNase E binds closely to Hfq interaction sites on sRNAs, thereby displacing Hfq from the sRNA-target RNA pair. This is followed by cleavage that occurs maximally 13 nucleotides downstream of the pairing site. The model is consistent with the RNase E cleavage 6 nucleotides downstream of the MicC-*ompD* sRNA-mRNA duplex [93, 96].

In addition to Hfq, RNase E can cooperate with several other known RNA-binding proteins to specifically regulate sRNA stability. One example is RapZ, which was identified in *E. coli* as an adaptor guiding RNase E for processing of the sRNA, GlmZ, as part of the mechanism controlling amino-sugar metabolism. As this metabolic pathway provides essential components required for cell envelope biogenesis, it must be synchronized with other cell division processes and part of a highly interconnected regulatory network. It has been hypothesized that RapZ guides RNase E through modification of the structure of the sRNA so it can be recognized by RNase E, or by functioning as an interaction platform by delivering the sRNA to RNase E [98, 99]. Another protein cooperating with RNase E is CsrD, an RNA-

binding protein that destabilizes the sRNAs CsrB and CsrC in an RNase E-dependent way and it was suggested that CsrD might induce structural changes in the RNAs that make them more susceptible for attack by the ribonuclease [100]. RNase E cleavage sites have been mapped in RsmZ, an analog of CsrB [101]. Most likely, there are more proteins like RapZ and CsrD that bind sRNAs and target them for degradation by RNase E and other enzymes.

The CTD of RNase E can also interact with regulators such as the inhibitory proteins, RraA and RraB, which bind the CTD at distinct sites [102]. RraA was shown to modulate degradosome activity by altering its composition through interaction with RhlB and the RNA binding regions of the CTD [98]. RraB binds within aminoacids 694-727 of the C-terminus of RNase E and also influences the degradosome composition in vivo [103]. In *Pseudomonas aeruginosa* the RNase E CTD is targeted by a phage protein Dip that inhibits its activity during phage infection [104, 105]. Crystallographic and biophysical data suggest that the interaction site resides within the RNA binding region of the CTD.

Substrate preferences of RNase E

Given the pivotal role of RNase E in RNA processing, turnover and RNA-mediated regulation, the question naturally arises whether the enzyme has cleavage preferences and if these might encode information on the lifetime of the substrate. One salient signature of RNase E cleavage sites is that they are AU-rich and single-stranded [106–108], with strong preference for U at position +2 with respect to the scissile phosphate. U+2 is predicted to make favourable and conserved interactions with the enzyme [12]. The site of cleavage by RNase E was originally mapped to a consensus sequence A/GAUUA/U [5] in single stranded region [5, 109, 110]. This consensus sequence has been confirmed and extended by the recent mapping of over 22,000 cleavage sites in Salmonella [12]. This preference for single-stranded substrates is in accord with the crystallographic data, as the catalytic site cannot fit double-stranded substrates. Recently it was noted that RNase E cleavage sites are flanked by stem-loop structures [111].

A notable feature of RNase E is its 5′-end dependence for certain substrates, for which it strongly prefers 5’ mono- over the tri-phosphorylated group [34, 112]. This feature explains why stable 5′-stem loops that mask the 5’ end from the enzyme protect mRNAs from decay. Crystallographic studies show that the terminal phosphate in a

single-stranded region is recognised by hydrogen bonding interactions with R169 and T170 in a 5'-sensor domain, and this interaction is proposed to favour a closed conformational state that boosts enzyme activity (Figure 2 and 3A). Comparison of crystal structures of holoenzyme with an RNA substrate analogue bound and apoenzyme shows that RNase E seems to be in an open form in the latter that closes upon substrate binding [113]. This movement enables optimal orientation of substrate for catalysis and is favoured by the interaction of the 5' end with the sensing pocket (Figure 3A). While the 5'sensing pocket can accommodate 5'-triphosphate, due to steric hindrance the enzyme cannot close upon the substrate and in consequence the catalytic activity is impeded. Studies with short oligonucleotides indicate that the apparent boost in catalytic efficiency for substrates with 5' monophosphate compared to those with a 5' hydroxyl group arises principally from the reduced Michaelis-Menten parameter, K_m [114], suggesting that the effect of 5'-end sensing is mostly to contribute to substrate binding.

A second, potentially large class of RNase E substrates exists for which the enzyme activity is not affected at all by the chemical status of the 5' end [106, 114, 115]. The existence of this class has led to the hypothesis of two potential pathways for substrate recognition by

RNase E: 5'-end-dependent and internal entry [116]. The first pathway relies on 5'-monophosphate recognition described above. Data from experiments with substrates with complex secondary structure suggest that the NTD can engage such substrates as a potential mechanism that contributes to the 5' bypass mode of operation [114]. Moreover, for some substrates, the internal entry pathway could be favoured by interactions of RNA substrates with the NTD and arginine-rich segments present in the CTD [39, 117]. While two different ways of recognising the substrate exist, neither of those pathways is strictly essential and the pathways may work in a cooperative manner [21, 116]. It is interesting in this regard to note that the truncation of the C-terminal domain of RNase E is lethal when combined with mutations in the 5' sensing pocket in the catalytic domain, suggesting that the pathways of degradation involving 5' end activation and RNA fold recognition are not redundant and there is a mechanistically important interplay between the C-terminal and N-terminal domains during substrate recognition and cleavage [21].

Early studies of RNA processing of complex substrates by RNase E indicate a role for secondary structure recognition by the enzyme. The 9S gene product is cleaved by RNase E twice to yield the 5S precursor that is further trimmed by nucleases to give the mature 5S

ribosomal RNA [15, 110, 118]. Evidence indicates that a secondary structure in the 5'-region of 9S is essential for its recognition by RNase E [110]. More recently, high-throughput sequencing analysis on transcriptome-wide scale in *E. coli* revealed that, in many mRNAs which are RNase E substrates, a stem-loop is present upstream of the cleavage site [111]. It was also found that RNase E cleaves *ompD* mRNA in the presence of a small non-coding RNA, MicC, which guides mRNA degradation in a specific manner [96]. Those two RNAs form a duplex by imperfect base-pairing upstream of the cleavage site, and changes to this duplex influence RNase E activity (K. Bandyra *et al*. in preparation). These findings indicate that structural motifs in RNA substrates might be crucial for recognition by RNase E to help align single-stranded regions for cleavage [119, 120].

Encountering and acting upon substrates

RNases act on any RNA to which they have access and a match to their specificity. As they have redundant activities, the ribonuclease that performs the first cleavage will depend on which first encounters the substrate in a permissive conformation [7, 121]. As RNase E seems to be the main enzyme for initiating the onset of degradation, it must

have some type of privileged access. Since degradosomes are tethered on the cytoplasmic membrane during aerobic growth, RNAs destined for processing or decay must be delivered to them, or encounter them by random diffusion.

It has been noted that the overexpression of certain transcripts results in rapid degradation by RNase E, most likely because they are produced faster than could be accommodated by the ribosomes and therefore are exposed [122]. Regulatory RNA-binding proteins can modulate the efficiency of translation initiation by directly competing with ribosomes for binding to the ribosome interaction region or by initiating a change in the secondary structure of the mRNA sequence near this region [123–125]. The resulting reduction in translation initiation efficiency often decreases mRNA stability as well. Moreover, emerging data suggest that codon optimality may also regulate mRNA degradation pace by influencing the rate of ribosome elongation [126]. Data support a model in which mRNA degradation is prevented by efficient translation, and operates through close coordination of transcription with translation. However, it might be expected that at some stage in committed translation, the transcript is no longer needed as a template, and consequently the mRNA would move off a polysome into the degradation pathway. One model for substrate

access is that RNase E might encounter the emerging 5' end of transcripts as it spools off the end of a polysome. This could be activated by an sRNA and would lead to a process of co-translational decay [127] (Figure 4A). Another mode of degradation might occur when the rates of translation initiation efficiency or elongation are reduced, so that the spacing of the translating ribosomes on the mRNA is less compact. In this case, it is more likely that RNase recognition sites in the mRNA become exposed causing transcript decay [128] (Figure 4A). Such a degradation mode would demand the cooperation of rescue mechanisms like non-stop decay.

Cellular localization of RNase E and the degradosome.

Adjacent to the N-terminal catalytic domain is an amphipathic α-helix that tethers the RNA degradosome to the bacterial cell membrane in *E. coli* and is expected to affect the way that the four natively unstructured C-terminal regions would extend outwards the tetrameric catalytic centre (Figure 4B). Strikingly, the functionally analogous (but not homologous) enzyme of *Staphylococcus aureus*, RNase Y, is also membrane associated [129, 130]. Under anaerobic

conditions, RNase E become destabilized and may come off the membrane [85].

Although the association of RNase E with the cytoplasmic membrane is required for optimal cell growth in *E. coli*, for other bacteria the RNA degradosome is not membrane localized. For example, in the α-proteobacteria *C. crescentus*, RNase E forms patchy foci that associate with the nucleoid instead of the membrane [131].

The membrane-associated *E. coli* RNase E generates transient foci that form on transcripts *in vivo* [32]. These foci may be cooperative degradation centers formed by several degradosome particles, and they share remarkable similarities and functional analogy with the eukaryotic ribonucleoprotein (RNP) granules formed by RNA binding and processing enzymes [132]. These RNP granules are microscopic structures resembling phase-separated droplets and are proposed to act as 'nano-organelles' that are partitioned from the cytoplasm without the requirement for a lipid membrane. The liquid-liquid phase separation is postulated to be mediated by disordered regions of RNA-binding proteins that can form new interactions within such droplets. This phase-separation not only brings about compartmentalization of the enzymes and RNA binding proteins, but also influences their specificities for nucleic acids. In the context of the degradosome,

extensive disordered regions in the C-terminal tail of RNase E could promote phase separation through self-interaction or distributed contacts with RNA and association with unstructured regions of other degradosome components [97]. Thus, clustering of the ribonuclease on the cytoplasmic membrane may be a 2-dimensional analogue of the phase transition behaviour proposed for RNP granules and could yield highly cooperative activities on a bound substrate. The CTD of RNase E has great plasticity to accommodate numerous RNA species of different sequence and structure, and could contribute to the formation of such proposed granule-like foci. The CTD might help to intercept polysomes, free RNA, or structured RNA precursors for interaction with the degradosome, and orchestrate the constantly alternating RNA universe.

Figure 4. Model of the degradosome interaction with polysomes and cellular membrane. A. Speculative model of degradosome interaction with the polysome. RNase E can gain access to translated transcripts (dark blue) upon sRNA action (top panel), when an sRNA (red) in complex with Hfq (orange) targets the translation initiation region (TIR, black) and by inhibiting assembly of ribosomes provides access for RNase E. The enzyme can also gain access to translated mRNA on its own (bottom panel). Figure adapted from [133]. B. Association with the inner membrane (grey) is mediated by an amphipathic helix (dark grey) localised in the C-terminal domain of RNase E. RNase E – purple, RhlB – green, enolase – yellow, PNPase – blue.

Summary and Perspectives

Analyses of bacterial RNA metabolism continue to reveal its numerous links to key regulatory processes. Many of the mechanisms that enable efficient RNA processing and degradation in bacteria have analogous processes in organisms of other life domains including metazoans. RNase E has been a key paradigm in understanding the complexity of RNA mediated regulation and metabolism in bacteria. RNA-binding proteins, which often act in concert with RNase E, can act as global regulators to help orchestrate complex behavior. Detailed profiling of cellular targets of the sRNA chaperone Hfq, the translational repressor CsrA, the ProQ protein and cold-shock proteins have shown that there are extensive posttranscriptional networks in bacteria [38, 134–139]. Many of these bacterial RNA binding proteins impact on bacterial virulence [38] (see also 'Regulatory RNAs in virulence and host-microbe interactions').

There is growing appreciation of the contribution of RNA metabolism in mediating complex behaviour of individual cell and cooperative communities. This behaviour is also manifested during bacterial infection – for example, in the rapid response provided by

sRNAs in pathogenic bacteria to coordinate invasion steps and adjust quickly to demanding and hostile environment inside the host [140]. Although the understanding of the processes of RNA metabolism and riboregulation is growing, there is still scope to discover some extraordinary solutions that nature has developed to improve cellular efficiency and capacity to adapt, develop and evolve.

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Table 1. Components of the bacterial RNA degradosome and analogous or homologous assemblies from archaea and eukaryota

	RNases	RNA helicases	Exosome-like proteins	Metabolic enzymes	Chaperones	Other proteins
Escherichia coli	RNase E	RhlB	PNPase	enolase	Hfq*	RapZ, CsrD, RraA, RraB
Pseudomonas aeruginosa	RNase E	DeaD	PNPase		Hfq	Dip
Caulobacter crescentus	RNase E, RNase D	RhlB	PNPase	aconitase		
Saccharomyces cerevisiae	Rrp44**	Mtr4, Ski2	Rrp41, Rrp45, Rrp46, Rrp43, Mtr3, Rrp42, Rrp40, Rrp4, Csl4			Ski complex
Mitochondrial exosome	Dss1	Suv3				

*Interaction with Hfq might be indirect and mediated by RNA

**Hydrolytic ribonuclease component of the exosome, a member of the RNase R family and unrelated to RNase E.