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Machinery, mechanism, and information in post-transcription control of gene expression, from the perspective of unstable RNA

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Summary

Throughout all the domains of life, and even among the co-existing viruses, RNA molecules play key roles in regulating the rates, duration, and intensity of the expression of genetic information. RNA acts at many different levels in playing these roles. *Trans*-acting regulatory RNAs can modulate the lifetime and translational efficiency of transcripts with which they pair to achieve speedy and highly specific recognition using only a few components. *Cis*-acting recognition elements, covalent modifications, and changes to the termini of RNA molecules encode signals that impact transcript lifetime, translation efficiency, and other functional aspects. RNA can provide an allosteric function to signal state changes through the binding of small ligands or interactions with other macromolecules. In either *cis* or *trans*, RNA can act in conjunction with multi-enzyme assemblies that function in RNA turnover, processing and surveillance for faulty transcripts. These enzymatic machineries have likely evolved independently in diverse life forms but nonetheless share analogous functional roles, implicating the biological importance of cooperative assemblies to meet the exact demands of RNA metabolism. Underpinning all the RNA-mediated processes are two key aspects: specificity, which avoids misrecognition, and speedy action, which confers timely responses to signals. How these processes work and how aberrant RNA species are recognised and responded to by the degradative machines are intriguing puzzles. We review the biophysical basis for these processes. Kinetics of assembly and multivalency of interacting components provide windows of opportunity for recognition and action that are required for the key regulatory events. The thermodynamic irreversibility of RNA-mediated regulation is one emergent feature of biological systems that may help to account for the apparent specificity and optimal rates.

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Introduction

At the sequence level, genomic information is analogous to a programming language that is translated into code through the process of biogenesis of functional proteins. In this perspective, the genomic sequence might be evaluated for its 'entropy', based on concepts for evaluating signal communication (Shannon and Weaver, 1949). However, such an analysis does not seem to capture the logical twist that gene products not only arise from but also interact and interpret the genomic sequence. Accordingly, there must be a greater richness in the encoded information that underpins not only this self-reference but also the vast interconnections of biological systems (Smirnov, 2022). Classical genetic models, such as the 'one gene; one enzyme', have been long recognised as being insufficient to capture the extensive interconnectedness of gene products in the context of the bustling and crowded cellular environment. At this macroscopic level, the act of communication is deeply interwoven with – and impacts upon – the information itself

(Al-Hashimi, 2023). From a systems perspective, biological information hinges on understanding which signals are transmitted and for what purpose – ultimately contributing to the organism's fitness.

Encoded genetic information extends beyond the direct mapping of codons to amino acids, as captured in the iconic central dogma (Crick, 1970), encompassing additional regulatory and storage functions by the information intermediate, RNA (Figure 1). For instance, codon usage in protein-encoding transcripts influences RNA secondary structure, translation elongation rates, and ultimately protein folding and expression (Komar *et al.*, 2024; Waudby *et al.*, 2019). Codon usage biases are found in all taxa, implicating its general importance in biological fitness (Plotkin and Kudla, 2011). The three-dimensional structure of RNA transcripts further encodes information, influencing rates of translation initiation, elongation and termination, and other aspects of molecular recognition (Berkovits and Mayr, 2015; Ganser *et al.*, 2019). The secondary structure in an RNA can influence the potency of small regulatory RNA (e.g., miRNA, described below) by reducing accessibility and affinity to target sites. This serves as an example of how codon usage could not only alter the target site sequence but also potentially affect its accessibility to regulatory small RNA. Yet, another subtle aspect of the encoded information is RNA's capacity to switch conformational states, which enables allosteric propagation of signals, whereby the binding of a partner at one site impacts activity or interaction at distant sites.

RNA also encodes information in the features that are recognised by degradation machinery, controlling transcript stability and lifespan. Additionally, RNA transcripts can present sequences targeted by regulatory RNAs – via base-pairing complementarity – which can modulate the lifetime and translational efficiency of the transcripts. These regulatory RNAs can interlink different pathways into elaborate regulatory networks (Nitzan *et al.*, 2017; Papenfort and Storz, 2024). In plants, small RNA-directed DNA methylation regulates also at the gene transcription level, usually resulting in repression. Furthermore, sequence-encoded physicochemical properties of RNA, including the propensity for self-interaction, can contribute to the formation of nanoscale compartments (Tauber *et al.*, 2020). Emerging roles for secreted RNAs suggest that they may connect environmental cues and past cellular events to gene regulatory mechanisms (Maori *et al.*, 2019) or, in the case of pathogens, manipulate host gene expression (Sahr *et al.*, 2022). Consequently, RNA-encoded information influences gene expression at multiple timescales, from seconds to durations extending beyond cell division.

Found in all life forms, regulatory RNAs expand the reach of post-transcriptional control by modulating translation and transcript lifetime, through processes referred to as RNA-mediated regulation (hereafter, 'riboregulation'). When a regulatory RNA binds its target – often helped by facilitators of riboregulation – it frequently triggers degradation by multi-enzyme assemblies. These 'nanomachines' are not necessarily related by evolutionary divergence from common protein folds, and likely arose independently. How the machines are modulated by RNA and how they find targets with speed and precision are central questions to exploring their biological function.

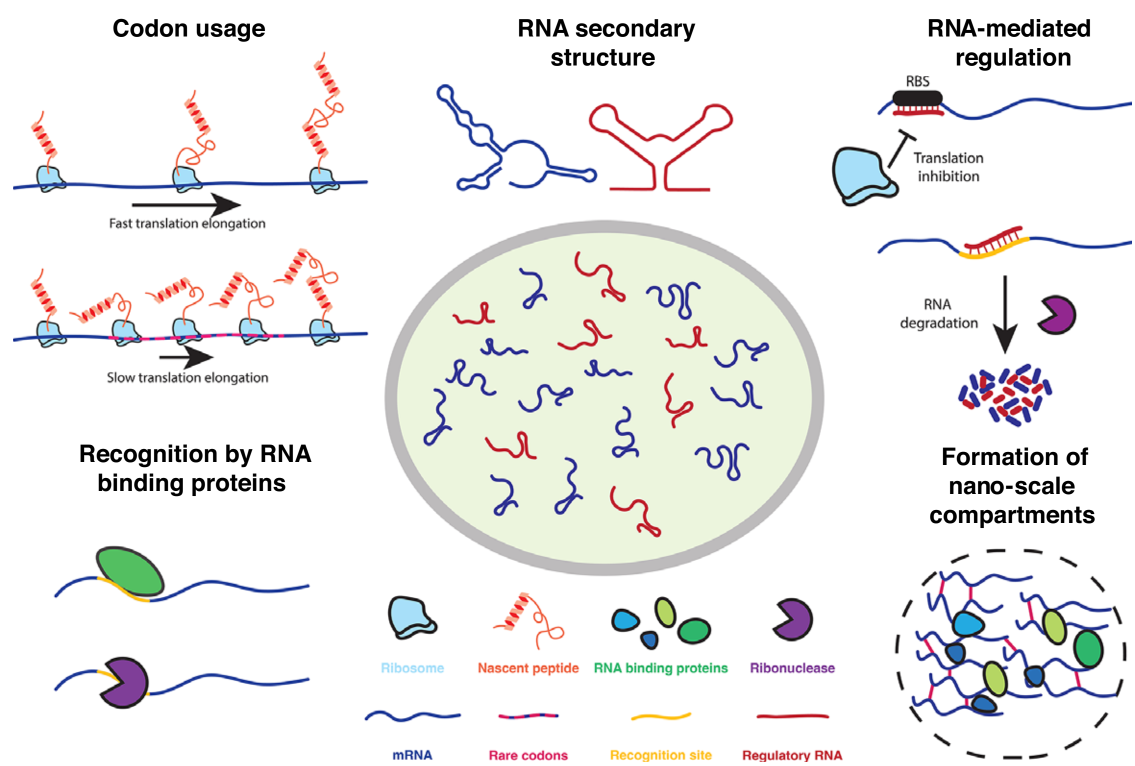

Figure 1. The hidden information of a genome, from an RNA perspective.
The central panel depicts a distribution of RNA species for protein-coding (blue) and regulatory RNA (red). RNA in the cell is seldomly free, but instead engaged in ribonucleoprotein complexes or handover from one ribonucleoprotein complex to another or recognised for turnover by enzymes (lower left panel). Sequence influences RNA fold and conformability (capacity to switch states) (upper middle panel). Structural and sequence features in transcripts can encode information for recognition by equilibrium binding proteins to form ribonucleoprotein complexes, or preferred cleavage sites for ribonucleases to silence or remodel a transcript (lower left). Regulatory RNAs can find tune translation initiation rates or trigger degradation of targeted transcripts with (partial) base-pairing complementarity (upper right). Codon usage in an mRNA can impact translation rates, with consequences for co-translational folding of nascent polypeptides (upper left). RNA can also contribute to the formation of nanoscale sub-compartments in the cell comprising conformationally and compositionally heterogeneous ribonucleoprotein assemblies (lower right).

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In considering the efficacy of riboregulation, one important consideration is its specificity. In an equilibrium scenario, the specificity of molecular interactions for cognate versus non-cognate partners can be attributed to relative binding energies, with discrimination based on the relative binding energies. However, most cellular processes are not at equilibrium, and many are effectively irreversible (Wong and Gunawardena, 2020). Other contributions must be considered in understanding specificity in the cell. *In vivo*, kinetic control and competition with other potential binders heavily influence regulatory outcomes. Often, dissociation constants, which are ratios of *off*- and *on*-rates of a binding interaction, do not differ greatly between different binding partners, whereas *on*-rates can be significantly distinct, explaining why some binders are more effective competitors. In multivalent systems, the microscopic *on*-rates for the stepwise binding interactions can provide windows of opportunity for competitors to rapidly exchange with an already-bound RNA (as seen, for example, in the hexameric Hfq described further below and shown in Figure 4). Cellular systems often rely on mechanisms like proofreading to enhance specificity, analogous to fidelity mechanisms in translation and signalling pathways (Boeger, 2022; Hopefield, 1974; Ninio, 1975). Such out-of-equilibrium processes, essential to sustaining cellular life, underscore the stepwise irreversible and energetically costly nature of biological information processing.

This review explores various factors influencing post-transcriptional regulation of genetic information, covering the timescales, subcellular localisation, and biological consequences of different events in RNA lifecycles. It also examines physicochemical features – such as RNA conformation, conformational flexibility, and chemical modifications – that affect recognition by RNA-binding proteins (RBPs). Additionally, the review discusses key elements of riboregulation, including regulatory RNA molecules, their protein partners, and the RNA degradation machinery, with examples drawn from all domains of life.

RNA lifetimes, cleavages, and regulatory consequences

In all extant organisms, the turnover of mRNA and other RNA species provides a critical component in the control of gene expression. It allows rapid adaptative responses to signals and changes in metabolic state (Palumbo *et al.*, 2015) as well as temporal coordination of gene expression dynamics that have been conceptualised as a 'transcriptome vector field' (Qiu *et al.*, 2022). In bacteria, mRNA half-lives are typically 2 to 5 minutes (Anderson *et al.*, 2010; Steglich *et al.*, 2010) but can be as short as seconds (Jenniches *et al.*, 2024). Ribosomal RNA, tRNAs, some small regulatory RNAs (sRNAs), and mRNAs can have half-lives longer than bacterial generation time (Durand *et al.*, 2015; Hamouche *et al.*, 2021; Khemici *et al.*, 2015) and can, therefore, contribute to multi-generational effects whereby RNAs can be inherited by the daughter cell from the mother cell or, for some species, through formation of dormant spores. Archaea RNA lifetimes have been more difficult to measure, but a distribution of lifespans has been reported in the time scale of minutes (Andersson *et al.*, 2006). In single-celled and metazoan eukaryotes alike, a nascent RNA can persist from hours to years, for those sustained in storage, but can be reduced to minutes in response to appropriate signalling (Choi *et al.*, 2024).

RNA degradation can arise through spontaneous chemical processes or through self-cleavage, as seen, for example, in the catalytic ribozymes, but these do not match the rates and specificity required to meet cellular demand. Instead, protein enzymes – ribonucleases – are the powerful natural catalysts that have evolved to confer suitable rates and targeting that are key to controlling RNA decay. The ancient origins of some of the key enzymes highlight the critical roles they play in the evolution of complex regulatory systems (Rehwinkel *et al.*, 2006). However, their activities must be guided and controlled, as suggested by the observation that some of the most effective biological toxins cleave RNA indiscriminately, resulting in rapid cell death (Blower *et al.*, 2011; Cruz and Woychik, 2016). On the other hand, once the initial cleavage is made by a ribonuclease (that has been well trained for the cellular context), degradation must go to completion because the accumulation of even the smallest fragments can be deleterious (Kim *et al.*, 2019). The cooperation of these enzymes and their accessory factors ensures that, once cleavage is initiated, the intermediates are rapidly reduced to single nucleotides. Ribonucleases are finely tuned and have co-evolved as part of a system to provide cleavage at a suitable rate, at a defined cleavage point in the case of maturation of precursors, or to completion in the case of decay. In this perspective, RNA is itself a distinctive class of substrate that can evolve to match enzyme requirements. Access to ribonucleases can be either through stochastic exposure or facilitated by an active remodelling of the RNA from its protected state that presents it for cleavage. For most of their lifetimes, RNAs are engaged in complexes with proteins and other macromolecules that confer protection. The composition of these RNA-protein assemblies is dynamic and varies throughout the RNA life cycle (Choi *et al.*, 2024). Some factors are required to protect certain RNAs, while others specifically target other RNAs for degradation.

Degradation involving ribonucleases is initiated by exoribonucleases or from an internal cleavage by endoribonucleases. Those two classes of enzymes can often cooperate to rapidly degrade a substrate as cleavage by endoribonucleases can lead to entry sites for exoribonucleases. Once initiated, cleavage of a bacterial RNA by an endoribonuclease can result in degradation of the entire RNA molecule, in the generation of two stable RNA molecules, or in differential degradation of either the upstream or downstream fragment through exoribonuclease entry (Le Scornet *et al.*, 2024).

Controlled cleavage can be part of a maturation process of mRNA or the biogenesis of regulatory RNA in diverse organisms. In bacteria, cleavage events may result in stabilisation of the processed mRNAs and differential expression of co-transcribed genes encoded by polycistronic messages from operons, which are the major gene expression units in bacterial genomes. For RNase Y of the firmicutes, substrate RNAs are cleaved at preferred sites (Khemici *et al.*, 2015; Marincola *et al.*, 2012; Marincola and Wolz, 2017), with efficiency driven by primary nucleotide sequence immediately downstream of the cleavage site and by secondary structure a few nucleotides further downstream (Le Scornet *et al.*, 2024). In the pathogen *Staphylococcus aureus*, RNase Y cleaves the mRNA of a virulence regulation operon, resulting in differential levels of the encoded proteins (Marincola *et al.*, 2012). In the phylogenetically divergent *Escherichia coli* and other gamma-proteobacteria, a similar mechanism has arisen by convergent evolution, where programmed mRNA decay by secondary structure recognition by the conserved RNase E is involved in differential cleavage in operons (Dar and Sorek, 2018). As in the case of RNase Y, the secondary structure in the RNA near the cleavage site signposts differential degradation of adjacent protein-encoding transcripts. Structural models propose that RNase E can recognise stem-loop structures to direct cleavage upstream or downstream (Bandyra *et al.*, 2018; Islam *et al.*, 2023).

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RNA degradation machines and their accessory factors

In eukaryotes, there exists a rich diversity of specialised machinery involved in RNA degradation and processing. These machineries include assemblies such as the cytoplasmic and nuclear exosomes that act on a variety of RNA substrates in both destructive and constructive roles, being involved not only in transcript turnover but also in the maturation of pre-ribosomal RNA (Figure 2) (Keidel *et al.*, 2023; Kögel *et al.*, 2022). Other salient examples are deadenylase complexes such as CCR4-NOT that act on poly(A) tails of coding transcripts (Tang *et al.*, 2019; Tang and Passmore, 2019). This assembly is a key control hub, as demonstrated by its targeting by effector proteins of pathogenic bacteria to remodel host expression (Levdansky *et al.*, unpublished; Shimo *et al.*, 2019). Numerous accessory assemblies can also be found that help with decay, such as the nuclear exosome targeting (NEXT) complex and the poly(A) exosome targeting (PAXT) complex, which direct non-functional and polyadenylated transcripts, respectively, to the nuclear exosome (Schmid and Jensen, 2019). Transcript decay, in addition to allowing kinetic control of gene expression, also functions to counter the deleterious effects of errors in mRNA biogenesis, as occurs, for example, in nonsense-mediated decay (NMD). The NMD machinery degrades transcripts with premature termination codons but can also be targeted by upstream open reading frames (Kishor *et al.*, 2019). Because the NMD machinery can degrade regulators of developmental and stress response pathways, it contributes to complex metazoan processes (Li *et al.*, 2015; Lou *et al.*, 2015), and its dysfunction is associated with genetic disease (Supek *et al.*, 2021). The NMD components are not limited to organisms that splice transcripts and may have coincided with regulatory complexity that accompanied the diversification of metazoan lineages (Behm-Ansmant *et al.*, 2007).

Analogous machines of RNA metabolism also exist in bacteria, and like their eukaryotic counterparts, they play roles in both turnover and maturation. In bacterial lineages, RNA degradation machines have arisen independently. A key example is a comparison of model organisms of bacilli and gamma-proteobacteria, which are highly divergent bacterial lineages. RNase Y, mentioned earlier, represents a major family of bacterial RNA decay ribonucleases found in many firmicutes, including the model organism *Bacillus subtilis*, for which the enzyme is well studied, and the pathogens *S. aureus, Bacillus anthracis*, and *Listeria monocytogenes* (Errington and Aart, 2020; Kovács, 2019). RNase Y makes multi-enzyme assemblies, and studies of the endoribonuclease in *S. aureus* and *B. subtilis* indicate that the enzyme interacts with the glycolytic enzyme enolase and the ATP-dependent DEAD-box RNA helicase CshA (Giraud *et al.*, 2015; Lehnik-Habrink *et al.*, 2010; Redder, 2018; Roux *et al.*, 2011) (Figure 2). These interactions are thought to be transitory since they are lost upon isolation from cell extracts. In enterobacteria, the conserved RNase E endoribonuclease is the key component of the multi-enzyme RNA degradosome that is central to RNA processing and decay (Figure 2). One of the components of the degradosome is the exoribonuclease polynucleotide phosphorylase (PNPase), which is an ancestor of the core of the multi-enzyme exosome found in eukaryotes and some archaea (Bathke *et al.*, 2020; Viegas *et al.*, 2020). Other canonical components are ATP-dependent helicases from the DEAD-box family and enzymes from central metabolism, such as enolase (Bandyra and Luisi, 2018) (Figure 2). A third major ribonuclease decay system in bacteria involves RNase J, which belongs to the wider metallo-β-lactamase family, with homologs that function in RNA metabolism found in all domains of life (Clouet-d'Orval *et al.*, 2015).

Although these RNA degradation machineries evolved independently, they share similarities. For example, helicases are often

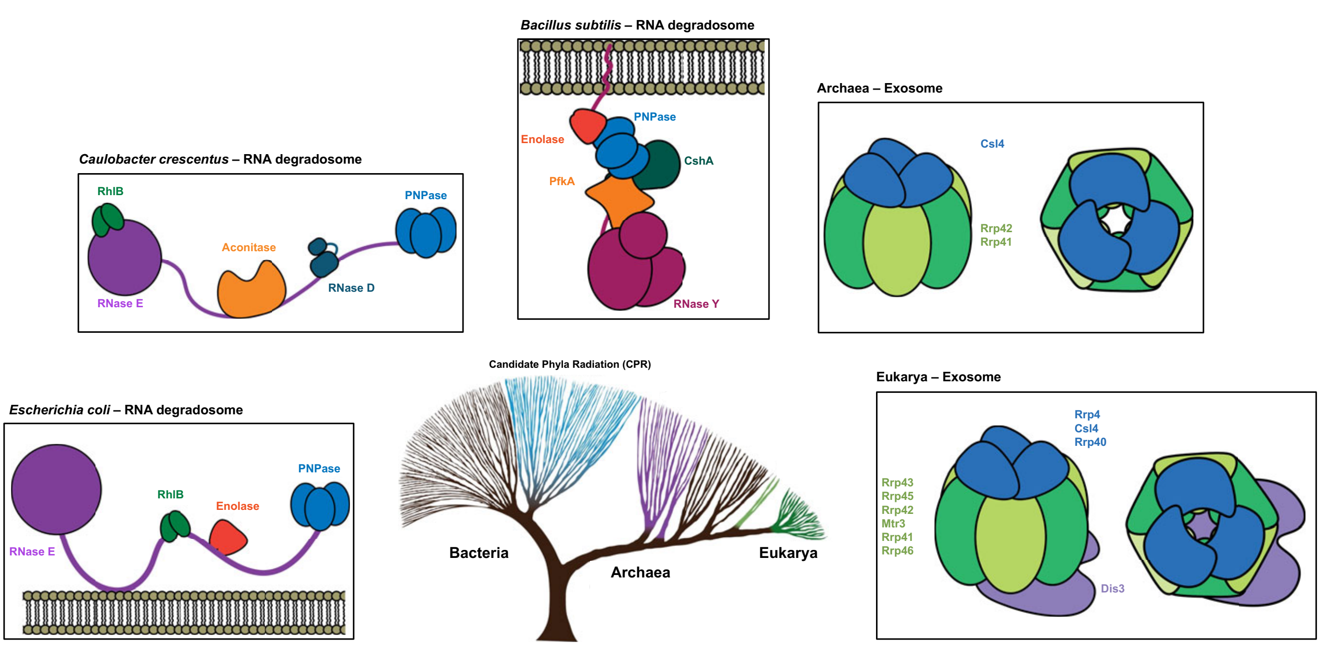

Figure 2. The tree of life and machinery of RNA turnover and riboregulation.
Examples of ribonuclease complexes in all domains of life (note their divergence in the tree of life). Current models for the tree propose that the eukaryotic lineage arose once in an endosymbiosis event. The membrane association of the degradosome is found in some Gram-negative bacteria, such as *Escherichia coli*, and Gram-positive bacteria, such as *Bacillus subtilis*, whose degradosomes are based on ribonucleases that have no shared common folding ancestor (RNase E and RNase Y, respectively) (Aït-Bara and Carpousis, 2015; Hunt *et al.*, 2006). However, not every bacterium presents membrane-bound RNA degradosome: in the α-proteobacterium *C. crescentus*, RNase E is cytosolic (Bayas *et al.*, 2018). The exosomes of archaea and eukarya share an ancient common ancestor with polynucleotide phosphorylse (PNPase), a component of bacterial RNA degradosomes (Symmons *et al.*, 2002). The tree of life was adapted from Spang and Ettema, 2016.

part of the bacterial RNA decay systems as well as the eukaryotic exosome, indicating a common requirement throughout to couple RNA unwinding to the RNA degradation machinery (Bandyra and Luisi, 2018; Hardwick and Luisi, 2013). The broad evolutionary landscape of machines that have emerged independently and converged onto similar functional roles underscores the importance of RNA metabolism in biological function. RNA turnover and riboregulation have arisen with multi-cellular complexity in metazoans and with the capacity for complex, multi-scale responsiveness in single-cell organisms.

Riboregulation and atlases of the regulatory terrains

RNA-mediated regulation and the key participating factors are well characterised in the three domains of life (Gorski *et al.*, 2017). Eukaryotic micro RNAs (miRNAs) and small non-coding RNAs (sncRNAs) are involved in gene silencing including degradation of target mRNAs and translation inhibition (Truesdell *et al.*, 2012). The sncRNAs participate in RNA interference, not only through post-transcriptional gene silencing but also through transcriptional gene silencing by chromatin modifications (Martienssen and Moazed, 2015). These regulatory RNA molecules are central to developmental processes and responses to environmental changes in metazoans, whose genomes encode numerous miRNAs, and the human genome is proposed to encode more than 2000 (Kozomara *et al.*, 2019). miRNAs are transcribed as precursors containing hairpin loop structures (pri-miRNAs) that first undergo processing in the nucleus by a complex of the RNA duplex-specific hydrolytic endoribonuclease RNase III Drosha and its partner DGCR8 and their homologs (O'Brien *et al.*, 2018). The cleavage product of Drosha, pre-miRNA, is then transported to the cytoplasm, where its loop is cleaved by another RNase III endoribonuclease, Dicer, resulting in a mature miRNA duplex. One of the two strands in the mature miRNA duplex is then loaded into a multiprotein assembly to form a miRNA-induced silencing complex (miRISC) (Iwakawa and Tomari, 2022)(Figures 3A,B). The Dicer enzyme is also implicated in the biogenesis of transfer RNA-derived small RNAs (tsRNAs), which can direct transcriptional silencing of target genes in the nucleus in a distinctive pathway. The process involves the ribonuclease Ago2 protein from the argonaut family, and is proposed to involve cleavage of the nascent transcript (Di Fazio *et al.*, 2022). In RNA interference, there can be interplay between RNA decay and riboregulation, particularly within the small interfering RNA (siRNA) pathway in organisms that encode RNA-dependent RNA polymerase (RdRP). The primary Dicer product, siRNA, guides RISC to its target and can subsequently recruit RdRP following target RNA cleavage. This recruitment facilitates the synthesis of dsRNA from the target transcript. The resulting dsRNA, now a Dicer substrate, undergoes further processing into secondary siRNAs, amplifying the RNAi response and reinforcing such specific RNA decay. This feedback loop illustrates how riboregulation can drive RNA decay, which in turn enhances the same regulatory mechanism.

In bacteria and archaea, numerous sRNAs have been identified that are often generated in response to stress, metabolic change, or programmes of host infection (Gorski *et al.*, 2017; Papenfort and Storz, 2024; Wagner and Romby, 2015). Bacterial sRNAs can either inhibit or promote translation of their mRNA targets (Figures 3C, D). Some sRNAs also encode small proteins that can contribute to another layer of regulatory complexity (Aoyama and Storz, 2023). Bacterial sRNAs can have a significant impact on gene expression by buttressing transcriptional regulation and linking different regulatory modules to support complex phenotypes. For example, in the clinical pathogen *Pseudomonas aeruginosa*, a sRNA regulates the switch from chronic to acute infection (Cao *et al.*, 2023). In *Salmonella*, an sRNA acts as a post-transcriptional timer of virulence gene expression during host infection (Westermann *et al.*, 2016). A small RNA secreted by the pathogen *Legionella pneumophila* mimics host miRNA to manipulate immune response (Sahr *et al.*, 2022). A complex RNA-mediated regulatory cascade can be involved in phage defence (Tabib-Salazar and Wigneshweraraj, 2022).

Bacterial sRNAs can be expressed from independent promoter elements or processed from 3′ ends of protein-encoding transcripts or non-coding RNA precursors (Adams and Storz, 2020; Chao *et al.*, 2017). sRNA processing is mainly through endoribonucleases RNase III, which cleaves double-stranded RNAs, and the conserved RNase E, described earlier, which prefers single-stranded substrates (Bechhofer and Deutscher, 2019; Svensson and Sharma, 2021). Cleavage by these enzymes helps to generate many chaperone-dependent sRNAs (Chao *et al.*, 2017; Chao and Vogel, 2016; Miyakoshi *et al.*, 2015; Updegrove *et al.*, 2015). sRNAs derived from mRNA 3′-ends frequently function in autoregulation (Hoyos *et al.*, 2020) and in cross-regulating the same pathways as the protein-coding transcript from which they are released (Miyakoshi *et al.*, 2015). In this way, gene regulation is achieved whereby an mRNA directly influences its expression or that of another mRNA without changes in transcription. This type of cross-regulation also occurs in eukaryotes (De Mets F *et al.*, 2019; Melamed *et al.*, 2016).

The power of riboregulation for networking and its link with metabolic processes are illustrated by the case of riboswitches, which are RNA molecules that bind specific metabolites and undergo conformational stabilisation that impacts gene expression. The ligands can trigger structural change co-transcriptionally (Lou and Woodson, 2024) and cross-couple with transcriptional pausing (Widom *et al.*, 2018). Riboswitches are likely to be an ancient mode of regulation that may have originated at the early stages of the origin of life (Kavita and Breaker, 2023). The link between metabolism and riboregulation is further consolidated with findings that bacterial sRNAs support regulation of central carbon metabolism by modulating translation initiation and degradation of target mRNAs in metabolic pathways (De Mets F *et al.*, 2019; Miyakoshi *et al.*, 2019; Papenfort and Storz, 2024). Metabolic enzymes are often encoded in operons, and those can be modulated by sRNAs that are likely to extend or complement the physiological function of the operon. A salient example is a non-coding sRNA, SdhX, produced by RNase E-dependent processing from the 3′-UTR of the *sdhCDAB-sucABCD* operon that encodes three enzyme assemblies catalysing successive reactions in the tricarboxylic acid cycle (De Mets F *et al.*, 2019). SdhX helps in adjusting carbon flux by negatively regulating acetate kinase levels, thereby providing a link between the expression of enzymes in the tricarboxylic acid cycle and acetate metabolism pathways that confer the capacity for growth on acetate. Thus, riboregulation can contribute to cross-regulation between similar pathways, and these and other findings illustrate how mRNA 3′-UTRs provide opportunity for evolution of regulatory RNA networks in bacteria (Miyakoshi *et al.*, 2015; Updegrove *et al.*, 2015).

RNA is also used to guide targeted RNA decay in bacterial innate immunity. The well-studied RNA-guided DNA targeting is used by bacterial and archaeal CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated genes) systems, which provide defence against invading mobile genetic elements through CRISPR RNA (crRNA)-guided Cas effectors (Hille *et al.*,

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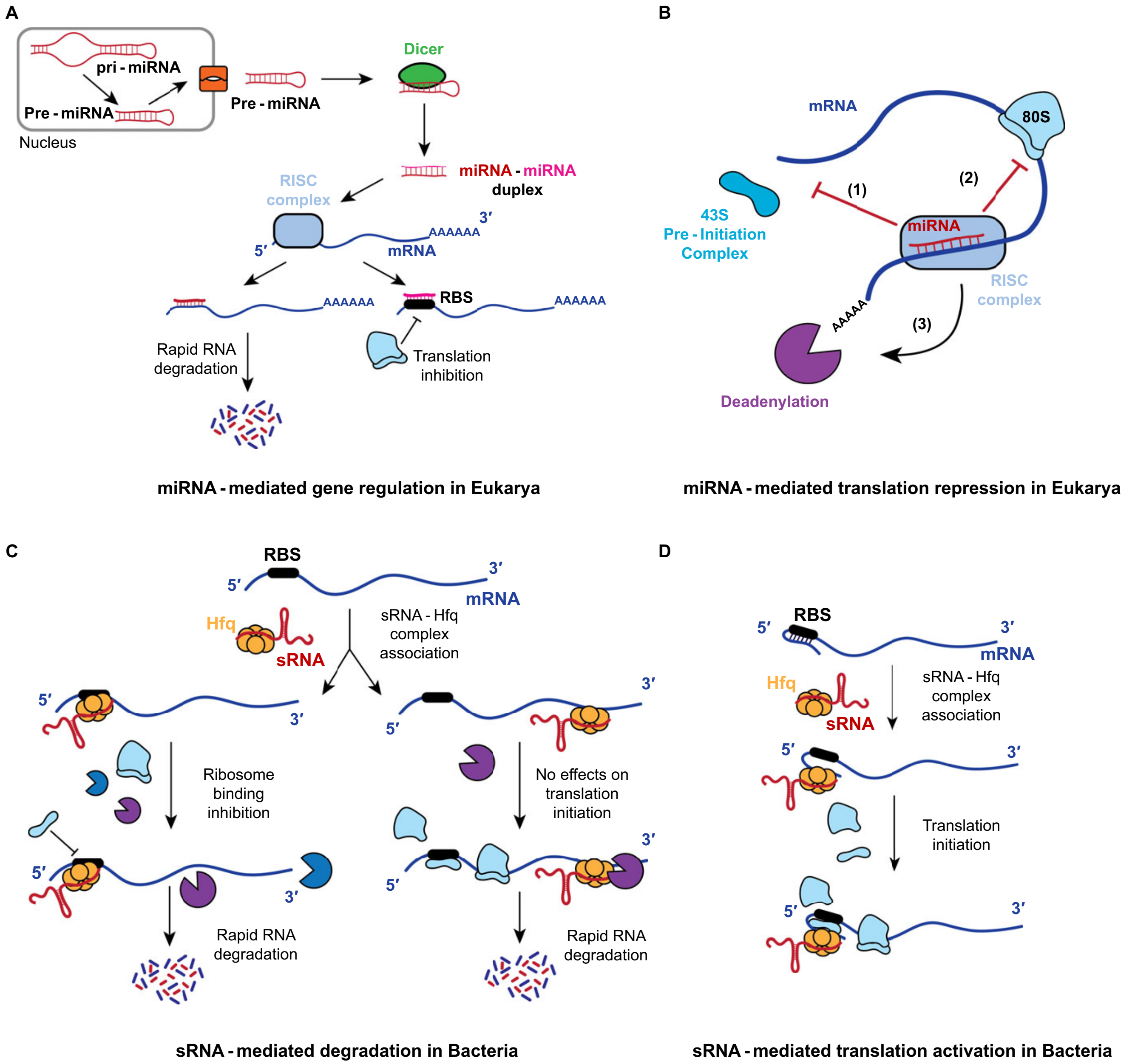

Figure 3. Schematic representation of regulatory roles of small regulatory RNAs in Bacteria and Eukarya.
The lifetime of a transcript affects the rates of information transfer, and regulatory RNAs can modulate this lifetime. (A) Schematic representation of miRNA maturation and modes of action in eukarya. Pri-miRNA (red) is transcribed in the nucleus and is converted to pre-miRNA (red), which is the substrate that is transported into the cytosol. In the cytosol, the complex Dicer (green) engages with the pre-miRNA generating a miRNA duplex (red and pink). The miRNA interacts with the RISC complex (light blue) bound to the mRNA target (blue). In this context, the miRNA (red) can either lead to degradation of the mRNA target (on the left) or mediate translation initiation (on the right, pink miRNA). (B) Schematic representation of miRNA-mediated translation repression. The miRNA-induced silencing complex (RISC) binds the 3′-UTRs of mRNAs. Translation repression via miRISC-mediated gene silencing occurs in a multitude of steps, in which 43S PIC recruitment can be targeted (1), slow-down of translation (2), and/or deadenylation and subsequent mRNA decay can be promoted (3) (Meyer *et al.*, 2024). (C) Examples of sRNA-mediated degradation in bacteria. The binary complex formed by the sRNA (red) and the RNA chaperone Hfq (orange) interacts with the mRNA target (blue) in two possible scenarios: on the left side of the panel, the complex Hfq:sRNA binds to the ribosome binding site (RBS, black) and therefore blocks the binding of ribosomes, inhibiting translation and leaving the mRNA exposed to the attack of ribonucleases (dark blue for exoribonucleases and purple for endoribonucleases), which rapidly degrade both the mRNA and the sRNA; on the right side of the panel, the complex Hfq:sRNA binds internally to the transcript, allowing translation of the mRNA to occur and subsequently delivering the target to RNase E for degradation of both the mRNA and the sRNA after translation. (D) Example of sRNA-mediated translation initiation in bacteria. The RBS (black) may not be accessible to ribosomes because involved in the formation of secondary structures. The complex Hfq (orange): sRNA (red) by interacting with the region upstream of the RBD can release the ribosome binding domain from the secondary structure element, making it accessible to the ribosome and mediating translation initiation.

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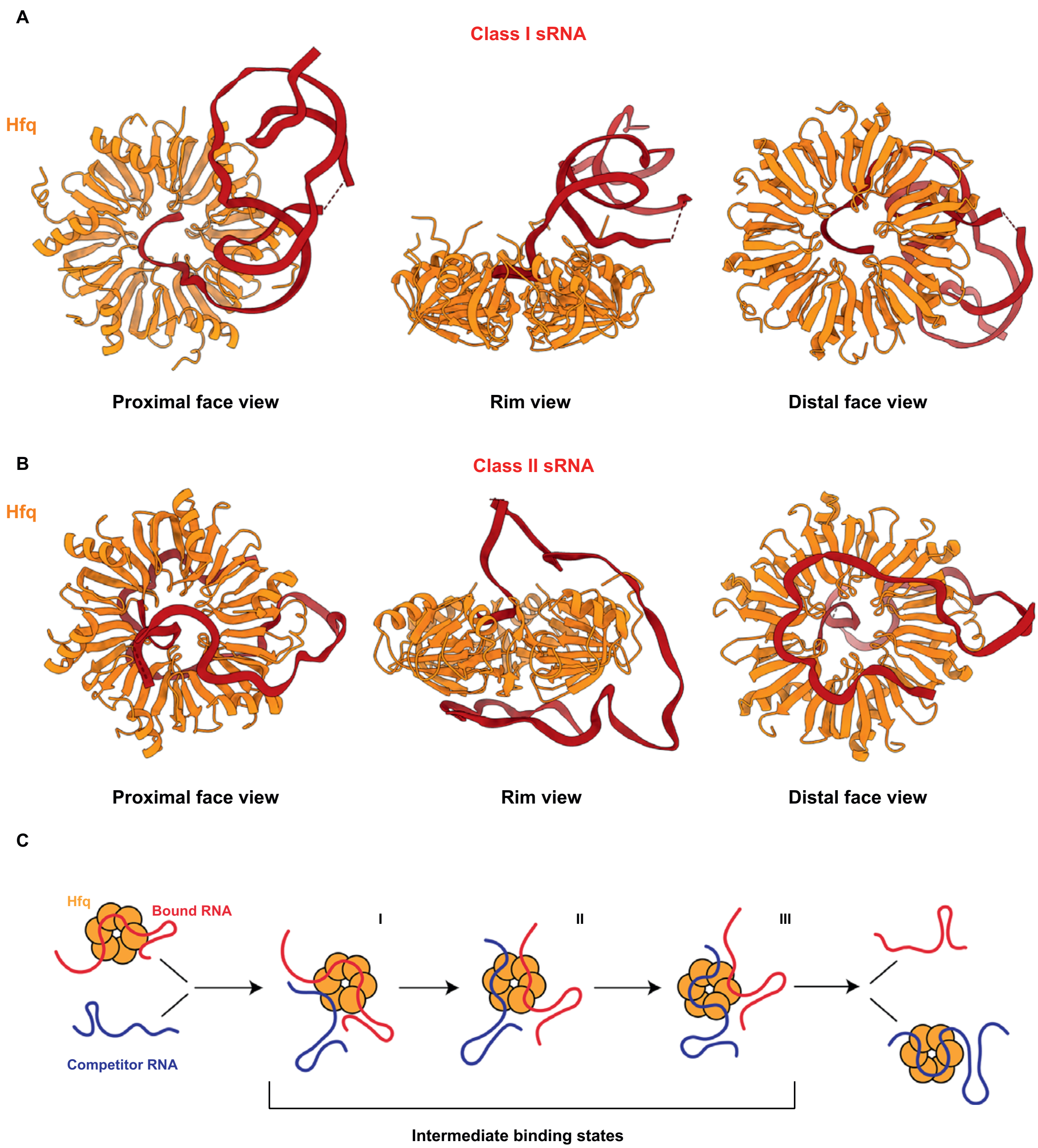

Figure 4. The bacterial RNA chaperones Hfq and its modes of RNA interaction.
The hexameric Hfq (orange) exposes three RNA-binding surfaces: proximal face (left), rim (middle) and distal face (right). (A) Class I sRNAs interact with Hfq through the proximal face and the rim (PDB: 4V2S) (Dimastrogiovanni *et al.*, 2014). (B) Class II sRNAs bind to the proximal and distal faces of Hfq. (PDB: 7OGM) (Dendooven *et al.*, 2021). (C) Multivalency can increase binding through chelate effects, but also on the kinetics of exchange, and have effects on riboregulation. Schematically represented here is the hexameric Hfq (orange) exchanging the bound RNA (red) through stepwise interactions of the protomers with the competitor RNA (blue). In step I, the competitor RNA (blue) mediated contact with only one Hfq monomer, while most of Hfq is engaged in binding with the previously bound RNA (red). Through multivalency, the competitor RNA interacts with subsequently larger portions of Hfq (step II and III), leaving the previously bound RNA with fewer interaction with the RNA chaperone, and resulting in replacement the RNA originally bound.

2018). Notably, RNA-guided DNA targeting mechanisms are also found in eukaryotes (Saito *et al.*, 2023). RNA-guided DNA/RNA degradation is not the sole mechanism for CRISPR-Cas to confer immunity against foreign genetic elements in prokaryotes. The Type III-E is a recently identified atypical Type III system, and its ribonucleoprotein (RNP) complex can direct RNA-guided RNA cleavage at specific sites (Özcan *et al.*, 2021; van Beljouw *et al.*, 2021).

RNA has also been found to potentially modulate the activity of enzymes directly. Riboregulation by specific RNAs has been proposed to influence the glycolytic enzyme enolase during embryonic stem cell differentiation (Huppertz *et al.*, 2022), and to affect the activity of serine hydroxymethyltransferase (SHMT1), which interconverts serine and glycine in one-carbon metabolism (Spizzichino *et al.*, 2024). In another example, sRNAs in complex with the RNA chaperone Hfq (see next section) have been proposed to suppress the exoribonucleolytic activity of PNPase, which is a component of the RNA degradosome (Dendooven *et al.*, 2021). The suppression is relieved upon pairing with a cognate partner RNA that remodels the ribonucleoprotein complex; thus, the RNA/Hfq complex helps to toggle the enzyme between destructive and chaperone modes. The above examples illustrate the potential of RNA for 'riboreprogramming' protein activity so that it gains a new function.

Facilitators and effectors of riboregulation

Protein partners of regulatory RNAs are structurally diverse and likely arose repeatedly in evolution (Stenum *et al.*, 2023). Representative proteins that facilitate riboregulation and are well characterised, such as the Argonaute and Pumilio proteins in eukarya and RNA chaperones in Proteobacteria (Gorski *et al.*, 2017; Swarts *et al.*, 2014). These proteins can each bind hundreds and, in some cases, perhaps thousands of RNAs. How they serve as generalists for RNA interaction is an intriguing puzzle in molecular recognition.

In bacteria, riboregulatory facilitators include chaperones, such as Hfq, ProQ, and carbon storage regulatory (Csr) proteins (Holmqvist and Vogel, 2018; Melamed *et al.*, 2020). These proteins promote interactions between sRNAs and mRNAs, remodel RNA structure, and affect RNA stability. ProQ belongs to an extensive protein family (the FinO-domain family), whose members are present in numerous β- and γ-proteobacteria. In the model bacterium *E. coli* and other related bacteria, Hfq stabilises sRNAs against turnover and can facilitate the base-pairing matching of many different sRNA-mRNA pairs (Santiago-Frangos and Woodson, 2018; Wagner and Romby, 2015). As one sRNA can regulate multiple targets, and multiple sRNAs can regulate a single target, a highly interconnected regulatory network results that is dependent on Hfq availability. Perhaps it is not surprising that mutations in Hfq have pleiotropic effects (Gorski *et al.*, 2017).

Hfq is a member of the Sm/LSm superfamily of RBPs, which can be found in almost every organism. The bacterial Hfq forms a hexamer that presents three faces within the core for interaction with RNA (Figure 4A,B). The 'proximal face' is close to the amino-terminal end of Hfq, the 'distal face' lies on the opposite side of the Hfq hexamer, and the 'rim region' separates the proximal and distal faces and provides additional RNA-binding sites (Figure 4A,B). Intrinsic transcription terminators, found at the 3′ end of many operon mRNAs, bear a stem-loop structure followed by a uridine-rich stretch, and are preferred targets of Hfq on the proximal face (Otaka *et al.*, 2011). The distal face binds up to six occurrences of an A-R-N motif (A: adenine, R: purine, N: any nucleotide) that can be found in mRNA targets or more complex sRNAs that wrap over all three surfaces of Hfq (Robinson *et al.*, 2014). Emanating from the conserved core is an intrinsically disordered carboxyl-terminal domain that is variable in size and sequence but acts synergistically with the other RNA-binding faces on the conserved core and contributes to the specificity of its RNA annealing activity (Kavita *et al.*, 2022; Santiago-Frangos *et al.*, 2016, 2017; Santiago-Frangos *et al.*, 2019). The hexameric architecture of Hfq provides multivalency for RNA interaction, which can yield strong overall binding through chelate cooperativity, but also provides a mechanism for exchange of RNAs on the surfaces on short time scales (Fender *et al.*, 2010; Roca *et al.*, 2022) (Figure 4C). The stepwise binding reduces the activation barrier for the exchange, despite the overall high binding affinities, which are in the nanomolar range for most RNAs.

Kinetic aspects of riboregulation, *in vitro* and *in vivo*

Searching for a match between a riboregulatory and a target seems akin to finding a needle in a haystack. From the moment RNA enzymes are loaded with guide RNAs, a process must follow that ensures the exploration of a large excess of non-specific DNA or RNA before the target sequence is encountered. How is this achieved with biologically meaningful rates, and how is misrecognition of off-targets avoided?

Bacteria offer a convenient system to explore temporal and specificity aspects of riboregulation. Models for random 3D diffusion in a simplified, unhindered environment predict that target site binding by regulatory RNAs in bacteria occurs in several minutes (Flegg, 2016; Małecka and Woodson, 2024). However, evidence indicates that bacteria respond to sRNA induction within 2 minutes or less of receiving an environmental signal (Papenfort *et al.*, 2006). This discrepancy might be accounted for by a facilitated diffusion process, analogous to that proposed by Berg, Winter, and von Hippel (1981) to explain how DNA-binding proteins encounter duplex DNA through a combination of three-dimensional search and local one-dimensional sampling. Facilitator proteins can support both processes for regulatory RNAs, as well as facilitate the matching of regulatory and target RNAs.

The RNA chaperone Hfq provides a model system for the action of the facilitator proteins, and the detailed kinetic analysis of Hfq and RNA engagements provides broader insights into the process of riboregulation in other systems. As mentioned above, most sRNAs are chaperoned by Hfq in the model bacterium *E. coli* and other bacteria (Figure 4). While RNA association with Hfq is diffusion-limited *in vitro*, the formation of RNA-RNA-Hfq complexes is much slower. A first step involves the fast binding of Hfq to (A-R-N)-repeat motifs in the mRNA, which has $k_{on} \sim 1\text{–}10\times10^7$ $M^{-1}s^{-1}$, close to diffusion-limitation (Hopkins *et al.*, 2011; Roca *et al.*, 2022). In a second step, the Hfq-RNA complex can recruit a second strand with $k_{on} \geq 10^8\ M^{-1}s^{-1}$ and a compaction brings sites from the mRNA to the rim of Hfq, where sRNA pairing can occur. Hfq can transfer an sRNA between sites on a single mRNA without dissociating from the mRNA, which has some analogy to monkey-bar transfer proposed for transcription factor diffusion on duplex DNA (Watson and Stott, 2019). Single-molecule fluorescence energy transfer results show that Hfq bridges the two RNAs in the sRNA-Hfq-mRNA complex.

Studies of Hfq using FRET (Förster (fluorescence) resonance energy transfer) reveal a mode of linear scanning and a compaction of the target mRNA to bring sRNAs to distant sites from the Hfq binding site through segmental transfer of sRNA between sites in a mRNA (Małecka and Woodson, 2024). The net effect is an iterative scanning of small RNA targets by Hfq that allows for rounds of scanning, base-pairing, and duplex unzipping until the sRNA-Hfq-mRNA complex finally dissociates. The efficiency of forming sRNA-mRNA-Hfq complexes improves when sRNAs are prebound to Hfq. sRNAs interact with more Hfq binding surfaces, likely requiring extensive conformational changes in the RNA, achievable only when the protein is unoccupied. Single-molecule studies show that some complexes dissociate, possibly due to RNAs not being fully base-paired. In such cases, RNAs rarely leave Hfq together; instead, the RNA that joined last is the first to leave (Małecka and Woodson, 2024). The model proposes that compaction and segmental transfer, combined with repeated cycles of base-pairing, enable the kinetic selection of optimal sRNA targets. Interactions with arginines bristling the surface of Hfq allow target RNAs to slide past the rim, presenting different nucleotides to the

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sRNA for base pairing. In this model, nucleotides between the rim and the A-R-N motif form a loop that shrinks or grows, depending on which bases are engaged on the rim. Another example where one-dimensional diffusion improves search time is the encounter of guide RNAs with targets in the CRISPR/Cas9 phage immunity system (Globyte *et al.*, 2019).

The speed of riboregulation *in vivo* suggests that substrate capture may facilitate the process, and possible mechanisms have been supported by experimental studies for the central bacterial RNase E to diffuse on substrates to reach downstream mRNA sites (Banerjee *et al.*, 2024; Richards and Belasco, 2019). RNase E can be activated by groups on the 5′ terminus on the RNA substrate, and the diffusion model holds that the enzyme scans from there until a high-cleavage sequence is encountered. Another mode of substrate capture is envisaged involving an opening and closing of the intrinsically disordered arms of the multi-enzyme RNA degradosome, somewhat like the tentacles of a sea anemone (Dendooven *et al.*, 2021). This mode may facilitate the capture of sRNA/chaperone complexes that can flexibly match transcripts for complementarity. Once a match is made, rapid remodelling favours handover to the catalytic centre to initiate degradation of both tagged mRNA and the sRNA regulator.

The fidelity and efficiency of riboregulation

The base-pairing regions of regulatory RNAs, often referred to as the 'seed', are typically short as seen in the 6-8 nucleotides embedded in the 21-mers for miRNAs or often less than 10 nucleotides for bacterial sRNAs (Santiago-Frangos and Woodson, 2018). In comparison, bacterial transcription factor sequence motifs have an average length of 16 base pairs, and in eukaryotes of about 8 base pairs (Wunderlich and Mirny, 2009). sRNAs do not require perfect matching to a target. Instead, structure and base-stacking are more likely to be the key factors for the efficacy of regulatory RNA action. A high-throughput screening study using a library of synthetic sRNAs with varying seed region lengths showed that, in the presence of the RNA chaperone Hfq, 12 nucleotides are sufficient for regulation and processing by RNase E (Brück *et al.*, 2024). For some sRNAs, however, longer seed regions may be necessary for efficient target regulation. When using the scaffold of a structurally complex sRNA, synthetic seeds of over 35 nucleotides are needed to achieve strong repression of a target mRNA (Brück *et al.*, 2024). In comparison, for a scaffold based on structurally simpler sRNA, a seed of 12 nucleotides was sufficient to regulate the target (Hoynes-O'Connor and Moon, 2016). This might be due to requirements to unfold the RNA. The longer base-pairing might compensate for weak RNA-Hfq interactions or the presence of structured RNA regions (Małecka and Woodson, 2021). In *E. coli*, the sRNA SgrS regulates the *ptsG* mRNA by imperfect base-pairing that involves 23 of the 31-nt long SgrS seed region (Maki *et al.*, 2010; Vanderpool and Gottesman, 2004). This can give high precision for matching, making off-target interactions comparatively rare. Another contributing factor is the interactions of the Hfq chaperone with mRNA (Faigenbaum-Romm *et al.*, 2020). In terms of applications, efficient repression of target mRNAs can be achieved *in vivo* using antisense peptide nucleic acids (PNAs) conjugated to cell-penetrating peptides that are 9-mer to 10-mers (Goltermann *et al.*, 2019; Popella *et al.*, 2022).

Whereas seeds at the RNA ends are less restricted topologically for making pairs, some seeds are in loops (Solchaga Flores *et al.* 2024). In these cases, matching to make a duplex could present a topological problem because the duplex formation requires remodelling of structural parts to allow free rotation. Base-stacking, as well as complementarity of the pairing, is also likely to be an important factor in the energy of equilibrium binding of seeds to targets, but also in the rates at which the pairing is made and, potentially, rejected. Strong binding of RBPs raises the puzzle of slow *off*-rates that may be outside the seconds range needed for riboregulation. The Hfq protein is a salient example, where RNAs bind in the nanomolar K_D range. As described, the high affinity is due to the chelate cooperativity of the arranged protomers in the oligomeric quaternary structure, but the exchange rates of competing RNAs can be high despite the strong affinities, due to stepwise replacement of individually weak interactions (Santiago-Frangos and Woodson, 2018) (Figure 4c).

Kinetic proofreading in riboregulation

Linus Pauling (1957) noted that some enzyme reactions exhibit specificity far beyond the theoretical expectations based on measured relative binding energies for cognate and non-cognate substrates. To explain the puzzling fidelity of these and other molecular recognition processes, in which the free energy of equilibrium binding is not sufficient to account for discrimination, models were proposed by Hopfield (1974) and Ninio (1975) invoking the concept of kinetic proofreading. This process involves an irreversible reaction cycle that decreases errors at the expense of net entropy change (Boeger, 2022) and effectively involves a delay step between the initial recognition event and its downstream effect that changes the free energy difference. Specificity is enhanced not by increasing the energetic difference between cognate and non-cognate associations but by applying the difference both before and after the delay step. Following the delay step, dissipation of free energy favours dissociation of the enzyme-substrate complex over the association and return of the suspended delay mechanism to its initial state. Therefore, rebinding of the substrate tends to occur prior to, and not after, the delay step. Kinetic proofreading has been invoked to explain the fidelity of transcription and translation that exceeds the energy difference of pairing cognate and near-cognate codons at equilibrium (Boeger, 2022). In translation, discard pathways are accelerated by the irreversible step of GTP hydrolysis by the elongation factor EF-Tu (bacteria) and its homologs in eukaryotes and archaea. A kinetic proofreading step has been proposed for pre-mRNA splicing quality control, energised by the ATPase action of the RNA helicases of the splicing machinery (Egecioglu and Chanfreau, 2011) and is also likely to occur in the multi-step process of ribosome assembly (Baßler and Hurt, 2019).

RNA-mediated regulation is also a non-equilibrium, effectively irreversible process through impact on RNA turnover. A possible example of proofreading might be provided by the case of the endoribonuclease RNase E, which is known to target single-stranded RNAs at AU-rich sites in different bacteria. sRNA degradation may occur after binding to the target mRNA due to coupled degradation by RNase E (Massé *et al.*, 2003) or remodelling so that the 3′ end is unprotected (Dendooven *et al.*, 2021). sRNAs with a 5′ seed region may be more susceptible to processing by the endoribonuclease RNase E, especially if the seed region sequence bears AU-rich motifs serving as cleavage sites. Cleavage has been seen to rapidly remove the seed region (Bandyra *et al.*, 2012; Bandyra *et al.*, 2024), which would remove the capacity to direct pairing, and could occur as an effective surveillance process, whereby inadequate pairing destroys the sRNA but not the mismatched transcript. Although wasteful, this could improve the overall fidelity of the system. The metabolic costs at the systems

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level would resemble an energy-dependent discard pathway, akin to what is seen in kinetic proofreading.

RNA surveillance might also involve such proofreading based on the potential cooperative interplay between helicases and RNA-binding sites in ribonucleases, where defective RNA or ribonucleoprotein complexes with an unstable structure would be unwound or remodelled and subsequently directed to ribonucleases for degradation. Folded RNA would however withstand helicase activity and be released. This model has been proposed, in the context of the gamma-proteobacterial RNA degradosome, for the cooperation of the RNA helicase RhlB and flanking RNA-binding segments in RNase E (Chandran *et al.*, 2007), which would act in a 'proofreading mode'. The partial unwinding or remodelling mediated by the helicase may also be coupled with the processing of structured precursors.

Turnover of RNA with 3′-end tailing is similarly a non-equilibrium process. Aspects of riboregulation such as pairing and turnover might have aspects for kinetic proofreading that ensure achieving fidelity and specificity to overcome the limitations of molecular discrimination. Certain eukaryotic RNA degradation processes have the appearance of a futile cycle that consumes energy, but perhaps these processes may, in effect, be energy-dependent discard pathways that contribute to molecular discrimination. For instance, the pathway for nonsense-mediated decay is guided by the ATPase activity of a helicase (UPF1), with an impact on the kinetics and efficiency of NMD (Chapman *et al.*, 2024; Kishor *et al.*, 2019). Mutations in the helicase that prevent ATP hydrolysis result in loss of decay target discrimination (Lee *et al.*, 2015). Also, RNA degradation is part of the process for nuclear import of the decay machinery (Haimovich *et al.*, 2013). In these and other processes, the energy consumption through ATP hydrolysis or RNA degradation itself is a licensing step for subsequent steps that impact the kinetics and, potentially, the fidelity of the processes.

RNA conformation, 'conformability', and dynamics, in recognition and allostery

Perhaps the most challenging and subtle aspect of decoding information in an RNA molecule is its conformational space, the depth and distribution of the energy minima in that space, the rates of transitions between states, and whether that complex landscape has any biological meaning. Intuitively, the conformation of RNA and its 'conformability,' which is the capacity to adapt shape to optimally bury surface and match chemically complementary faces with partners, must be important aspects of molecular recognition of the nucleic acid. Like other biological macromolecules, RNA molecules undergo motions in ranges of picoseconds to seconds, representing a timescale of 12 orders of magnitude (Ganser *et al.*, 2019; Roy *et al.*, 2023). These motions encompass large-scale conformational adjustments for shape-fitting and co-transcriptional and co-translational folding. Conformational selection is likely an important aspect of molecular recognition of RNA (Liberman and Wedekind, 2012; Vicens *et al.*, 2011). Experimental studies by NMR implicate conformational ensembles as being important in cellular activity. One example is transactivation of the human immunodeficiency virus 1 (HIV-1) genome, which is driven by a short-lived RNA conformational state of a conserved and structured RNA element located at the 5′ end of the retroviral genome (Ken *et al.*, 2023). The motions in an RNA or ribonucleoprotein complex can also propagate structural changes that can communicate allostery signals. Examples of allostery can be found in the stepwise assembly of the splicing machinery that removes introns from transcripts, in the RNA-ligand complex formation in riboswitches (Peselis *et al.*, 2015) and state switching of ribosomes during translation (Walker *et al.*, 2020).

From the perspective of energy, RNA longer than 10 bases tends to become compact but in a dynamic equilibrium of conformations (Vicens and Kieft, 2022), supported by long-distance paring (Lu *et al.*, 2016; Schultes *et al.*, 2005). The poly(A) tail that regulates eukaryotic mRNA stability is recognised by deadenylase enzymes primarily based on its stacking signature (Tang *et al.*, 2019; Tang and Passmore, 2019), but this can be remodelled (Schäfer *et al.*, 2019). Co-transcriptional folding can be an important aspect of regulatory RNA action (Rodgers *et al.*, 2023). In analogy to protein folding (Streit *et al.*, 2024), the transcriptional machinery may decrease the entropy penalty of co-transcriptional folding of the nascent transcript. The *glmS* ribozyme riboswitch can respond to its ligand during transcription to regulate mRNA decay at an early stage of mRNA synthesis (Lou and Woodson, 2024). Structural analysis by cryoEM has provided a detailed view of how ligand-activated folding of a nascent riboswitch RNA is coupled with transcription elongation in bacteria (Chauvier *et al.*, 2023). Environmental factors can be envisaged to have a context-dependent impact on this RNA folding, for example, through macromolecular crowding (Daher *et al.*, 2018), and the local environment is likely to contribute to the effectiveness of co-transcriptional RNA folding processes.

For RNA to be accessed, the intrinsic RNA structure may need to be remodelled. An extended state conformation for RNA, which might be a requirement for local recognition or action of ribonucleases with preference for single-stranded regions, would have an energetic cost. Translating ribosomes, ATP-dependent helicases, and other RNA remodelling proteins partially unwind structured regions (Bhaskaran and Russell, 2007; Rouskin *et al.*, 2014; Yang *et al.*, 2007), while translation inhibition causes mRNAs to decrease in end-to-end length *in vivo* (Adivarahan *et al.*, 2018; Khong and Parker, 2018). Thus target RNA structure can impact on scanning, recognition, and the subsequent regulatory activity that determines the fate of the target RNA. Target site accessibility is likely to impact on regulatory RNA potency and the avoidance of off-target effects.

Modification of chemical identity and impact on function

In organisms from all domains of life, RNA is covalently modified, with an impact on lifetime and recognition. More than 170 modifications of RNAs have been identified, mostly on tRNAs (McCown *et al.*, 2020), but some on long non-coding RNAs and mRNAs. 5′-end caps added to nascent RNA protect from exonucleases and regulate subsequent processing steps. An N^7-methylguanosine (m^7G) cap is a common modification of nascent transcripts in eukaryotes. Non-canonical 5′-end moieties have been described in prokaryotes and eukaryotes, such as the ubiquitous coenzyme NAD^+ (nicotinamide adenine dinucleotide) and 3′-desphospho-coenzyme-A, each introduced as a first nucleotide during transcription and implicated in subsequent RNA metabolism (Bird *et al.*, 2016; Cahová *et al.*, 2015; Doamekpor *et al.*, 2022; Jiao *et al.*, 2017; Vvedenskaya *et al.*, 2018). A summary of the most important RNA modifications can be found in Figure 5.

As a few salient examples, N^6-methyladenosine (m^6A) is one of the most ubiquitous modifications of eukaryotic RNAs and controls the processing, export, splicing, and metabolism of cellular RNAs. m^6A modification acts as a general mechanism to control RNA half-life (Rücklé *et al.*, 2023). The biological effects of m^6A are

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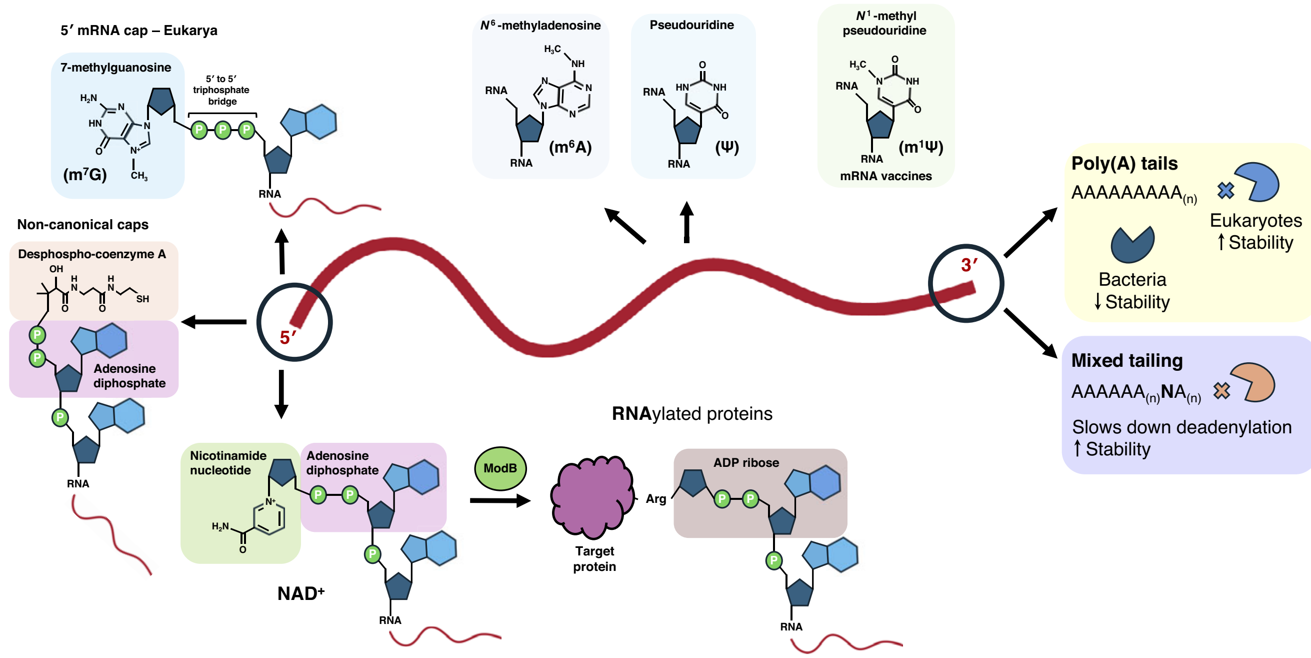

Figure 5. Schematic view of some of the most common RNA modifications.
An N^7-methylguanosine (m^{7}G) cap is a common modification of nascent transcripts in eukaryotes. Other 5′-end moieties, including 3′-desphospho-coenzyme A and NAD$^+$, have been described in prokaryotes and eukaryotes. NAD-capped-RNAs can be used by the bacteriophage T4 ADP-ribosyltransferase ModB as a substrate to link RNA chains to acceptor proteins. Modified nitrogenous bases, including N^6-methyladenosine (m^{6}A) and pseudouridine (Ψ), can also be found in naturally occurring RNA molecules and have been critical to the development of mRNA vaccines (m1Ψ). Polyadenylation is another key signal that impacts the lifetime of mRNAs. Poly(A) tails, added at the 3′ end of eukaryotic mRNAs are crucial elements for export from the nucleus, translation initiation, and mRNA stability. The heterogenous composition of tails acts as a 'speed bump' to slow deadenylation of transcripts, increasing their stability. In bacteria, Poly(A) tails can act as a signal for RNA degradation.

largely mediated by specific m^{6}A RBPs, which recruit other protein complexes to affect RNA processing (Liao *et al.*, 2018). For instance, YTHDC1 binding to m^{6}A RNA in Chromatin-associated regulatory RNA (carRNA), affects the transcription of genes and promotes their degradation via the nuclear exosome targeting (NEXT) complex (Liu *et al.*, 2020). *Syn-* and *anti-*conformations of the N^6-methyl group are favoured for single-stranded and duplex forms, respectively, and affect the presentation for recognition by partners and decrease the stability of double-stranded regions (Roost *et al.*, 2015). m^{6}A methylation increases flexibility and solvent accessibility in hairpin stems (Zhou *et al.*, 2016), where, without disrupting these elements of secondary structure, it modulates local RNA structure and increases accessibility of adjacent bases for RBPs (Jones *et al.*, 2022). Pseudouridine is also a common RNA modification in all domains of life and has been found in bacterial mRNAs (Schaening-Burgos *et al.*, 2024). *In vitro*, pseudouridine can inhibit ribonuclease activity (Islam *et al.*, 2021), so the modification could have a potential role in modulating transcript lifetime.

Polyadenylation is another key signal that impacts the lifetime of eukaryotic mRNAs. PolyA tails added after the stop codon are crucial elements for export from the nucleus, translation initiation, and to signal RNA degradation in either nucleus or cytoplasmic compartments. Modification in the 3′ polyA tail has been found to impact transcript lifetimes in trypanosome parasites. The heterogenous composition of tails also acts as a 'speed bump' to slow deadenylation of transcripts and is a consequence of the stochastic incorporation of non-adenosine nucleotides by polyA polymerases of the TENT family (Lim et al., 2018). Regulatory RNA elements help to recruit the polymerase and are exploited by viruses to selectively stabilise their transcripts (Seo *et al.*, 2023).

The effectiveness of mRNA vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has made synthetic mRNA technology a promising avenue for treating and preventing disease. Key to this technology is the incorporation of modified nucleotides such as N1-methylpseudouridine (m1Ψ) into the mRNA to increase antigen expression and reduce immunogenicity. The modification increases the average length of polyA tails on the vaccine transcripts against the viral spike protein through recruitment to a TENT family polyA polymerase associated with the endoplasmic reticulum (Krawczyk *et al.*, 2022), but the recognition mechanism is yet to be defined.

Finally, it is interesting to note that RNA itself can be covalently linked to proteins (Wolfram-Schauerte *et al.*, 2023; Yilmaz Demirel *et al.*, 2024). ADP-ribosyltransferases transfer an ADP-ribose fragment from NAD to acceptor proteins (Figure 5). A bacteriophage T4 ADP-ribosyltransferase ModB accepts NAD-capped-RNA as a substrate, resulting in the covalent linkage of entire RNA chains to acceptor proteins in a process termed 'RNAylation' (Wolfram-Schauerte *et al.*, 2023; Yilmaz Demirel *et al.*, 2024). ModB specifically RNAylates its host protein targets, such as ribosomal proteins, at arginine residues. RNAylation has been proposed to play roles in the interaction between phages and bacteria (Wolfram-Schauerte *et al.*, 2023).

RNA metabolism and sub-cellular compartmentalisation

In eukaryotic cells, the nuclear envelope separates transcription and translation, but in prokaryotes, the lack of this membrane barrier allows mixing of all steps of gene expression, from transcription to translation and decay (Wolfram-Schauerte *et al.*, 2023). Nonetheless, some prokaryotes appear to have effective compartments for RNA degradation, where the machinery can be membrane-bound

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so that the machinery for RNA metabolism is separated from the nucleoid. This compartmentalisation results in a delay between transcription and degradation for some transcripts and is required for the orderly biogenesis of ribosomes in *E. coli* (Hadjeras *et al.*, 2023; Mackie, 2013). The chromatin around transcripts encoding transmembrane machines, such as secretion systems, is proposed to come into proximity with the membrane in a process known as transertion, which couples transcription, translation and membrane insertion (Bakshi *et al.*, 2015; Kaval *et al.*, 2023; Roggiani and Goulian, 2015). Transertion may also occur for other bacterial membrane proteins.

In eukaryotes, ribonuclease activity can be localised at the endoplasmic reticulum membrane, as seen with the transmembrane inositol-requiring enzyme 1 (IRE1), which has dual serine/threonine-protein kinase and ribonuclease activity (Walter and Ron, 2011). At a more general level, eukaryotic mRNA transcripts that encode non-membrane proteins are not evenly distributed across the cytoplasm, and the sub-cytoplasmic location of translation has been observed to control protein output (Berkovits and Mayr, 2015; Horste *et al.*, 2023). Functionally related groups of transcripts are enriched in membrane-free compartments, with localisation patterns correlated with gene architecture and RBPs interacting with the 3′ untranslated regions.

Some RBPs can cluster into membrane-less organelles upon interaction with RNA molecules via liquid-liquid phase separation (LLPS) (Boeynaems *et al.*, 2018; Lin *et al.*, 2015). Ribonucleoprotein and RNAs have high local concentration within LLPS bodies (Guzikowski *et al.*, 2019) where physicochemical features have been proposed to impact RNA secondary structure (Nott *et al.*, 2016). The physical-chemical conditions necessary for RNA-containing foci to form are not completely understood, but it has been shown that proteins with RNA-binding domains and intrinsically disordered regions tend to form punctate bodies (Banani *et al.*, 2017; Berkovits and Mayr, 2015; Horste *et al.*, 2023; Protter *et al.*, 2018). Biomolecular condensates are readily identified in eukaryotic cells, as seen in nucleoli, P-bodies or stress granules (Figure 6A). These are ribonucleoprotein assemblies that are compartmentalised without a lipid membrane, and which facilitate specific cellular processes. The phase separation appears to occur in the nucleoplasm compartment which likely helps the assembly of RNA genomes of viruses (Haller *et al.*, 2024).

Like eukaryotic P-bodies, the recently discovered bacterial ribonucleoprotein bodies organise the mRNA decay machinery (Figure 6B). The intrinsically disordered RNase E C-terminal domain is proposed to be necessary and sufficient for LLPS and the formation of bacterial ribonucleoprotein bodies (Al-Husini *et al.*, 2018; Strahl *et al.*, 2015). The C-terminal domain is also highly charged, and charge screening might be involved in the formation of the phase-separated bodies (Holmstrom *et al.*, 2019). In the Gram-negative bacteria *Caulobacter crescentus*, the formation of LLPS bodies depends on the interaction of the RNA degradosome with RNA targets, particularly sRNAs, antisense RNAs and poorly translated mRNAs, and it is released by RNA degradation (Al-Husini *et al.*, 2018). The scaffold domain of the RNA degradosome, in *C. crescentus* and many other bacterial species, is punctuated by RNA-binding sites and is intrinsically disordered, suggesting that it could be a good mediator for the formation of liquid-liquid phase-separated bodies (Figure 6B). RNase E foci in *C. crescentus* colocalise with genes encoding ribosomal RNA (Al-Husini *et al.*, 2018) and could be co-transcriptional processing centres. Formation of degradosome foci has also been observed in *E. coli*, forming transient clusters driven by and dependent on RNA turnover (Strahl *et al.*, 2015). Truncation of the C-terminal domain of RNase E, which is the scaffold of the degradosome, lowers the fitness of *E. coli* and *C. crescentus* (Al-Husini *et al.*, 2018; Nandana et al., 2024), and impacts on symbiotic relations of bacteria with plants (Mallikaarachchi *et al.*, 2024), implicating the potential biological importance of cluster formation. Phase separation is also observed for the bacterial RNA chaperone Hfq under stress conditions (Goldberger *et al.*, 2022; McQuail *et al.*, 2020; McQuail *et al.*, 2022; McQuail *et al.*, 2024).

Co-transcriptional and co-translational degradation, and potential modulation by RNA

For the model bacterium *E. coli*, evidence has accumulated over decades that transcription is coupled to translation (Blaha and Wade, 2022; Qureshi and Duss, 2024), that mRNA degradation can commence during ongoing transcription (Chen *et al.*, 2015), and that translation affects mRNA degradation (Deana and Belasco, 2005). Co-transcriptional and co-translational mRNA degradation can potentially halt the synthesis of unneeded proteins in response to changing cellular requirements. For the *lac* and *trp* operons, RNA from genes near the promoter decay before the more distal genes are transcribed (Cannistraro and Kennell, 1985; Morikawa and Imamoto, 1969; Morse *et al.*, 1969). Translation can be coupled to transcription in archaea (Weixlbaumer *et al.*, 2021). However, in some bacteria, such coupling is not so important because the comparatively greater speed of transcription uncouples it functionally from translation. For example, in the firmicute *Bacillus subtilis*, RNA polymerase can translocate faster than the ribosome, so that the ribosome is uncoupled from transcription (Johnson *et al.*, 2020; Zhu *et al.*, 2021).

The ribosome binding site (RBS) affects the loading of ribosomes on the transcripts and hence can influence transcription–translation coupling. In *E. coli*, the RBS sequence can determine the fate of mRNAs as it modulates the probability of premature transcription termination, which occurs in the absence of transcription–translation coupling (Kim *et al.*, 2024). Recent evidence suggests that sub-cellular localisation of RNase E (or its homologs) and premature transcription termination, which arises in the absence of transcription–translation coupling, are key determinants that explain how different genes and species have evolved to regulate transcriptional and translational coupling to mRNA degradation (Kim *et al.*, 2024).

One of the pathways that leads to transcription termination in bacteria involves the transcription termination factor Rho, an ATP-dependent hexameric helicase that has been shown to interact with RNase E in *C. crescentus* (Aguirre *et al.*, 2017). The cooperation between these two enzymes has been proposed to result in the high degradation rates and greater probability of premature transcription termination observed in this species (Kim *et al.*, 2024). Rho-dependent termination has also been proposed to mediate co-transcriptional regulation by sRNAs, when transcription-coupled translation of the mRNA targets is reduced upon sRNA binding (Reyer *et al.*, 2021). Furthermore, co-transcriptional binding of a sRNA–Hfq complex represses a target transcript more efficiently and faster than post-transcriptional binding, possibly because it prevents the formation of a structure that otherwise promotes translation by enabling access to the RBS (Rodgers *et al.*, 2023). In archaea, the protein FttA mediates factor-dependent transcription termination by cleaving RNA co-transcriptionally through endonucleolytic cleavage followed by 5′→3′ exonucleolytic activity (Sanders *et al.*, 2020). In a process that mediates factor-dependent transcription

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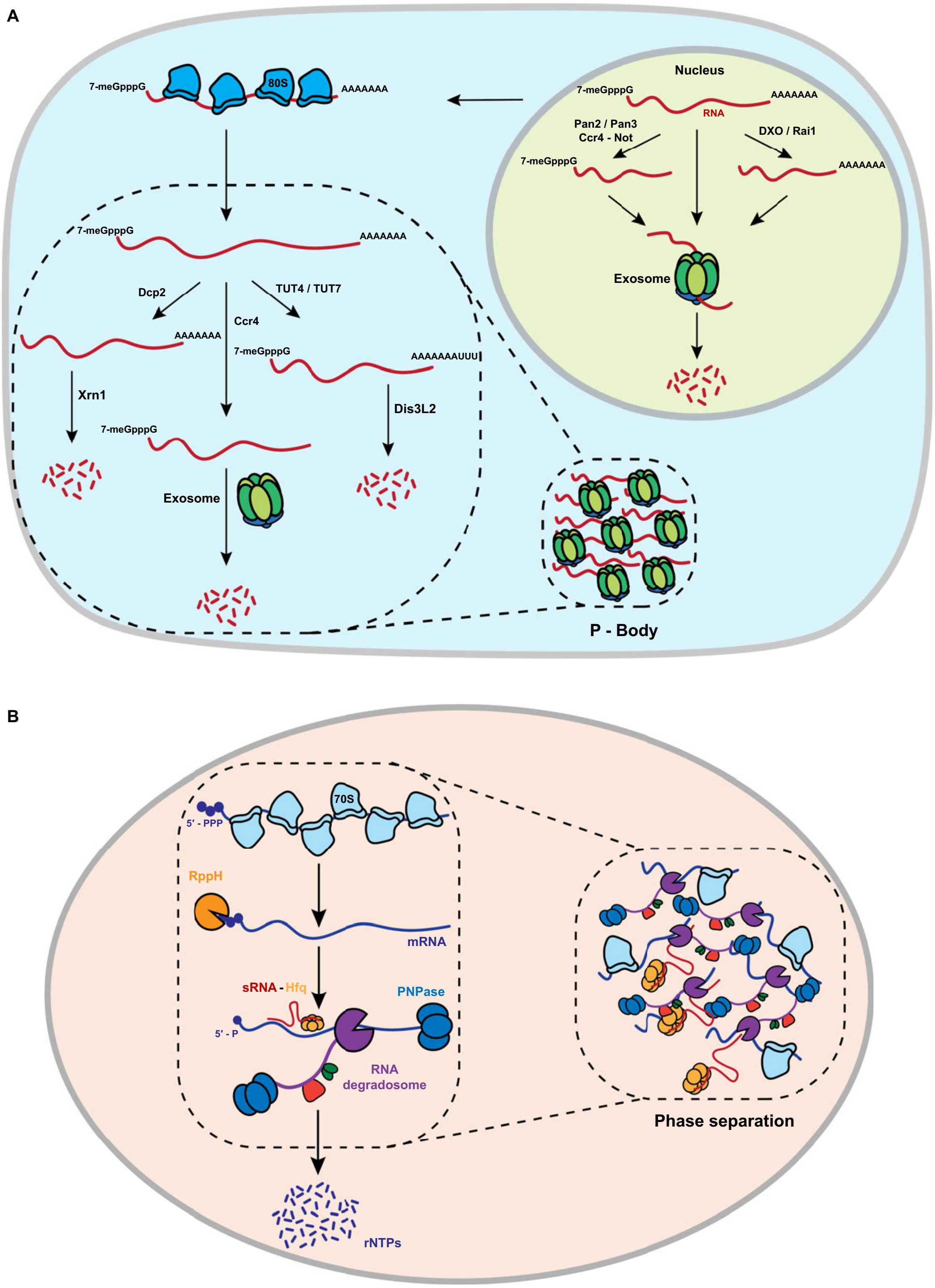

Figure 6. Compartmentalisation of ribonucleoprotein effectors in eukaryotes and bacteria.
(A) The cytosolic exosome in eukarya is known to facilitate the formation of P-bodies. (B) The scaffold domain of the RNA degradosome is intrinsically disordered and punctuated by RNA-binding domains, providing opportunities for liquid-liquid phase separation in bacteria. The scaffolding domain of the RNA degradosome could be a key player in the formation of bacterial RNP bodies, which have been proven to be very important in organising RNA turnover in the cell, posing a strong evolutionary force in maintaining the disordered and flexible features along evolution.

termination in all three domains of life, FttA 5′→3′ translocation on the nascent RNA triggers transcription termination by applying a mechanical force on the transcription elongation complex (TEC). The interaction between FttA and the TEC is bridged by the archaeal transcription factor Spt5, from the NusG/Spt5 protein family, conserved in bacteria, archaea, and eukaryotes (You *et al.*, 2024).

https://doi.org/10.1017/S0033583525000022

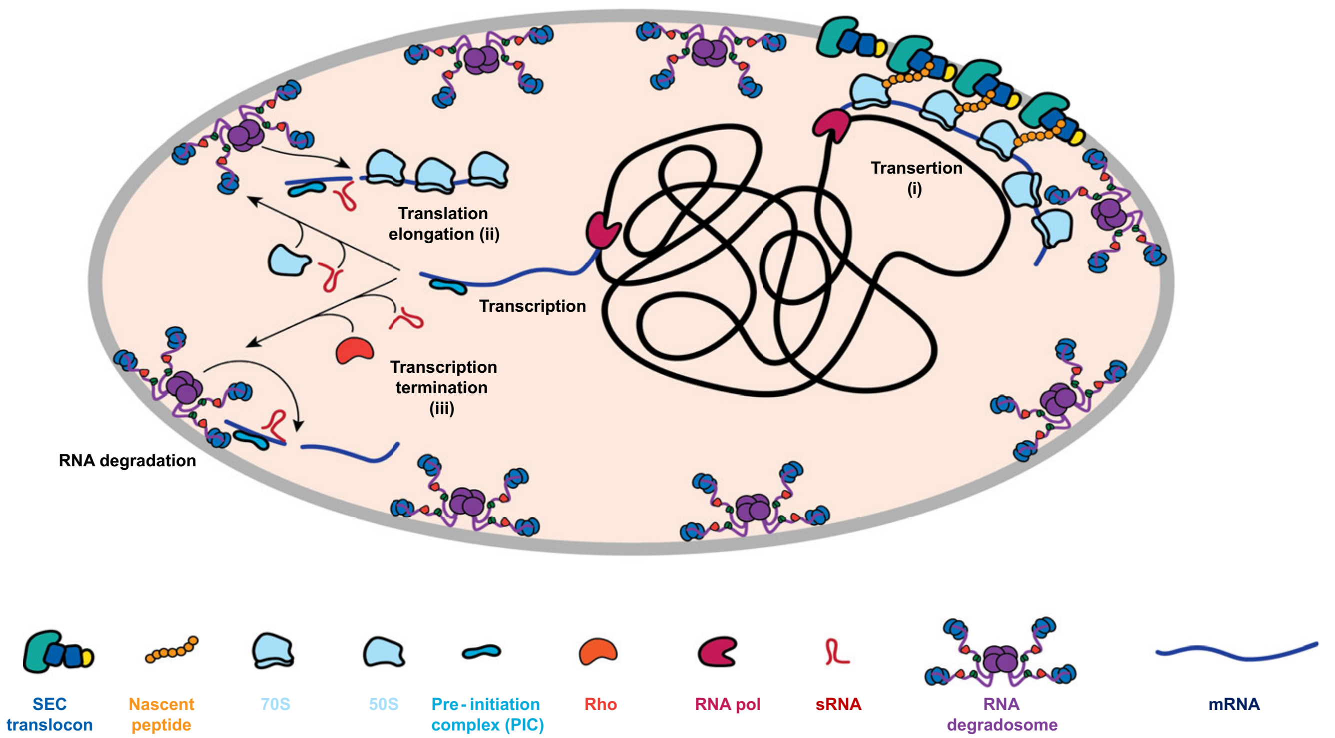

Figure 7. Schematic models for degradation of mRNAs, coupled to transcription and translation, in *Escherichia coli.*
The physical localisation of the RNA degradosome to the membrane poses a spatial layer to the regulation of gene expression and the hypothesis of RNA surveillance. In this cartoon, three models are presented where the RNA degradosome could access transcripts engaged with ribosomes and translating polysomes. First, genes encoding for some membrane proteins are known to be transcribed and translated in proximity to the membrane, following a process called transertion (i) (Kaval *et al.*, 2023). When transertion occurs, the RNA degradosome bound to the inner membrane is close to the translation site and can interact with polysomes scanning for unbound mRNA to cleave. Second, the degradosome could be interacting with polysomes and once the mRNA has been translated, upon binding of Hfq:sRNA complexes, it could cleave the mRNA. This mode is supported by the *in vivo* observation of RNA cluster formation by degradosomes in the presence of polysomes (ii) (Hamouche *et al.*, 2021). Finally, the RNA degradosome could act to turn over transcripts that might be incomplete through transcription termination (iii) (Bandyra *et al.*, 2024).

In bacteria related to *E. coli* and *B. subtilis*, where the key ribonucleases are localised to the membrane (*i.e.*, RNase E and RNase Y, respectively), mRNA degradation takes place exclusively on the membrane once mRNAs are released from the gene loci (Kim *et al.*, 2024). Co-transcriptional mRNA degradation can occur for inner membrane proteins but appears to occur infrequently for most other genes. The lack of co-transcriptional degradation would be advantageous when more proteins need to be made per transcripts, but in principle, could be triggered by sRNAs (Sedlyarova *et al.*, 2016). In *C. crescentus* and other bacteria, where major ribonucleases and RNA degradosomes reside in the cytoplasm, mRNA degradation may start during transcription (Kim *et al.*, 2024). One question that arises is how co-transcriptional or co-translational degradation is achieved with specificity to silence defined genes but does not result in global repression. This may be a passive mechanism but could be accelerated by tagging with small regulatory RNA (Figure 7) (Bandyra *et al.*, 2024).

The mRNA degradation machineries occur across prokaryotes and eukaryotes and can participate in co-translational degradation (Huch *et al.*, 2023). For example, the deadenylase CCR4-Not complex senses slow elongation through its weak interaction with the E-site of the ribosome and enhances the degradation of slowly translated mRNAs (Buschauer *et al.*, 2020). The importance of this weak interaction was illustrated by Jorgensen and Kurland (1990), who suggested that the strength of mRNA association with the ribosome was related to the rate of both proofreading errors (which arise from incorporation of the wrong amino acid) and processivity errors (which arise from the ribosome skipping a codon, frameshifting, or falling off the mRNA). Relatively weak association of mRNA with the ribosome is important for the process of codon selection and proofreading.

In eukaryotes, physical interactions of RNA polymerases with processing machinery enable coordinated splicing of introns, 3′-end cleavage, and RNA folding. Intron retention can prevent 3′-end cleavage in a nascent transcript and cause transcriptional readthrough, which is a hallmark of eukaryotic cellular stress responses (Shine *et al.*, 2024). Single-molecule methods indicate translation-dependent destabilisation of mRNAs (Dave *et al.*, 2023). The mechanisms could account for processes of co-translational decay (Herzel *et al.*, 2022; Huch *et al.*, 2023). Co-translational decay is proposed to involve the recruitment of the 5’→3’ exoribonuclease Xrn1 which follows the terminal translating ribosome identified in yeast and other eukaryotic species (Pelechano *et al.*, 2015; Tesina *et al.*, 2019). The dynamic folding of RNA during transcription is a key aspect of co-transcriptional gene regulation (Schärfen and Neugebauer, 2021). RNA structures in equilibrium and intermediate folds can sense temperature changes or other physicochemical cues, and helicases can remodel them to influence different processing steps. The co-transcriptional folding is likely to impact access and recognition by decay machinery.

In eukaryotic cells, the identification and degradation of defective RNAs and enormous numbers of spurious transcripts may not necessarily involve recognition of specific signatures (Bresson and Tollervey, 2018). It has been proposed that transcripts are subject to 'Decay by Default,' but transcripts with correct and timely maturation gain features that protect them from a fate of degradation by the decay machinery, such as caps and poly(A) tails. In this perspective, RNA polymerase II is in a constitutive surveillance-ready state, with recruitment of protective factors preventing RNA decay. In this scenario, a transcript will be automatically destroyed unless protected. Interactions of the CCR4-Not complex with proteins that lead to maturation or nuclear export might provide an opportunity for deadenylation of transcripts if the maturation is too slow or faulty. These processes may, in effect, be kinetic proofreading events, discussed in the earlier subsection, and contribute to fidelity.

Summary and perspectives

Riboregulation is an inherently non-equilibrium process that can be highly specific for defined targets while also operating rapidly. Multidentate interactions, energy-coupled processes and transient assemblies are the key to robust control mediated through riboregulation in the cellular context. These processes are difficult to capture, but progress has been made recently to follow them temporarily, spatially, and in structural detail. Single-molecule methods have provided insights into the stepwise development of encounter complexes, the remodelling of RNA species on chaperones and on the microscopic rate constants for these processes. Such analysis can reveal if rate constants are accelerated in energy-coupled processes, as occurs in canonical kinetic proofreading. Predictive methods based on machine learning and diffusional models have made highly accurate models for equilibrium complexes, including ribonucleoproteins (Abramson *et al.*, 2024), and this approach is anticipated to be useful for exploring transient, kissing complexes that underpin riboregulation. sRNA-mRNA pairs that are remodelled for ribonuclease action, helicase-RNA complexes on route to remodelling, and Michaelis-Menten-like enzyme species are difficult to capture experimentally. The predictive models can lead to testable hypotheses to explore the determinants of the kinetics and specificity and to engineering a series of trapped intermediates for experimental analysis.

There is also the prospect of engineering for systems biological applications. Antisense PNAs have proven useful as programmable antibiotics by targeting essential or resistance genes of specific target organisms (Popella *et al.*, 2021, 2022). Antisense PNAs (that are 9-mers or 10-mers) have been conjugated to cell-penetrating peptides and found to be sufficient for repression of target mRNAs in complex microbiomes (Goltermann *et al.*, 2019). PNAs can be rapidly modified by changing the antisense sequence, and species-specific targeting might be achievable with such an antisense strategy, leaving most of the microbiome intact. RNA-RNA interactome analysis in hypervirulent *Klebsiella pneumoniae*, an emerging pathogen causing invasive infections in humans, identified a sRNA regulator of cell division (Ruhland *et al.*, 2024) and a potent sRNA inhibitor of bacterial infection in mice (Wu *et al.*, 2024). These observations indicate the potential of targeting and engineering riboregulatory processes for therapy and complex systems design.

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