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Review

# Regulation of bacterial gene expression by non-coding RNA: It is all about time!

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## SUMMARY

Commensal and pathogenic bacteria continuously evolve to survive in diverse ecological niches by efficiently coordinating gene expression levels in their ever-changing environments. Regulation through the RNA transcript itself offers a faster and more cost-effective way to adapt than protein-based mechanisms and can be leveraged for diagnostic or antimicrobial purposes. However, RNA can fold into numerous intricate, not always functional structures that both expand and obscure the plethora of roles that regulatory RNAs serve within the cell. Here, we review the current knowledge of bacterial non-coding RNAs in relation to their folding pathways and interactions. We posit that co-transcriptional folding of these transcripts ultimately dictates their downstream functions. Elucidating the spatiotemporal folding of non-coding RNAs during transcription therefore provides invaluable insights into bacterial pathogeneses and predictive disease diagnostics. Finally, we discuss the implications of co-transcriptional folding andapplications of RNAs for therapeutics and drug targets.

## INTRODUCTION

In order to survive and thrive, bacteria must constantly tune their metabolism and overall gene expression to adjust to their ever-changing environment and ecological niches. Because of the competition between species, it is crucial for their survival that bacteria adapt quickly to transient nutritional resources as well as external threats such as antibiotics and toxins.[1]

In all living organisms, gene expression starts with the synthesis of RNA molecules from the genomic DNA through the process of transcription, ultimately giving rise to the biosynthesis of proteins through translation by the ribosomes.[2] In bacteria, a single multi-subunit RNA polymerase (RNAP) enzyme is responsible for the synthesis of all RNA transcripts within the cell.[3] The core enzyme forms a conserved architecture[4] comprising all of the regulatory functions necessary for the efficient and accurate synthesis and folding of the transcripts during all phases of transcription, namely initiation, elongation, and termination. RNAP is subject to multiple types of regulatory processes that, in combination, determine the overall levels of expression of all genes.[5,6]

RNA-mediated regulation of gene expression is a vital element in all living organisms, including bacteria. It plays a significant role in a variety of biological processes such as cell division,[7] virulence,[8,9] adaptation to environmental changes, and stress responses,[10–12] among others. The different RNAs involved in regulation include both coding RNAs (i.e., that are translated into proteins) and non-coding RNAs (ncRNAs) that are not translated but still play key functional roles within the cell. These latter regulatory ncRNAs have been discovered to play pivotal roles in gene expression and function in all organisms, contributing directly to bacterial fitness and pathogenicity.

Studies of ncRNAs in bacteria have revealed fascinating levels of complexity, both in transcriptional and post-transcriptional processes (Figure 1). A central feature of these regulatory functions is their precise timing and coordination of molecular events. Regulation often involves distinct structures that a single-stranded RNA can readily adopt based on its unique folding free energy landscape with many alternative folds,[13] the 5′ to 3′ directional folding process itself that may favor specific subsets of these structures and, ultimately, the RNA's engagement with cellular processes such as target binding and regulation by other macromolecular complexes. Particularly intriguing is the phenomenon of co-transcriptional folding—the folding of an RNA transcript as it is being transcribed, possible due to its relatively high speed (microseconds for entire stem-loops) compared to the slower (tens of milliseconds per nucleotide) timescale of transcription—which plays significant roles in shaping the function of ncRNA molecules (Figure 2).[14] Maintaining a temporal balance between transcription progress, folding, and RNA functional action is key to the survival of bacteria. Slight changes in the timing of transcription (too fast or too slow) therefore can have deleterious effects, leading to competitive disadvantages or even loss of viability. Examining the importance of the relative timescales of transcription and RNA folding therefore will allow for a deeper and more nuanced understanding of critical biomolecular processes. Beyond the fundamentals, advances in our understanding of the underlying timing mechanisms[15] and their downstream implications might unlock transformative improvements in the design and delivery of antibiotics for bacterial disease treatment.

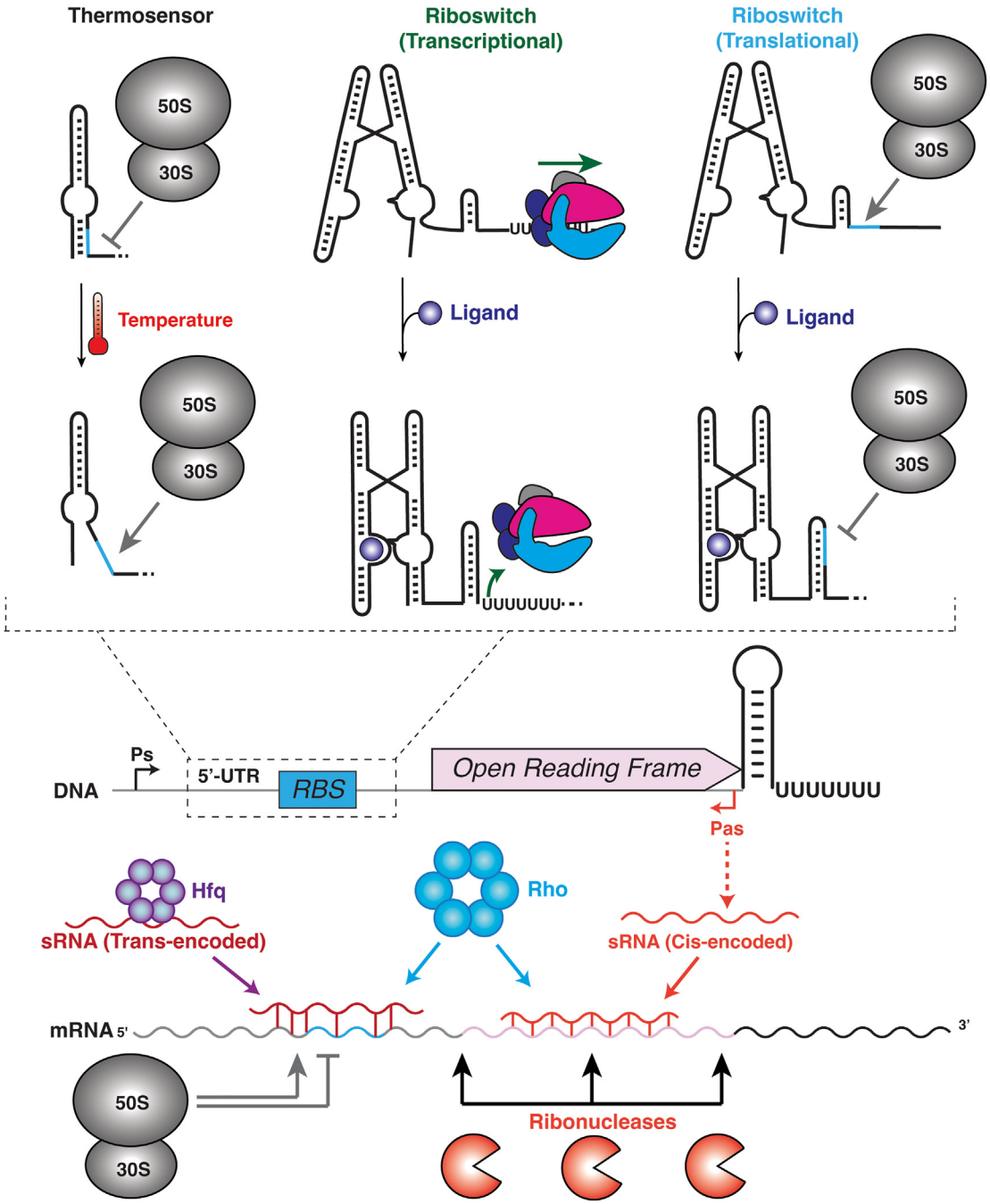



**Figure 1. Types of RNA-mediated regulation through ncRNAs**

*Cis*-encoded ncRNA elements are located in the 5′-untranslated region (5′-UTR) of mRNAs. RNA thermosensors change secondary structure through the effect of temperature, which affects the translation of mRNAs. Riboswitches are structural elements that change conformation upon binding a specific metabolite or ion (termed ligand), which in turn allows for the modulation of gene expression at the level of mRNA transcription (transcriptional riboswitch) or translation initiation (translational riboswitch). *Cis*-encoded antisense RNAs base-pair with the target mRNA and can induce degradation by ribonucleases (RNases). *Trans*-encoded ncRNAs usually interact by imperfect base-pairing with the target mRNA, often aided by RNA chaperone proteins like Hfq, ProQ, or CsrA. This interaction can either result in altered mRNA stability, through RNase degradation, or translation efficiency, by altering the accessibility of the ribosome binding site (RBS). $P_S$ = sense promoter; $P_{AS}$ = antisense promoter.

In this review, we aim to focus on the role and importance of ncRNAs in bacteria, the co-transcriptional folding process, and the particular significance of the relative timing of these events. We cast a spotlight on potential links with disease and therapeutic applications and lay out future directions for research in this promising area of study.

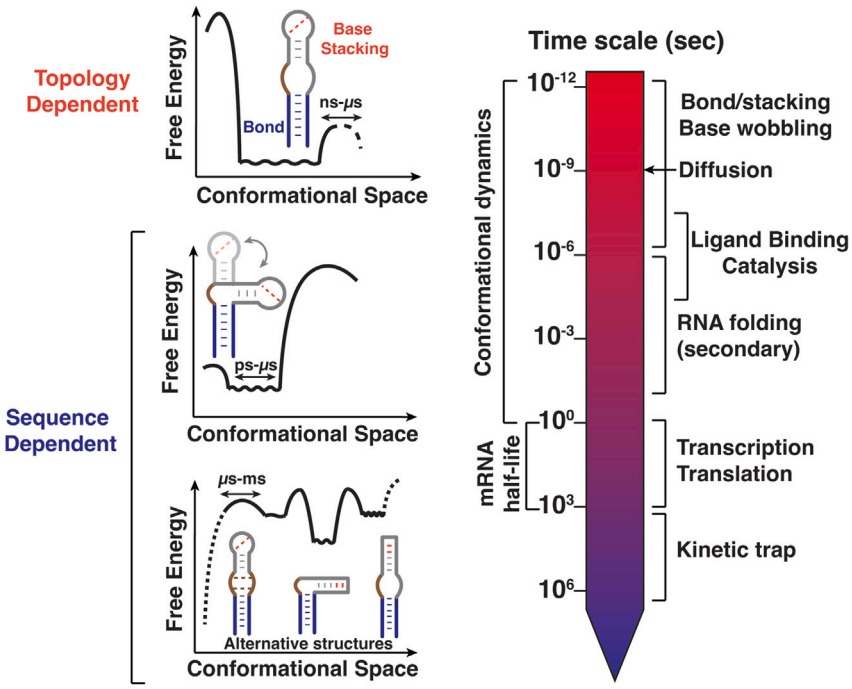



**Figure 2. Dynamic ensembles and free energy landscape define the timescale for ncRNA function**

Left panel: Representative RNA free energy landscape of an RNA hairpin. The different conformations include a co-axially stacked conformation (top), a folded flexible conformation (middle), and different alternative structures (bottom). The relative energy stability of each conformation is represented by the depth of the associated free energy minimum (black line). Right panel: Time-chart of dynamic processes in RNA.

## ncRNAs: MAESTROS OF BACTERIAL GENE REGULATION AND INFECTION

ncRNAs have emerged as significant players in gene regulation in all domains of life, including bacteria. Unlike coding RNAs, which are translated into proteins, ncRNAs are not recruiting the ribosome for translation, but instead typically perform regulatory functions at the transcriptional or post-transcriptional level (Figure 1). There are diverse classes of ncRNAs, varying in length and structure, with roles encompassing regulation of basal gene expression, stress responses, bacterial virulence, and adaptation to environmental changes.

Based on their sizes, two broad types of ncRNAs are distinguished: small ncRNAs, defined as less than 200 nucleotides in length, and long ncRNAs (lncRNAs) greater than 200 nucleotides. Small ncRNAs in bacteria are typically involved in the direct regulation of gene expression. Commonly, but not exclusively, they act through base-pairing with one or multiple target mRNAs to influence their stability or translation, allowing a fast and efficient gene expression response to environmental changes. Examples of these include small RNAs (sRNAs), acting in *trans*, and *cis*-regulatory elements such as riboswitches and thermosensors, which respond to cellular and/or environmental cues to modulate gene expression through their intrinsic structural changes (Figure 1).[16] In addition, while they are not translated into protein, some ncRNAs contain short open reading frames (leader peptides) that recruit the ribosome and thereby participate in gene regulation.[17] Conversely, transcription attenuation is a regulatory mechanism used by bacteria to regulate the production of proteins.[18] It operates during the transcription process and involves premature termination of the synthesis of the mRNA transcript. This attenuation mechanism often uses a leader sequence in the mRNA that forms a particular secondary structure acting as a premature stop signal. Like riboswitches, this is a critical process allowing bacteria to adapt and respond to their environment by controlling gene expression levels.

In contrast, lncRNAs contain more complex structural features establishing local to long-range interactions crucial for maintaining their regulatory functions in stability, organization, and functionality of the bacterial genome.[19,20] However, in the case of bacterial lncRNAs, the current knowledge is restricted to a handful of studies that investigated the functional roles of such long transcripts during pathogen challenge or stimulation with pathogen-associated molecular patterns with their hosts.[21,22] While the functions of bacterial lncRNAs may be confined to specific biological pathways, the molecular mode of action and potential accessory partners by which the function is mediated generally remains to be explored.

Bacterial small ncRNAs operate at all levels of gene regulation including transcription, translation, mRNA stability, and foreign gene silencing, requiring them to use various mechanisms to execute their regulatory functions (Figure 1). In *Escherichia coli* (*E. coli*), about 200 distinct *trans*-acting sRNAs 50–500 nucleotides in length are found in genomic locations distinct from their targets.[23] They typically share partial complementarity with their target mRNAs and potentially establish base-pairing to or near the target Shine-Dalgarno (SD) sequence (usually 10–25 base pairs long) to prevent ribosome binding, thus inhibiting translation initiation and/or activating RNA degradation.[24,25] In many cases, due to their limited complementarity as well as the competing secondary structures of both regulator and target, *trans*-encoded sRNAs engage their target with the help of chaperone proteins such as Hfq[26,27] or ProQ[28–30] to promote hybridization and sRNA-mediated regulation (Figure 1).

*Cis*-acting ncRNAs, in contrast, are encoded in the same DNA region as their putative target, usually embedded in the 5′ untranslated region of the corresponding target gene. They are often highly structured RNA motifs such as riboswitches and RNA thermometers that remodel their downstream expression platforms in response to the upstream aptamer domains engaging external (*trans*-acting) cues that include cellular metabolite and ion levels, and/or temperature, and modulate translation initiation,[31] transcription termination,[32,33] or mRNA

stability (Figure 1).[34,35] So far, ~55 riboswitch classes of known function have been discovered through sequence and structure conservation, combined with mechanistic studies, and perhaps many more are still expected to be found in niche or difficult-to-culture bacterial species.[36]

In addition to the regulation of gene expression through base-pairing with their target mRNAs, ncRNAs have evolved and adapted to also interact with a variety of regulatory proteins.[37–39] For example, ncRNAs can modulate their activity by mimicking and thus efficiently competing with their cognate targets. One intriguing instance is the Csr/Rsm regulatory network, a global carbon storage regulator common in pathogenic bacteria. In this two-component system, sRNAs can directly bind the carbon storage regulator A protein to sequester it from interacting with its cognate mRNA target.[40–42] This can result in the activation of the translation of previously blocked transcripts.

## The diverse roles of ncRNAs in bacterial disease make them attractive intervention targets

Since ncRNAs play important roles in bacterial physiology, their ubiquitous nature and variety of functions, particularly in regulatory processes, make them significant contributors to bacterial adaptability and infection virulence. Additionally, the dysregulation of ncRNAs, such as alterations in their expression through mutations, can lead to bacterial infection and disease.

One notable example is the ncRNA molecule RNAIII in *Staphylococcus aureus* (*S. aureus*), often associated with various human and animal diseases ranging from minor skin infections to severe diseases such as pneumonia and meningitis.[43] RNAIII is the key effector of the *agr* quorum-sensing system and controls the expression of numerous virulence factors through a sophisticated network of regulatory proteins and RNAs.[44,45] Interestingly, a prophage-induced sRNA, *sprY*, has been discovered to form a complex with RNAIII *in vivo*, decreasing hemolytic and virulence activity of *S. aureus*.[46] Another example is the sRNA *gcvB* identified in *E. coli* and *Salmonella enterica* (*S. enterica*), which indirectly impacts pathogenicity.[47] *GcvB* regulates the transport and assimilation of amino acids, which in turn could be related to virulence gene expression.[48–50] Thus, despite not being always directly involved in virulence, ncRNAs can significantly impact bacterial pathogenicity and the progression of disease through regulatory networks that act at multiple levels during the bacterial life cycle.[51]

Recently PinT, an sRNA found in *S. enterica* activated by PhoP within the infected host, was found to control the timing of the expression of invasion-related effectors and virulence genes, causing changes in both coding and non-coding host transcripts.[52,53] The bacterial pathogen Salmonella uses a type 3 secretion system to infect intestinal cells, but ceases expressing this system upon intracellular survival. Research supports that PinT plays a significant role in transitioning Salmonella from invasion to intracellular survival based on regulation of its virulence genes by the PhoPQ system.

Given their critical roles in regulating bacterial pathogenicity and the specific association that certain ncRNAs have with diseases, these molecules offer promising potential as disease biomarkers. That is, they can be used in the identification of bacterial strains or specific virulence states, paving the way for personalized treatment strategies. For instance, by measuring the expression level of a particular ncRNA in a patient, it may be possible to detect the presence of a specific bacterial infection or even predict its severity. The fact that ncRNA levels can rapidly change in response to environmental or metabolic shifts[54,55] makes them particularly attractive as real-time disease reporters. In a recent study, an sRNA encoded by the gene *sicX* was discovered to be strongly induced in *Pseudomonas aeruginosa* (*P. aeruginosa*) by low-oxygen conditions, in turn post-transcriptionally regulating anaerobic ubiquinone biosynthesis.[9] Upon deletion of this gene, the authors found that *P. aeruginosa* switches from a chronic to an acute life cycle in multiple mammalian models of infection. Therefore, expression and abundance of this sRNA constitute one new biomarker for bacterial infection and could be exploited diagnostically.

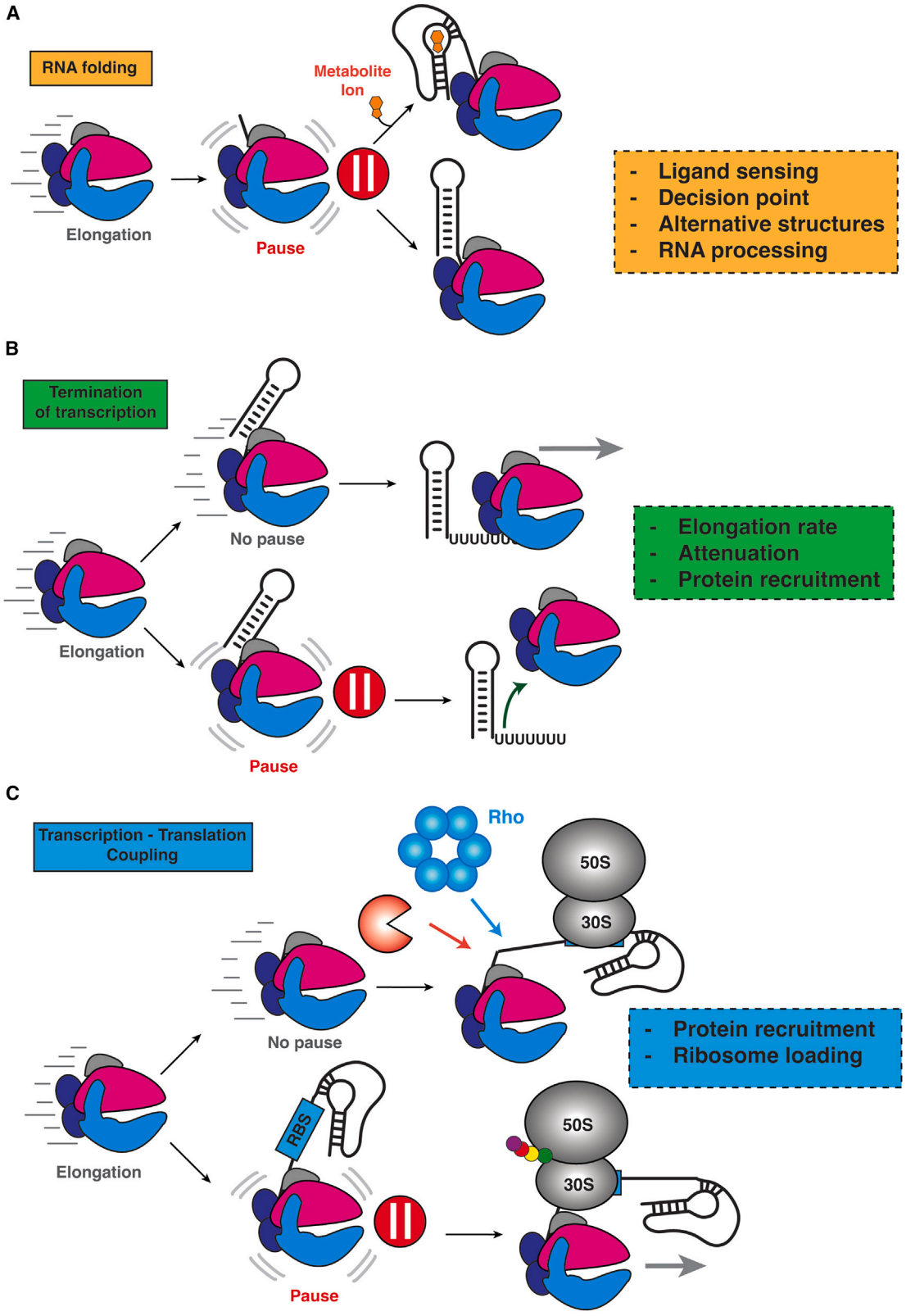
A better understanding of the molecular mechanisms of ncRNA involvement in disease can lead to the development of novel therapeutic strategies. For instance, molecules that inhibit the action of disease-related ncRNAs or essential genes regulated by ncRNAs could provide a new class of antibiotic compounds to combat bacterial infections. This notion is exemplified in the context of riboswitches since they bind a specific ligand and generally control the expression of essential genes specific to a bacterial species. For instance, analogs of the natural ligand with antibiotic properties were found targeting the guanine,[56–58] flavine mononucleotide (FMN),[59,60] and thiamine pyrophosphate[61] riboswitches. Conversely, antisense oligonucleotides targeting riboswitches found in pathogens such as *Listeria monocytogenes* or *S. aureus* have also been successfully deployed,[62,63] expanding our arsenal to fight the ever-expanding occurrence of antibiotic-resistant strains.[64]

To fully realize these prospects, comprehensive catalogs of ncRNAs and their regulatory networks in bacterial pathogens will need to be established.[65,66] Furthermore, the development of technologies for the sensitive detection of ncRNAs in clinical samples will be instrumental. The complex choreography of ncRNA expression, encompassing spatial and temporal controls, heralds an exciting frontier in understanding and combating bacterial diseases.

## Utilizing ncRNAs themselves as promising therapeutics

Recent years have seen significant strides in harnessing the power of ncRNAs for bacterial disease treatment.[67,68] Apart from a diagnostic target, novel therapeutic technologies—such as those utilizing widespread bacterial CRISPR-Cas defense systems against bacteriophages—are capitalizing on the regulatory capabilities of ncRNAs.[69] In particular, CRISPR-Cas9, guided by specific ncRNA sequences, can be directed to target and edit bacterial genomes. In an elegant example, this technology has been harnessed to develop "anti-virulence" therapies that disarm pathogens without killing them, aiming to minimize the evolutionary pressure for resistance development.[70] Another advancement has been the use of RNA interference technology in bacteria, wherein short ncRNA molecules are designed to inhibit the expression of specific genes by base-pairing with their cognate mRNAs and preventing their translation.[71]

Despite these practical examples and the constant increase in our understanding of ncRNA-mediated regulatory pathways

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in bacteria, further work is needed to be able to surgically target specific pathogenic species rather than killing a patient's entire microbiome with broad-spectrum antibiotics. RNA-based therapeutics lend themselves to more focused targeting due to their heightened information content relative to small-molecule drugs with more limited chemical features. A better understanding of ncRNA mechanisms and functions *in vivo*, and especially during bacterial infection, is critical for the development of new strategies and tools against the constantly increasing risk of a new epidemic of antibiotic-resistant bacterial strains.[64,72]

## ncRNAs FOLD CO-TRANSCRIPTIONALLY INTO INTRICATE STRUCTURES ON A RUGGED ENERGY LANDSCAPE

Whereas a plethora of ncRNAs such as sRNAs are thought to modulate gene expression at the post-transcriptional level, increasing evidence points toward a more complex regulation that operates during the transcription process itself. RNA molecules in general, and ncRNAs such as riboswitches in particular, are complex structures folded into unique three-dimensional shapes.[73,74] The intricate architecture of RNA molecules, ranging from simple base-pairing to forming binding pockets highly specific for cations or complex metabolites, is crucial for their function.[75–78] In addition, all RNA molecules undergo structural transitions that are either stabilized or destabilized under particular conditions with, in most cases, the alternative structures coexisting in the cell, at times at thermodynamic equilibrium (Figure 2).[79] Therefore, RNA structure can be described as a distribution of various conformations dictated by the nucleotide sequence (Figure 2). The many possible conformers and intermediate structures have their individual free energies and form an ensemble on a rugged (bumpy) free energy landscape.[13] Since thermodynamic stability (or low free energy) is favored, the proportion of each population is unequal, with some conformations more abundant than others in the ensemble. Almost isoenergetic sub-ensembles then give rise to multiple energy minima, most of which represent suboptimal structures within the landscape (Figure 2). Since the overall majority of conformations, however, exhibit higher (less favorable) free energies, the RNA landscape is dominated by only relatively few low-energy conformations, with the rest representing relatively unstable intermediates and outliers (sometimes referred to as "excited" states, even though they are still in thermal equilibrium).[80] Transition rates between these conformations depend on the height of the separating energetic barriers. With their much more favorable secondary structure energetics than proteins, the unproductive local energy minima of RNA molecules tend to produce kinetic traps. In some instances, this behavior manifests as very long-lived heterogeneities because of topological or other folding constraints (Figure 2).[81,82]

### Co-transcriptional folding as the key to unraveling RNA-mediated regulation

Work from the early 1980s established that RNA transcripts in the cell fold into secondary structures as they are being transcribed by RNAP,[83] rather than adopting their final structure upon transcription completion. Such a dynamic process termed co-transcriptional folding directly affects the available energy barriers of folding with the potential to kinetically trap fast folding, but energetically less favorable states in local energetic minima.[76,84,85]

Since transcription occurs sequentially from the 5′ to the 3′ end of the RNA chain,[86] newly synthesized RNA segments can start to fold before the downstream end of the transcript is available. As a result, co-transcriptional folding is linked intimately to the direction of the transcription process, which promotes dynamic, local, close-proximity interactions of the nascent RNA. Additionally, the rate of transcription, or the RNAP's velocity, directly affects RNA folding.[87–89] Indeed, while the transcription rate average ranges from 20 to 80 nucleotides per second, depending on the growth conditions of a given bacterial species,[90] local RNA secondary structure folds much faster, on a microsecond timescale, in turn raising opportunities for kinetically trapping conformations that then may take milliseconds to minutes or even hours to further evolve (Figure 2). In this context, transcriptional pausing,[5,91] or the temporary off-state of the RNAP during transcription, has been implicated as critical to the folding of RNA structures (Figure 3). Transcriptional pausing is a ubiquitous mechanism found in all domains of life that dictates vastly varying local speeds of the RNAP with profound impact on gene expression.[91] In eukaryotes, transcriptional pausing and co-transcriptional events have been found to influence RNA processing, such as mRNA splicing.[92–97] In bacteria, the interaction of the RNA transcript with RNAP and/or accessory proteins such as transcription factors can induce transcriptional pausing and modulate numerous biological processes, including transcription termination[98,99] protein recruitment,[100–102] RNA folding,[103,104] transcription-coupled DNA reparation,[105–107] and transcription-translation coupling.[108–110] The latter coupling is unique to bacterial species with their single cellular compartment.

For ncRNAs, which often perform functions based on their intricate structures, the process of co-transcriptional folding has recently been under intense study since it influences the final shape of the RNA transcript. Pioneering work on the catalytic *Tetrahymena* group I intron[111] and ribonuclease P[112] revealed that the enzymatic activity of denatured, then renatured synthetic RNAs is dramatically different from that obtained using nascent transcripts, consistent with the notion that the kinetics of transcription elongation are important for the ordered folding of nascent transcripts. Another example of this behavior is the FMN-sensing riboswitch from *Bacillus subtilis* (*B. subtilis*) that, upon ligand binding, regulates transcription elongation through

**Figure 3. RNA polymerase (RNAP) pausing as a fulcrum for cellular processes dictated by ncRNAs**

(A) Example of a pausing delay during transcription elongation that allows efficient ligand binding to a riboswitch, triggering RNA folding into a specific conformation.

(B) Transcriptional pausing is a mandatory step for efficient intrinsic transcription termination, favoring terminator hairpin folding.

(C) RNAP pausing in the vicinity of the ribosome-binding site/start codon allows proper coupling between the transcription and translation machineries in bacteria.

the formation of a terminator stem.[113–115] In this example, metabolite binding and stabilization of an alternative RNA conformation occur within key positions defined by RNAP pausing, further highlighting the critical importance of co-transcriptional processes for ncRNA function.

## Biology along timescales: The interplay of transcription and folding kinetics

One of the most fascinating aspects of ncRNA function is that it is deeply intertwined with RNA structure, which in turn is ultimately dependent on the transcription process. This involves the RNA molecule folding into its functional shape while still being transcribed, often with the 5′ and 3′ ends pairing at the very end of transcription,[116] perhaps to signal finality through topological closure. Notably, the entire transcription/folding process is dynamic, adaptable, and extends over a range of timescales that adjudicate the final ncRNA structure (Figure 2).[14] Understanding the kinetics of transcription relative to those of secondary and tertiary structure folding events thus becomes crucial for understanding ncRNA function. Consequently, co-transcriptional ncRNA folding is not just about space, but also about time. Nature exploits this inherent transcription-folding coupling, and characterizing the associated sequence of events in detail stands to open new avenues for the treatment of bacterial diseases.

The varying speed with which transcription proceeds affects the sequence in which sequential RNA regions emerge from the RNAP exit channel and start to interact, influencing the folding pathway. In general, slower transcription rates may provide more opportunity for regions transcribed early to fold onto themselves, with the potential to lead to alternative conformations compared to a renatured, thermodynamically more stable structure.[117–120] In contrast, faster transcription rates may give distal base-pairing interactions an opportunity to form, potentially driving the formation of a different structure (Figure 3A).[87,121] Therefore, the relative rate constants of RNAP progress during transcription and of RNA folding play a pivotal role in determining the final RNA structure and its ability to trigger downstream biological processes at a timescale suitable for the cell (Figure 2). For instance, in bacteria, intrinsic transcription termination depends on the formation of a longer terminator helix versus competing shorter anti-terminator helix and is intimately linked to transcriptional pausing (Figure 3B).[6,122,123] In this essential process, co-transcriptional folding—which itself is influenced by parameters such as temperature, ionic conditions, and cofactors—and the rate of transcription together dictate whether the RNAP is allowed to continue, thus integrating environmental cues into the regulatory outcome.

## Coordinating structure and function through kinetic competition

In addition to determining their structure and function, co-transcriptional folding enables nascent RNA transcripts to interact *in situ* with other cellular components (i.e., proteins, other RNAs, metabolites, ions) at the right time and place, empowering them with adaptability to cellular conditions. In fact, the spatiotemporal coordination of transcription, translation, and RNA degradation also have an important role in shaping the functionality of ncRNA structures in fine-tuning gene expression regulation. Many biological processes are tightly coupled, and the harmonized timing of these processes can lead to allosteric effects. Practical examples of such tightly coordinated processes among regulatory pathways include the time windows allowed for the ligand sensing by riboswitches,[115,124,125] recruitment of transcription factors,[100,126,127] stepwise assembly of proteins with ribosomal RNA during ribosome biogenesis,[128–131] and sRNA loading onto their mRNA targets.[132]

One notable example is the coupling between transcription and translation, which is unique to bacterial species because of their lack of subcellular compartmentalization. Transcription-translation coupling is defined by the translation of an mRNA into protein concomitantly with its synthesis by RNAP (Figure 3C). During this process, the pioneering ribosome closely follows and physically interacts with the downstream RNAP, spatiotemporally coupling the two processes through a mega complex termed the "expressome."[133–136] Through the resulting concomitant mRNA transcription and translation, the RNA transcript is protected by the ribosome from degradation by cellular ribonucleases[25] or premature transcription termination mediated by the ATP-dependent helicase termed Rho factor (Figure 1).[137,138] Alterations in the coupling of these processes can result in global changes in the cellular protein levels and modulate cellular responses to environmental stimuli (Figure 3C). Recently, single-molecule studies have unveiled an intriguing mechanism in which co-transcriptional folding of a riboswitch uncouples transcription and translation as a function of ligand binding.[108] In this study, surveying the transcription rate during transcription-translation coupling revealed that ligand binding to the preQ$_1$-sensing riboswitch from *B. anthracis* triggers a conformational change that leads to ribosome stalling in the translation initiation region, in turn holding the RNAP in place at a specific pause site and preventing downstream RNA transcription. While the molecular and structural details of this ncRNA-based regulatory mechanism remain unknown, its existence underscores the profound impact that small, but highly structured and ligand-responsive ncRNAs can have on the macromolecular complexes of gene expression.[139,140]

Another instance is the folding pathway of the fluoride-sensing riboswitch that integrates multiple signals to modulate gene expression.[100,141] Research on the transcriptional *crcB* fluoride riboswitch from *Bacillus cereus* revealed three interchanging conformations throughout the transcription process. A single fluoride anion locks the magnesium-induced state, governed by a single long-range, nesting base pair. Additionally, RNAP influences the free energy landscape, promoting riboswitch docking. Fluoride binding is an early step in folding the transcript into the docked conformation, a mechanism that leads to cellular fluoride detoxification. Ligand and transcription factor NusA both bind the co-transcriptionally folded RNA, affecting the process of downstream RNAP pausing and termination of transcription. The transcription factor binds reversibly, assessing the complex for co-transcriptionally folded RNA duplexes and altering the transcription rate based on the riboswitch's structure. This dynamic regulation is likely to allow for quick adaptive bacterial transcription.

Nature makes ubiquitous use of these features of RNA folding. Another example is the recent identification of the "anti-P1" intermediate present among translational riboswitches in

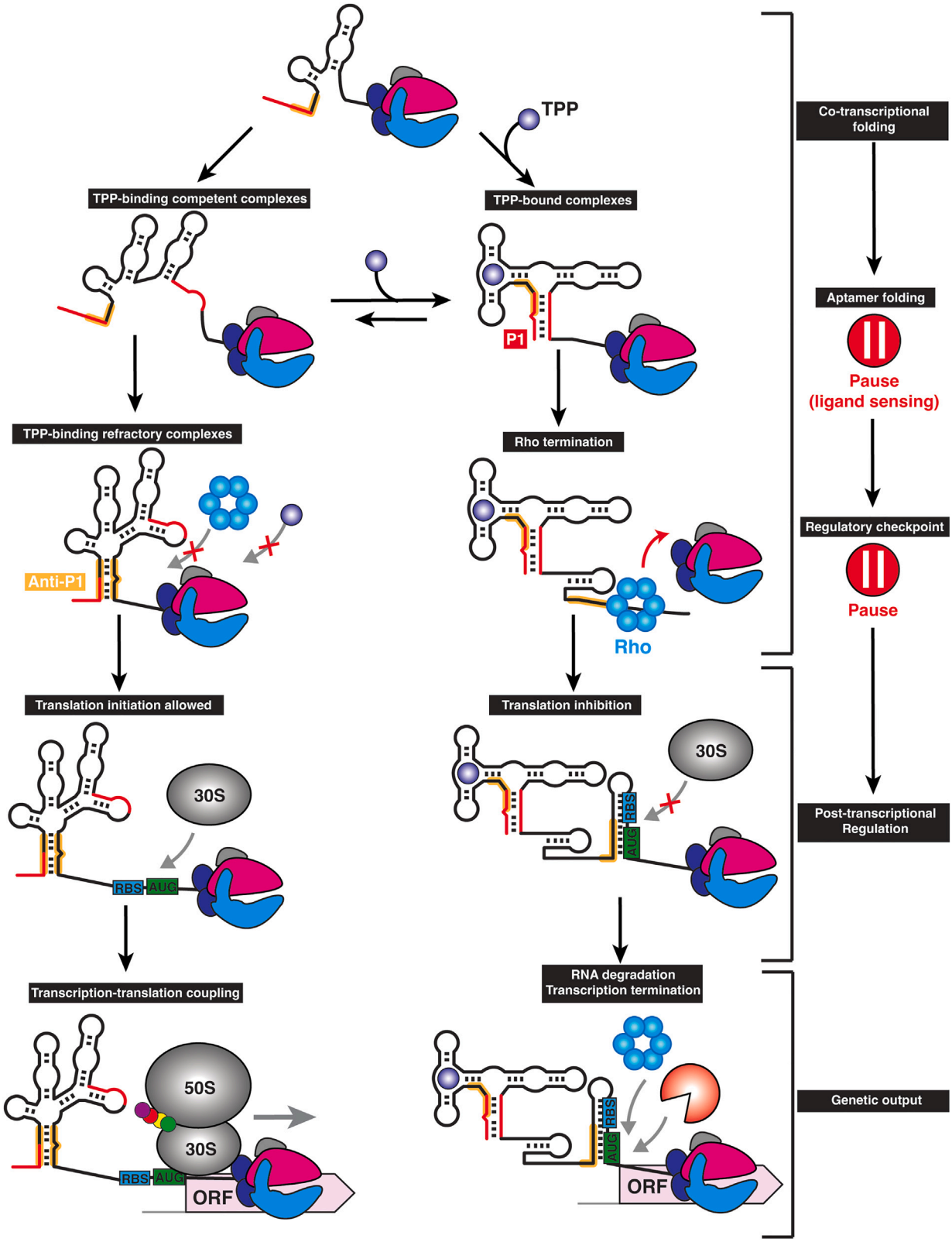


**Figure 4. The folding mechanism underlying the exemplary gene regulation by the *E. coli thiC* riboswitch**

In the absence of TPP (left), the riboswitch remains competent for efficient co-transcriptional ligand binding until the RNAP reaches the regulatory checkpoint pause site in the vicinity of the start codon. At this pause site, the energetically favored anti-P1 prevents folding of the riboswitch into the TPP-bound state and the

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*E. coli*.[117,124] Upon ligand binding, this regulatory switch induces the formation of an energetically favored sequestering stem in the expression platform that prevents ribosome access to the SD sequence. This conformational trap can be resolved prior to the regulatory decision by additional factors in the system, in this case by forming an alternative structure termed anti-P1 that instead holds the ribosome binding site "open" (Figure 4). Interestingly, this specific RNA structure that formed co-transcriptionally is central for regulation of gene expression at multiple levels. Indeed, while "holding" the aptamer in the ligand-free conformation, it also prevents spurious regulation by the Rho termination factor by sequestering its potential binding site on the mRNA (Figure 4).[124] Such induction of structural rearrangements by external factors or local RNA motifs that promote structural transitions is a primary mechanism employed by ncRNAs and particularly structured RNAs such as riboswitches.

At its most basic, the relative kinetics of ncRNA folding, ligand-induced conformational changes, transcription elongation and pausing, and translation initiation determine the fractional partitioning into distinct gene regulatory outcomes. Therefore, a thorough understanding of ncRNA function requires not only appreciating static structures but also—to equal measure—the dynamic features that are coordinated in time to lead to the desired spatiotemporal control of gene expression and thus capacity for cellular adaptation. Time-resolved probing[15] and the ensuing understanding of the temporal dimension of ncRNA biology will add further critical layers to the study of ncRNAs and possibly offer opportunities for versatile therapeutic strategies against bacterial pathogens.

### The impact of co-transcriptional folding on bacterial disease pathologies

Overall, bacterial ncRNAs display a remarkable complexity and sophistication, empowering flexible gene regulation. Their diverse roles and the precise timing of the regulatory events they affect enable their broad impact on bacterial fitness and virulence.

The importance of co-transcriptional folding extends beyond the folding of functional ncRNAs; it also plays a vital role in disease pathology. When the process of co-transcriptional folding does not proceed correctly due to cellular stress, it can lead to the production of misfolded RNAs that can lead to loss of function or even gain of undesired function and thereby harm the cellular machinery, leading to loss of cell viability. A classic example is observed in several human neurodegenerative diseases, where incorrect RNA folding can result in the formation of toxic aggregates.[142–144] Intriguingly, cohabiting bacteria can compete with one another by secreting and uptaking ncRNA fragments that act as growth suppressors in an apparent tug-of-war.[145] Moreover, single nulceotide polymorphisms can lead to dysregulation of co-transcriptional folding. For example, variants of RNA thermosensors were found by whole genome sequencing of the pathogenic bacterium *Neisseria meningitidis*, which causes invasive meningococcal disease such as septicemia and/or meningitis if it can enter the host bloodstream. Bacterial isolates that harbor such variants are characterized by a hypercapsulation phenotype and are dominant during invasive disease compared to the carrier state.[146] While the latter example is recent, it links ncRNA mutations to bacterial disease progression, possibly heralding similar discoveries for other pathogens.[9]

Analogous to mutations found in microRNAs and fragments of tRNAs that can lead to human cancer,[147–151] alterations of bacterial ncRNA sequences might change RNA folding kinetics, expression level, and/or functionality such as in target identification by an sRNA. Additionally, environmental factors such as a change in temperature, pH,[152] or ion availability[153,154] can also lead to alterations in the co-transcriptional folding process, underscoring the nuanced balance required for maintaining RNA functionality. In at least one case, it has been observed that ncRNAs can compensate for changes in, e.g., temperature by exploiting compensatory structural elements that dampen the effects of environmental changes for more robust riboswitching.[155]

On the therapeutic front, a better understanding of co-transcriptional folding paves the way toward developing unprecedented RNA-targeted therapies. Strategies aimed at correcting misfolded RNAs or employing small molecules that can stabilize the correctly folded RNAs are gaining momentum.[64,156,157] Intervention at the co-transcriptional level could allow for modulating bacterial gene expression with high specificity, highlighting the therapeutic potential of targeting co-transcriptional folding and related processes with antibacterial compounds. Future studies to probe the detailed mechanisms regulating co-transcriptional folding, the factors influencing it, and their roles in physiology and disease will be crucial toward addressing the ever-increasing threat of bacterial diseases.

## FUTURE PERSPECTIVES AND CONCLUSIONS

Despite the remarkable potential and recent advancements in our understanding ncRNA-based gene regulation and the underlying, finely balanced temporal coordination of events, several challenges persist. First, still more tools are needed to fully dissect the broad timescales of processes involved—ranging from microseconds to minutes—and understand their competition and harmonization. Second, in order to utilize these insights to derive fresh antibacterial strategies based on intervening ncRNAs that promise higher species specificity, more delivery methods will have to be developed to reliably transfer RNA molecules into bacterial cells, which tend to have robust protection mechanisms against such intrusions.[158–160] Third, for therapeutics applications, the inherently transient stability of RNA, susceptibility to targeted degradation, and the potential for off-target effects are significant concerns. Future research must address all of these challenges, develop more targeted delivery

direct termination of transcription by Rho factor, instead ultimately leading to transcription-translation coupling and protein synthesis. In the presence of TPP (right), folding of the riboswitch in the TPP-bound state leads to two gene regulatory responses. Transcription termination mediated by Rho factor occurs at the long-lived regulatory pause site near the start codon. Complexes that fail to terminate and instead carry on transcription into the coding region allow the formation of the sequestering stem, inhibiting translation initiation. In the downstream coding region, absence of translation can lead to RNA degradation by ribonucleases or transcription termination by Rho (polarity). Adapted from Ref.[124]

methods,[161,162] enhance the stability of RNA therapeutics,[163,164] and improve our understanding of potential side effects.

Advances in bioinformatics and artificial intelligence[165–168] as well as experimental techniques are expected to uncover still more ncRNAs, help characterize their functional roles and mechanisms, and aid in predicting the effects of their manipulation.[169] Recent improvements in structure prediction based on artificial intelligence have significantly improved our modern toolkits to address this question. Protein structure prediction problems have been solved for many proteins thanks to the recent advent of AlphaFold,[170,171] promising the discovery of new antibiotic targets. However, due to the limited number of RNA structures available and their insufficient quality, these deep learning methods are still challenging to apply to 3D RNA structures.[172] Here, the constant improvement of *in vivo* and *in vitro* structural probing methods[173–180] in terms of temporal and spatial resolution is expected to help build a comprehensive framework that integrates biological, computational, and clinical research, which will be crucial for a successful development of robust ncRNA-based, bacterium-specific, therapeutics. Recent advances in RNA structural probing methods such as selective 2′hydroxyl acetylation followed by primer extension or dimethyl sulfate-probing have the potential to further advance this goal. Indeed, through a combination with deep sequencing and computational analysis, these methods have unveiled unique RNA conformers among the vast population of transcripts probed.[177] More specifically, DANCE-MaP (deconvolution and annotation of ribonucleic conformational ensembles) presents an innovative tool for understanding gene regulation, specifically through its ability to elucidate the structural dynamics of RNAs like 7SK. As a global regulator of gene transcription, 7SK ncRNA sequesters the transcription factor P-TEFb. This function necessitates intricate shifts in RNA structure that, given their complexity, present a significant challenge for characterization. DANCE-MaP helps overcome this challenge by providing detailed views of these structural perturbations. In particular, DANCE-MaP enables the definition of per-nucleotide reactivity, direct base-pairing interactions, tertiary interactions, and thermodynamic populations for each state within an RNA structural ensemble. This technological advance has led to the discovery of a substantial structural switch within 7SK RNA, linking the dissolution of the P-TEFb-binding site to remodeling activity at distant release factor binding sites. The structural balance within 7SK RNA changes in response to cell growth and stress, providing the potential for targeted intervention to alter the expression of P-TEFb-responsive genes. By revealing the precise dynamics of 7SK RNA, DANCE-MaP offers support for how RNA structural dynamics can serve an integrating function for a variety of cellular signals controlling transcription. The power of DANCE-MaP lies in its potential to define RNA dynamics within the cellular context as a key to better understanding the mechanisms of gene regulation.

Time-resolved cryoelectron microscopy (cryo-EM) is another robust tool in the realm of structural biology that can be used synergistically with single-molecule level RNA dynamics data to enhance our insight into bacterial biology and antibiotic design. Cryo-EM visualizes macromolecular assemblies and even entire cells at high resolution and in a near-native state, capturing the structural dynamics of protein and nucleic acid assemblies. Whereas techniques like DANCE-MaP can reveal intricate details of RNA folding and interactions, cryo-EM can contextualize these interactions within larger molecular assemblies. The time-resolved aspect of this technique allows for the observation of conformational changes over time, offering a lens for viewing the dynamism of macrostructures.

In the context of antibiotic design, the integration of time-resolved cryo-EM and single-molecule RNA dynamics techniques can provide a more comprehensive understanding of the cellular processes that can serve as potential drug targets. For example, time-resolved cryo-EM can provide a view of the overall structure and motion of the bacterial ribosome,[179] a primary target of many antibiotics, while DANCE-MaP can delve into the details of RNA and small-molecule interactions with the ribosome, and how these features affect the function of the ribosome. In the fight against bacterial resistance, the resulting more detailed and nuanced understanding of bacterial biology attained by combining the above techniques inevitably will lead to new strategies for antibiotic design. The targeting of the precise locations and processes most essential for bacterial function, and therefore less prone to evolve resistance, will ultimately contribute to the preservation of human health.

An essential foundation in these endeavors will be the continued expansion of our understanding of co-transcriptional folding and the all-important time dimension that governs it.[14]

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## AUTHOR CONTRIBUTIONS

A.C. and N.G.W. conceived and wrote the manuscript; A.C. created the figures.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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