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The Impact of pH on Riboswitch RNA Structure and Ligand Binding

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biochemistry and Molecular Biophysics

by

Danea Palmer

Committee in charge:

Professor Tatiana Mishanina, Chair
Professor Thomas Hermann
Professor Colleen McHugh
Professor Navtej Toor
Professor Eugene Yeo

2026

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University of California San Diego

2026

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LIST OF ABBREVIATIONS

1M7	1-methyl-7-nitroisatoic acid
3WJ	three-way junction
4WJ	four-way junction
ASO	antisense oligonucleotide
ATP	adenosine triphosphate
BzCN	benzoyl cyanide
cDNA	complementary DNA
Co	cobalt
Cryo-EM	cryogenic electron microscopy
CTP	cytidine triphosphate
Cy3	cyanine3 dye
Cy5	cyanine5 dye
DMS	dimethyl sulfate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsRNA	double-stranded RNA
DU	dynamically undocked
FMN	flavin mononucleotide
GlcN6P	glucosamine-6-phosphate
GTP	guanosine triphosphate
K _d	dissociation constant

LB	Luria Broth
Li	lithium
MaP	mutational profiling
MD	molecular dynamics
Mg	magnesium
miRNA	micro RNA
Mn	manganese
mRNA	messenger RNA
Na	sodium
Ni	nickel
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PMO	phosphorodiamidate morpholino
PPMO	peptide-PMO
preQ ₁	pre-queuosine 1
PS	phosphothioate
RBS	ribosome binding site
RNA	ribonucleic acid
RNAi	RNA interference
RNAP	RNA Polymerase
ROS	reactive oxygen species
RT	reverse transcription

SAM	S-adenosylmethionine
SD	statically docked
SHAPE	selective 2' hydroxyl acylation analyzed by primer extension
smFRET	single-molecule Förster resonance energy transfer
sRNA	small RNA
ssRNA	single-stranded RNA
SU	statically undocked
TECprobe-VL	variable length transcription elongation complex RNA chemical probing
TIRF	total internal reflection fluorescence
T _m	melting temperature
TODP	transition occupancy density plot
TPP	thiamine pyrophosphate
UTP	uridine triphosphate
UTR	untranslated region
WHO	World Health Organization
ZTP	5-amino-4-imidazolecarboxamide riboside 5'-triphosphate

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VITA

- 2021 Bachelor of Science in Biochemistry, University of California Santa Barbara
- 2023 Master of Science in Biochemistry and Molecular Biophysics, University of California San Diego
- 2026 Doctor of Philosophy in Biochemistry and Molecular Biophysics, University of California San Diego

PUBLICATIONS

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*authors contributed equally to this work.

ABSTRACT OF THE DISSERTATION

The Impact of pH on Riboswitch RNA Structure and Ligand Binding

by

Danea Palmer

Doctor of Philosophy in Biochemistry and Molecular Biophysics

University of California San Diego, 2026

Professor Tatiana Mishanina, Chair

Riboswitches are 5'-untranslated regions of messenger RNA that bind a ligand and subsequently control bacterial gene expression. Riboswitches are comprised of a ligand-binding aptamer domain and an expression platform that controls transcription termination or affects translation initiation via controlling ribosome binding site accessibility. The widely conserved *yybP-ykoY* family of riboswitches bind manganese ions (Mn^{2+}) to affect the expression of genes

encoding Mn^{2+} transporter proteins. This dissertation explores the mechanism of one widespread Mn^{2+} -sensing riboswitch, *alx*, in *E. coli*, which activates the translation of the Alx Mn^{2+} exporter. The *alx* riboswitch uniquely activates translation *in vivo* in response to both alkaline pH and increased Mn^{2+} concentration, making it a novel example of RNA-based gene regulation. Prior studies have determined the static structure and dynamics of isolated Mn^{2+} -sensing aptamer domains. While valuable, these works did not include the riboswitch expression platform nor address why pH has a distinct effect on the *alx* riboswitch, but not on other *yybP-ykoY* family riboswitches. This dissertation addresses the question of how Mn^{2+} binding and cytoplasmic pH affect the structure and dynamics of the *alx* riboswitch in response to these two diverse cellular signals. Specifically, we employ a variety of techniques to study the structure, folding, and dynamics of the *E. coli alx* riboswitch. We use chemical probing techniques to understand how the structure and co-transcriptional folding of *alx* changes in response to Mn^{2+} and alkaline pH and compare these data to another *E. coli* Mn^{2+} -sensing riboswitch, *mntP*, that does not exhibit a pH response *in vivo*. We use single-molecule FRET to understand how pH affects the *alx* aptamer dynamics and Mn^{2+} -binding affinity. Finally, we employ *in vivo lacZ* gene reporter fusions to understand how these varying conditions affect gene expression *in vivo* and evaluate key mutants to determine the RNA elements that are responsible for the *alx* pH response. These data reveal that alkaline pH promotes a more open *alx* aptamer structure that is primed for sensing Mn^{2+} at lower concentrations compared to neutral pH. Additionally, we identify two key RNA loops that are responsible for the observed pH response. We also highlight that co-transcriptional folding and transcription kinetics are essential for the riboswitch to sense ligands and affect ribosome binding site accessibility. Given our observations, we present an antibiotic design strategy leveraging how

co-transcriptional folding intermediate RNA elements could be targeted by antisense oligonucleotides in cells to inhibit the expression of genes essential for bacterial survival.

Chapter 1 Introduction

Synopsis

This dissertation focuses on how two distinct cellular signals, metal-ion concentration and pH, affect gene expression by altering bacterial riboswitch RNA structure, folding, and dynamics. This work provides key insights into mechanisms of riboswitch gene regulation. Chapter 1 introduces riboswitches and the key experimental techniques employed throughout the study. Chapter 2 focuses on how ligand binding and pH affect RNA structure and folding pathways while Chapter 3 highlights how these signals impact RNA dynamics. Chapter 4 presents a perspective on how riboswitch folding pathways could be targeted by antibiotics to yield a more effective *in vivo* response.

Background

Nearly half of all characterized enzymes require metal ions for catalysis, but metal ions can be harmful for organisms if present in excess amounts.^{1,2} Therefore, tightly regulating the concentration of metal ions is essential for maintaining cellular processes and avoiding toxicity. In bacteria, manganese (Mn^{2+}) is a critical transition metal ion for countering oxidative stress by replacing oxidized iron cofactors in enzymes to partially rescue their activity (Fig. 1).^{2,3} Mn^{2+} also serves as a cofactor in superoxide dismutase enzymes that degrade harmful reactive oxygen species (ROS), and Mn^{2+} forms low molecular weight complexes with small molecules that break down ROS.⁴ Despite this critical

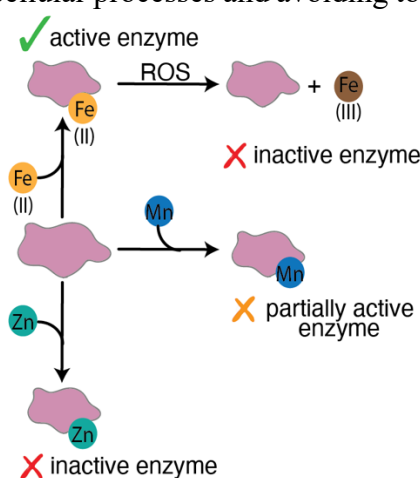


Figure 1.1. Model for enzyme mismetallation and partial rescue.

role, high enough concentrations of Mn^{2+} can be highly toxic for cells due to its ability to replace iron in protein active sites, yielding inactive enzymes.³ Mn^{2+} import and export are tightly regulated in bacterial cells to maintain the careful homeostasis appropriate for the environmental conditions.⁵

Bacterial cells regulate Mn^{2+} transport by altering the transcription and/or translation of Mn^{2+} transporter proteins based on the intracellular level of Mn^{2+} .⁵ This regulation depends on

metal-binding transcription factors, such as MntR or Fur, or Mn^{2+} -binding riboswitches.^{6,7} Riboswitches are 5'-untranslated regions of bacterial mRNA that directly bind ligands and undergo conformational changes to alter the transcription or translation of the downstream gene (Fig. 2).⁸ Many classes of metal-binding riboswitches have been characterized, including magnesium,^{9,10}

nickel,¹¹ cobalt,¹¹ manganese,⁷ sodium,¹² and lithium.¹³ The Mn^{2+} -sensing motif belongs to the *yybP-ykoY* family, which is the most widely distributed metal-ion binding riboswitch family.¹⁴ These Mn^{2+} -sensing riboswitches share a conserved aptamer domain that directly binds Mn^{2+} and have diverse expression platforms that regulate the transcription or translation of Mn^{2+} transporter proteins.^{7,15} The Mn^{2+} -binding aptamer domain is well characterized: crystal structures, single-molecule Förster Resonance Energy Transfer (smFRET), and molecular dynamics (MD)

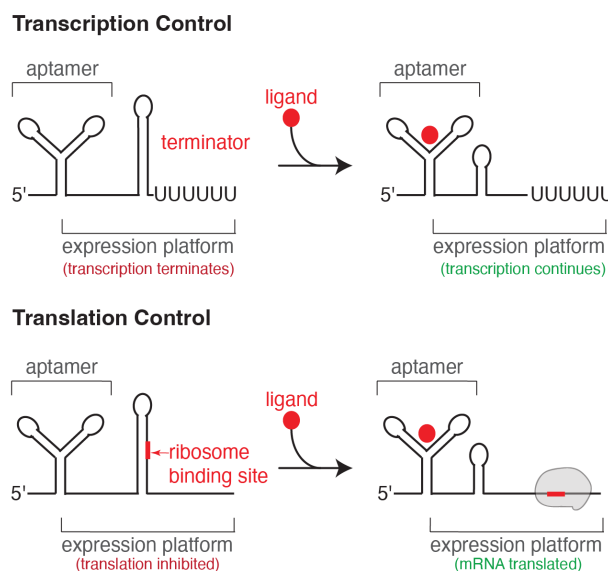


Figure 1.2. Model for ligand-dependent riboswitch control of transcription and translation in bacteria.

simulations provided insights into the metal-binding site, overall RNA fold, and Mn^{2+} -dependent RNA dynamics.^{7,15–17}

In *E. coli*, there are two Mn^{2+} -binding riboswitches that regulate the translation of two different genes, *alx* and *mntP*, both encoding Mn^{2+} exporter proteins.¹⁸ The *alx* gene was originally discovered as a gene upregulated by alkaline pH¹⁹, and the upstream riboswitch was later characterized as the pH-responsive element (PRE).²⁰ Subsequent study demonstrated that PRE, now named *alx*, is a member of the *yybP-ykoY* family and additionally upregulates gene expression in response to excess $[\text{Mn}^{2+}]$.⁷ However, *mntP* and other *yybP-ykoY* family riboswitches have not been shown to respond to alkaline pH, and the mechanism of the *alx* pH response remained uncharacterized.¹⁸ While static aptamer structures of Mn^{2+} -binding riboswitches are well understood, *alx* and *mntP* bind Mn^{2+} during transcription to affect the fold of the expression platform, an RNA region that was not well-studied in previous work. This dissertation addresses (1) how the structure of the full-length *alx* and *mntP* riboswitches are affected by Mn^{2+} binding and alkaline pH, (2) how the dynamics of the *alx* aptamer generate the observed *in vivo* pH response, and (3) which regions of the *alx* RNA are responsible for its unusual pH response.

Studying riboswitches

In vivo gene reporter assays can be used to evaluate how riboswitches affect gene expression when bacteria are grown under different conditions. The gene reporter construct used in this dissertation involves cloning the riboswitch of interest upstream of the *lacZ* reporter gene (Fig. 3). The *lacZ* gene encodes the β -galactosidase enzyme, which will enzymatically cleave an analog of its natural substrate to generate a yellow-colored product.^{21,22} The development of yellow color over time is used to quantify enzymatic activity, and thus riboswitch regulation of gene expression.

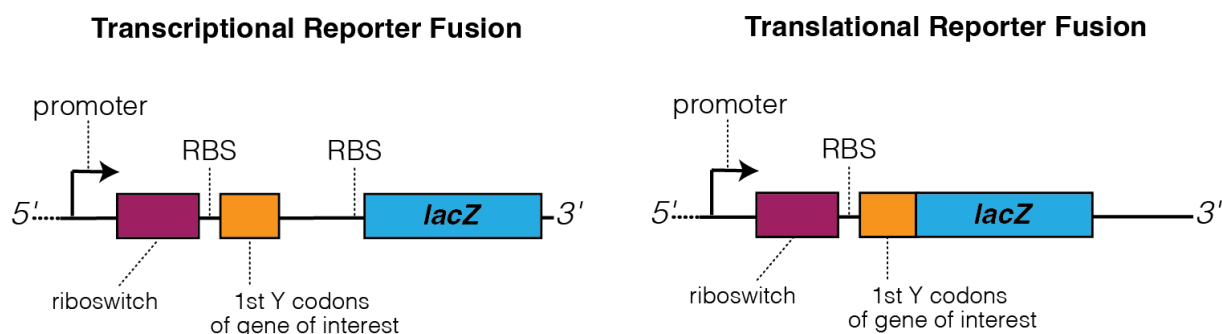


Figure 1.3. Construct design of *lacZ* reporters for measuring riboswitch control of gene transcription or translation in cells.

Chemical probing is an indirect approach to evaluate how riboswitch structures are affected by different conditions. One technique, selective 2' hydroxyl acylation analyzed by primer extension (SHAPE) followed by mutational profiling (MaP), yields information on which RNA regions are flexible, facilitating more accurate two-dimensional structure prediction using these experimentally determined constraints (Fig. 4).²³

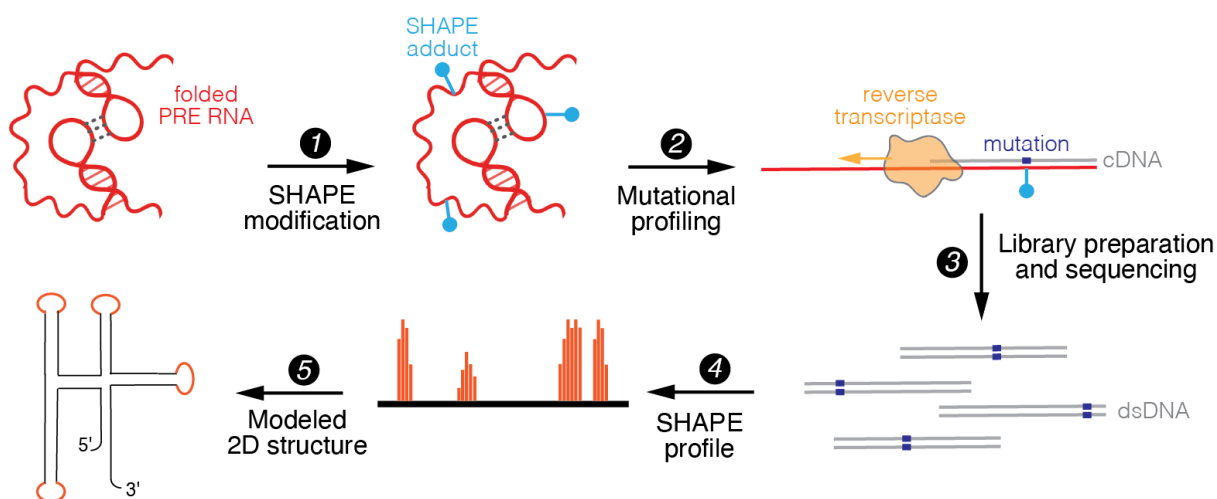


Figure 1.4. SHAPE-MaP method overview.

The dynamics of riboswitch aptamers begin to explain the mechanism and kinetics of ligand binding. For the *alx* riboswitch, these dynamics are especially interesting as a potential explanation for the pH-dependent gene expression observed *in vivo*. smFRET has been used to characterize the Mn^{2+} -binding kinetics of other aptamers within the *yybP-ykoY* family^{16,24,25}, and smFRET can be leveraged to additionally evaluate the effect of pH on *alx* aptamer dynamics and Mn^{2+} binding. This technique involves installing fluorophores on two distal “legs” of the aptamer.

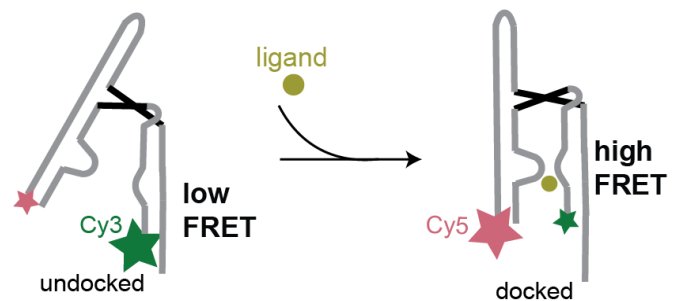


Figure 1.5. smFRET construct design and method overview.

Ligand binding promotes “docking” of the two legs together, generating a distinct

FRET signal that can be quantified in real time to understand the kinetics of ligand binding under various conditions (Fig. 5).

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Chapter 2 Structurally distinct manganese-sensing riboswitch aptamers regulate different expression platform architectures

Nucleic Acids Research

Manuscript Title

Structurally distinct manganese-sensing riboswitch aptamers regulate diverse expression platform architectures

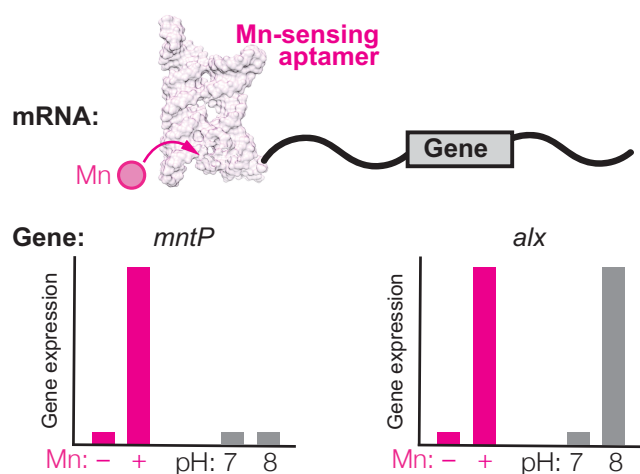
Authors

Christine N. Stephen¹, Danae E. Palmer¹, Clarisa Bautista, and Tatiana V. Mishanina^{1*}

¹Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA, 92093, USA

* To whom correspondence should be addressed. Tel: +1 858 246-5883; Email: tmishanina@ucsd.edu

Graphical Abstract



Abstract

Manganese (Mn)-sensing riboswitches protect bacteria from Mn toxicity by upregulating expression of Mn exporters. The Mn aptamers share key features but diverge in other important

elements, including within the metal-binding core. Although X-ray crystal structures of isolated aptamers exist, these structural snapshots lack crucial details about how the aptamer communicates the presence or absence of ligand to the expression platform. In this work, we investigated the Mn-sensing translational riboswitches in *E. coli* (*mntP* and *alx*), which differ in aptamer secondary structure, nucleotide sequence, and pH-dependence of Mn response. We performed co-transcriptional RNA chemical probing, allowing us to visualize RNA folding intermediates that form and resolve *en route* to the final folded riboswitch. For the first time, we report that sampling of metal ions by the RNA begins before the aptamer synthesis and folding are complete. At a single-nucleotide resolution, we pinpoint the transcription window where “riboswitching” occurs in response to Mn binding and uncover key differences in how the *alx* and *mntP* riboswitches fold. Finally, we describe riboswitch-specific effects of pH, providing insights into how two members of the same riboswitch family differentially sense two distinct environmental cues: concentration of Mn and pH.

Introduction

Transition metal ions play key cellular roles (e.g., serving as enzyme cofactors) but become harmful in excess due to misincorporation into biomolecules and metal-catalyzed formation of reactive oxygen species^{1,2}. To match the need for metal ion transporters, cells use the metal’s intracellular concentration to regulate expression of the metal’s transporters at the transcription and/or translation level³. Examples of this regulation include metal-binding transcription regulators^{4,5} or direct interaction of the metal with a riboswitch encoded in the mRNA^{6–8}. Riboswitches are 5’-untranslated regions of mRNA that promote or inhibit expression of the downstream gene by changing their folding upon binding a ligand⁹. A riboswitch is comprised of a ligand-binding aptamer overlapping with an expression platform, whose folding determines the

transcriptional and/or translational fate of the mRNA⁹. Many classes of metal-sensing riboswitches have been identified, such as magnesium⁷, nickel⁸, cobalt⁸, and manganese (Mn)^{6,10}.

The *yybP-ykoY* Mn-sensing riboswitch family is the largest metal-sensing family, with >1,000 unique representatives in different bacterial phyla¹¹. Crystal structures, single-molecule FRET (smFRET), and molecular dynamics (MD) simulations of isolated Mn-sensing aptamers provided valuable insights into the overall RNA fold, metal binding site, and RNA dynamics in the presence or absence of Mn^{12,13}. However, these studies lack the expression platform of the riboswitch – the “business end” that determines the gene expression outcome. Consequently, it remains unknown how Mn binding to the aptamer actuates the differential folding of the downstream RNA to affect its transcription or translation. Further, studies to date were performed on pre-synthesized RNA, missing the aspect of active transcription that is critical to riboswitch folding^{14–17}.

We addressed these gaps by investigating the two Mn-sensing translational riboswitches in *E. coli* which regulate translation of the *alx* and *mntP* genes, with the *alx* riboswitch upregulating gene expression in response to alkaline pH and increased Mn concentration ([Mn])^{18,19}. Binding of Mn to either the *mntP* or *alx* aptamers promotes a translationally active conformation in the expression platform, increasing expression of the Alx and MntP Mn exporters^{10,19,20} (see Fig. 1A for *alx* and *mntP* aptamer secondary structure diagrams). Multiple structures of Mn-sensing riboswitch aptamers established their core structural features, consisting of two coaxially stacked “legs” connected by a four-way junction (4WJ)^{6,12,21} (Fig. 1B). The right leg (L1, P1.1, P1.2, and P2; Fig. 1C) extrudes a conserved adenosine in L1 to form a cross-helix A-minor interaction with P3.1 in the left leg (L3, P3.1, P3.2, and P4; Fig. 1B). The L3 loop in the left leg stacks below the conserved adenosine in the A-minor interaction, forming a phosphate-rich metal-binding hot spot (Fig. 1B). X-ray crystal structures show two metal-binding sites in the aptamer, with one occupied

by Mg^{2+} (M_A) and the other by Mn^{2+} (M_B)^{6,12,21} (Fig. 1B). The P1.1 “switch helix” is a key element of the *yybP-ykoY* aptamer as its 3’-half alternatively pairs within the aptamer or the expression platform depending on whether Mn is bound, with consequences to folding of the rest of the expression platform¹².

Despite belonging to the same family, the *alx* and *mntP* riboswitch aptamers harbor intriguing structural differences. First, the *alx* aptamer contains an intrinsic termination site in the P4 helix²², which occurs before the metal-sensing pocket forms (Fig. 1A). The *alx* aptamer lacks the P2 helix, resulting in a three-way junction (3WJ) rather than a 4WJ in the *mntP* aptamer and all other aptamers studied by X-ray crystallography. Lastly, the L3 sequences in *alx* and *mntP* metal-sensing cores differ (Fig. 1B), raising the possibility that L3 tunes Mn affinity of the aptamer.

The *alx*-like riboswitches are exclusively present in the *Enterobacteriales* order, members of which are often found in environments that can alkalinize, such as water, soil, and in association with other organisms^{23,24}. Upon alkaline pH challenge, the *E. coli* cytosol alkalinizes and then recovers to pH 7.8²⁵; in turn, cytosol alkalinization increases cytoplasmic $[\text{Mn}]$ ¹⁹, potentially explaining *alx* translational activation at alkaline pH. Initial studies of the *alx* and *mntP* riboswitches put forward alternative secondary structure models based on chemical probing with pre-synthesized RNA^{10,18}, prompting us to ask (i) how these alternative structures fold and respond to Mn during transcription and (ii) why alkaline pH uniquely affects *alx* structure. To answer these fundamental questions, we applied modern RNA chemical probing methods to *alx* and *mntP*, where ribonucleotides are modified by either a selective 2’-hydroxyl (SHAPE) reagent or dimethyl sulfate (DMS) and detected by high-throughput mutational profiling (MaP)²⁶.

In this work, we performed DMS-MaP in a co-transcriptional format using established methods^{27,28}, getting to the heart of how Mn binding to the aptamer is “communicated” to the

expression platform. Unexpectedly, we uncovered evidence that both *alx* and *mntP* aptamer folding intermediates begin sampling for divalents before the full aptamer domain has emerged from RNAP, challenging the notion that a fully folded aptamer is required for ligand sensing. Further, a critical RNA loop that participates in Mn sensing diverges in sequence between *alx* and *mntP* and may hold the key to how pH differentially affects riboregulation by these riboswitches. Using gene reporter assays, we show that alkaline pH alone cannot rearrange the *alx* riboswitch into a translationally active conformation; instead, the *alx* riboswitch senses the increased [Mn] brought about by alkaline pH. Lastly, our co-transcriptional chemical probing data uncovered distinct behaviors of the *alx* and *mntP* expression platforms during folding, with *alx* expression platform sampling both active and inactive conformations regardless of the presence of Mn much more frequently than *mntP*.

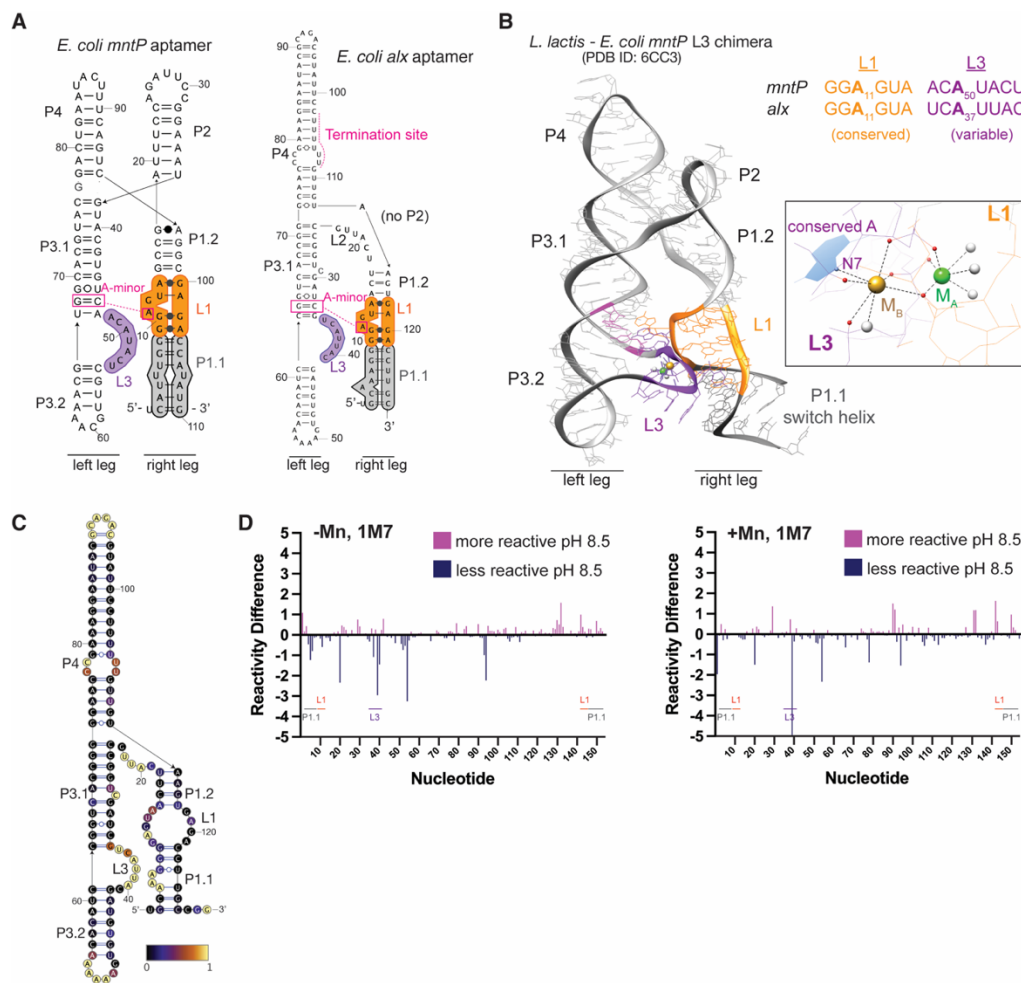


Figure 2.1. Introduction to the *E. coli alx* and *mntP* Mn-sensing translational riboswitches.

A. *mntP* and *alx* aptamer structure models showing the *mntP* 4WJ and *alx* 3WJ elements. The key Mn-sensing loops (L1 is orange and L3 is purple) and the P1.1 switch helix (gray) are highlighted. **B.** *L. lactis* Mn-sensing aptamer crystal structure with the *E. coli mntP* L3 sequence (PDB ID: 6CC3, chain A). “Left leg” and “right leg” refer to coaxially stacked helices in the aptamer. L3 (purple), L1 (orange), P1.1 (gray), and G-C receptor base pair in cross-helix A-minor (magenta) are highlighted. Mg^{2+} in the M_A site and Cd^{2+} in the M_B site (captured instead of Mn^{2+}) are green and yellow spheres, respectively. Sequence was renumbered to match the *mntP* riboswitch sequence. Sequence alignment of *alx* and *mntP* L1 and L3 are orange and purple, respectively. **C.** Secondary structure model of equilibrium-refolded (pH 7.2 -Mn) *alx* aptamer modeled based on reactivity with 1M7. **D.** Individual nucleotide reactivity differences when modified with 1M7 at neutral (7.2) vs alkaline pH (8.5), measured in the presence or absence of Mn. Here and elsewhere, the reactivity differences discussed in this study were substantial (i.e. in the range > 0.5 or < -0.5 , which are notably above values in relatively unreactive regions).

Material and Methods

The source and catalog number (if applicable) for all key reagents are provided as a Supplementary Table 1.

All oligonucleotides were purchased from either Integrated DNA Technologies or Eton Bioscience. A summary of all oligonucleotides used in this work including sequences, modifications, and purifications is presented in Supplementary Table 2.

Procedures for protein preparations, construction of plasmids and bacterial strains, and preparation of DNA templates are provided in the Supplemental Methods. Details for all DNA templates, strains, and plasmids are tabulated in Supplementary Tables 3-6.

All buffers for RNA chemical probing experiments were pH-adjusted at 25 °C.

Single-round *in vitro* transcription for Variable Length Transcription Elongation Complex

RNA structure probing (TECprobe-VL) experiments

To prepare the holoRNAP, *E. coli* RNAP (6 μ M) and s^{70} (12 μ M) were combined in 1x TECprobe transcription buffer (100 mM Tris-Cl, pH 7.2 or 8.5, 50 mM KCl, and 1 mM DTT) and incubated for 30 min at 37 °C. The single-round *in vitro* transcription reactions for TECprobe-VL experiments were assembled by first combining 1x transcription buffer, 40 nM DNA template (*alx* riboswitch, Template 3, Supplementary Table 3) or 50 nM DNA template (*mntP* riboswitch, Template 6, Supplementary Table 3) with randomly distributed internal biotin-11 dNMPs (AAT BioQuest/Biotium), 0.5 μ M holoRNAP, and 500 μ M ATP, UTP, CTP, and GTP. The transcription buffer omitted magnesium, which was a part of the start solution added later to initiate transcription elongation. Tris-Cl was added at a final concentration of 100 mM to minimize pH changes during the chemical probing reaction, as described previously²⁸. The reaction was incubated for 10 min at 37 °C to form open promoter complexes. Next, SAvPhire Monomeric Streptavidin (Sigma-Aldrich) was added to the reaction at a final concentration of 5 μ M, followed by incubation for 10

min at 37 °C to allow streptavidin binding to biotinylation sites on the DNA template. Transcription was initiated by adding 10X start solution (100 mM MgCl₂, 100 µg/ml rifampicin) to the reaction to a final concentration of 10 mM MgCl₂ and 10 µg/ml rifampicin to inhibit reinitiation. After addition of start solution, the final reaction volume was 60 µl. To test the effect of the Mn ligand on riboswitch structural rearrangements, MnCl₂ was added to a final concentration of 1 mM in the transcription reactions and was introduced alongside the start solution. The transcription reaction was incubated for 30 s at 37 °C before proceeding to the chemical probing, which is described in the next section.

Single-round *in vitro* transcription for the single-length TECprobe-SL experiments followed the same protocol as outlined above for variable length TECprobe-VL experiments. The DNA templates used for TECprobe-SL experiments contained a terminal biotin modification that was introduced by a 5' biotinylated reverse primer in the PCR (*alx* riboswitch, Template 7, Supplementary table 3).

***In vitro* RNA chemical probing during TECprobe-VL experiments**

Benzoyl cyanide (BzCN, Sigma-Aldrich) probing was performed by splitting the 60-µl transcription reaction into 25 µl aliquots and mixing with either 2.78 µl of 400 mM BzCN dissolved in anhydrous DMSO (Invitrogen) to a final concentration of 17.7 mM [(+) sample] or with anhydrous DMSO [(-) sample]. Since the BzCN probing reaction is complete within ~1 s, no additional incubation was required before quenching the reactions with 250 µl of TRIzol reagent (Invitrogen).

DMS probing was performed by splitting the 60-µl transcription reaction into 25 µl aliquots and mixing with either 2.78 µl of 6.5% v/v DMS (Sigma-Aldrich) in 200 proof ethanol [(+) channel] or with 200 proof ethanol [(-) channel]. The samples were incubated for 5 min at 37 °C.

The DMS probing reaction was quenched by adding 6.67 μ l of 14.5 μ M 2-mercaptoethanol and incubating the sample at 37 °C for 1 min. *In vitro* transcription reactions were then quenched by addition of 250 μ l of TRIzol reagent.

RNA purification post-TECprobe-VL probing

The RNA was purified from the quenched *in vitro* transcription reactions using the following protocol. First, 50 μ l of chloroform was added to the samples and the samples were mixed by vortexing followed by centrifugation at 18,000g and 4 °C for 5 min. Next, the aqueous layer was transferred to a new tube and nucleic acids were precipitated by addition of 105 μ l cold isopropanol and 1 μ l GlycoBlue Coprecipitant (Invitrogen). The samples were incubated at room temperature for 15 min and centrifuged at 18,000g and 4 °C for 15 min. The supernatant was discarded and the pellet containing the precipitated nucleic acids was washed by addition of 200 μ l 70% ethanol. Following the 70% ethanol wash, the samples were centrifuged at 18,000g and 4 °C for 2 min and the supernatant was discarded. The pelleted nucleic acids were resuspended in 25 μ l of 1x DNase buffer (10 mM Tris-Cl, pH 7.5, 2.5 mM MgCl₂) and mixed with 0.75 μ l of Turbo DNase (Invitrogen) to remove residual DNA template. The samples were incubated at 37 °C for 15 min and DNase digestion was quenched by addition of 250 μ l TRIzol reagent (Invitrogen). A second TRIzol extraction was performed as described above, except the pellet containing the DMS-modified RNA transcripts was resuspended in 5 μ l of 10% (v/v) DMSO.

RNA 3' adapter ligation onto the TECprobe-VL purified transcripts

The 9N_VRA3 adapter oligonucleotide (0139, Supplementary Table 2) was pre-adenylated with the 5' DNA Adenylation Kit (New England Biolabs) using the following protocol. A 100 μ l master mix was prepared that consisted of the 1X DNA Adenylation Buffer (New England Biolabs), 100 μ M ATP, 5 μ M 9N_VRA3 oligo, and 5 μ M Mth RNA Ligase (New England

Biolabs). The 100 μ l master mix was split into 4 aliquots (25 μ l each) in PCR tubes and incubated in a thermal cycler set to 65 °C for 1 h. To clean up the adenylation reactions, reactions were pooled to 50 μ l followed by addition of 500 μ l TRIzol (Invitrogen) and 100 μ l of chloroform to each 50 μ l pool. The samples were vortexed to mix and centrifuged at 18,000g and 4 °C for 5 min. The aqueous phase was transferred to a new tube and the adapter was ethanol precipitated. The pellet was resuspended in Tris-Cl, pH 8.0, and its concentration was quantified with the Qubit ssDNA Assay Kit (Invitrogen) and a Qubit 3 Fluorometer. The pre-adenylated adapter was diluted to 1 μ M, divided into small aliquots, and stored at -20 °C.

The RNA 3' adapter ligation reactions were performed using the following protocol. Purified RNA redissolved in 5 μ l of 10% (v/v) DMSO was combined with 1x T4 RNA Ligase Buffer (New England Biolabs), 0.5 U/ μ l SuperaseIN (Invitrogen), 0.05 μ M pre-adenylated 9N_VRA3 adapter, 15 % PEG 8000, and 5 U/ μ l T4 RNA Ligase 2, truncated KQ (New England Biolabs). The final volume for the ligation reactions was 20 μ l and the reactions were incubated at 25 °C for 2 h.

SPRI bead purification of adapter-ligated RNA

A solid-phase reversible immobilization (SPRI) bead purification protocol was used to deplete excess 9N_VRA3 adapter oligonucleotide. The SPRI beads were prepared with SpeedBead Magnetic Carboxylate Beads (Cytiva) using the protocol from Jolivet and Foley²⁹. The ligation reactions were combined with 17.5 μ l of water, 40 μ l anhydrous isopropanol, and 22.5 μ l of the SPRI beads. The samples were vortexed and incubated at room temperature for 5 min. The samples were then placed on a magnetic separation rack and the beads collected on the tube wall before disposing of the supernatant. Keeping the tubes on the magnetic stand, the beads were washed twice with 200 μ l of 70% ethanol. After discarding the second ethanol wash, the tubes were placed

in a dry bath set to 37 °C to evaporate the residual ethanol. The beads were resuspended in 25 µl of elution buffer (20 mM Tris-Cl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100, 10 mM MgCl₂) and the samples were incubated for 3 minutes at room temperature to elute the RNA. The samples were placed on a magnetic rack and beads collected on the tube wall before aliquoting the supernatant containing the RNA into a fresh tube.

***In vitro* TECprobe-VL cDNA synthesis and cleanup**

The reverse transcription reactions were performed via capture of RNA transcripts on streptavidin beads using a 5' biotinylated reverse transcription primer (0159, Supplementary table 2), which anneals to the 9N_VRA3 sequence that was appended to the intermediate riboswitch transcripts. Dynabeads MyOne Streptavidin C1 beads (Invitrogen) were equilibrated as described previously²⁸. 5 µl of 500 nM RT primer was combined with the purified RNA samples and annealed in a thermal cycler using the following program: 70 °C for 5 min, ramp to 50 °C at 0.1 °C /s, 50 °C for 5 min, ramp to 40 °C at 0.1 °C /s, 40 °C for 5 min, cool to 25 °C. The equilibrated streptavidin beads were placed on a magnetic stand to allow the beads to collect on the tube wall and the supernatant was discarded. The streptavidin beads were then resuspended with the samples containing RNA annealed to the 5' biotinylated RT primer and incubated at room temperature for 15 min on an end-over-end rotator. The samples were placed on a magnetic stand and the beads were allowed to collect on the tube wall before discarding the supernatant. The beads were resuspended in 19.5 µl of reverse transcription master mix and incubated in a thermal cycler set to 42 °C for 2 min before addition of the reverse transcriptase. Upon addition of 0.5 µl of SuperScript II Reverse Transcriptase (Invitrogen) to each sample, the 20 µl RT reactions contained 1x first strand buffer (50 mM Tris-Cl, pH 8.0, 75 mM KCl), 500 µM dNTPs, 10 mM DTT, 2 % formamide, 10 ng/µl Extreme Thermostable Single-Stranded DNA Binding Protein (ET SSB) (New England

Biolabs), 0.1 % Triton-X-100, and 3 mM MnCl_2 . The reverse transcription reactions were incubated at 42 °C for 180 min followed by a 70 °C incubation for 50 min to inactivate the SuperScript II and then cooled to 12 °C. Next, the RNAs were degraded by adding 1.25 U RNase H (New England Biolabs) and 12.5 U RNase I_f (New England Biolabs) to each sample and the samples were incubated at 37 °C for 20 min followed by a 70 °C incubation for 20 min to inactivate the RNases. The samples were placed on a magnetic stand and the beads were allowed to collect on the tube wall. The supernatant was discarded and the beads were resuspended in 75 µl of Tris Wash Buffer (10 mM Tris-Cl, pH 8.0, 0.05% Triton X-100) to wash the beads. The samples were placed on a magnetic stand and the wash was discarded. The bead-bound cDNA was resuspended in 25 µl of the Tris Wash Buffer and stored at -20 °C.

***In vitro* chemical probing of equilibrium-refolded intermediate transcripts**

Single-round *in vitro* transcription using randomly biotinylated DNA templates was executed as described for TECprobe-VL experiments above. To quench transcription, 600 µl of TRIzol was added to the reactions. Next, 120 µl of chloroform was added to the samples and the samples were vortexed to mix followed by centrifugation at 18,000g and 4 °C for 5 min. The aqueous layer was transferred into a new tube and nucleic acids were isopropanol precipitated as described for TECprobe-VL experiments except a larger volume of isopropanol was used to account for the larger sample volume. The pellets containing the intermediate RNA transcripts were resuspended in 30 µl of 1x TECprobe transcription buffer (100 mM Tris-Cl, pH 7.2 or 8.5, 50 mM KCl, 1 mM DTT), placed on a heat block set to 95 °C for 2 min, and snap-cooled on ice for 1 min. To each sample, 30 µl of 2x equilibration buffer (200 mM Tris-Cl, pH 7.2 or 8.5, 100 mM KCl, 2 mM DTT, 20 mM MgCl_2) was added, which contained either no Mn or 2 mM MnCl_2 (1 mM final in 60 µl reaction), and the samples were incubated at 37 °C for 20 min. The RNAs

were then probed with DMS as described for TECprobe-VL experiments, and the DMS-probed RNA was purified as described for TECprobe-VL experiments.

Preparation of TECprobe-VL dsDNA libraries for sequencing

cDNA libraries were PCR-amplified for high throughput sequencing using the following protocol. Separate PCR reactions were performed for the (+) and (-) samples, which contained 1x Q5 Reaction Buffer, 1x Q5 GC Enhancer, 200 μ M dNTPs, 250 nM RPI Indexing Primer (0163-0178,0235-0248, Supplementary table 2), 250 nM dRP1_NoMod.R reverse primer (0162, Supplementary Table 2), 10 nM SC1Brdg_MINUS or SC1Brdg_PLUS channel barcode oligo (0160,0161, Supplementary Table 2), 12 μ l of bead-bound cDNA library, and 0.02 U/ μ l Q5 High-Fidelity DNA Polymerase (New England Biolabs). Amplification was performed with the following thermal cycler program: 98 °C for 30 s, [98 °C for 10 s, 62 °C for 20 s, 72 °C for 20 s] x 22 cycles, 72 °C for 2 min, hold at 12 °C.

High-throughput DNA sequencing of TECprobe-VL libraries

Sequencing of the TECprobe-VL and equilibrium refolded intermediate transcript libraries was performed by Novogene Co. on a Novaseq X Plus system with 2 x 150 Paired End (PE) reads and a 20% PhiX spike in. The TECprobe-VL and equilibrium refolded intermediate transcript libraries were sequenced at a depth of ~30-60 million PE reads.

Sequencing of TECprobe-SL libraries was performed by Novogene Co. on a Novaseq X Plus system with 2 x 150 PE reads. No PhiX spike in was added since these libraries were sequenced in a partial lane. The TECprobe-SL libraries were sequenced at a depth of ~10 million PE reads. Sequencing data for TECprobe-VL and TECprobe-SL experiments is publicly available in the Sequencing Read Archive (SRA) under project number PRJNA1197522 and BioSamples are tabulated in Supplementary Table 7.

TECprobe-VL data processing and visualization

Sequence read pre-processing, target generation using `cotrans_preprocessor`, and `shapemapper2` run script generation were performed exactly as described in the published protocol using the publicly available TECprobe data processing tools (<https://github.com/e-strobel-lab/TECtools>). TECprobe visualization tools was used to assemble reactivity matrices and analyze the correlation between replicate data sets (https://github.com/e-strobel-lab/TECprobe_visualization). The `compile_sm2_output` script was used to assemble the reactivity profiles for intermediate transcripts into matrix format and to normalize the dataset. The normalized reactivity matrices were visualized in heatmap format using GraphPad Prism.

For the *alx* riboswitch, replicate data sets were required to obtain adequate sequencing depth for longer transcripts, as there was a strong bias in representation towards shorter transcripts for the amplified cDNA libraries. Plots with fraction of aligned reads per transcript length and alignment rates for *alx* co-transcriptional TECprobe-VL experiments are presented in SI Fig. 21. Additionally, the presence of two intrinsic termination sites in the *alx* riboswitch sequence (110 and 190 nt) results in a clear drop in sequencing depth for intermediate transcripts past these sites (SI Fig. 19A). For the co-transcriptional DMS-MaP libraries, 5-7 independent replicates were pooled per chemical probing condition to increase data quality for longer transcript lengths (see SI Figs 22-25 for individual and concatenated heatmaps). Plots with fraction of aligned reads per transcript length and alignment rates for *alx* equilibrium refolded TECprobe-VL experiments are presented in SI Fig. 26. For the equilibrium refolded *alx* TECprobe-VL experiments, 2 independent replicates were pooled per chemical probing condition to increase data quality for longer transcript lengths (see SI Figs 27-30 for individual and concatenated heatmaps). To further increase sequencing depth when necessary, multiple independent PCR amplifications were performed with

the same cDNA library and sequenced; reads were subsequently pooled together for data processing.

For the *mntP* riboswitch, two replicate data sets were concatenated and analyzed together after assessing the correlation between replicates using the `plot_cotrans_correlation` script available in TECprobe visualization tools. Plots with fraction of aligned reads per transcript length and alignment rates for co-transcriptional *mntP* TECprobe-VL experiments are presented in SI Fig. 31. Plots with fraction of aligned reads per transcript length and alignment rates for equilibrium refolded *mntP* TECprobe-VL experiments are presented in SI Fig. 32. To further increase sequencing depth when necessary, multiple independent PCR amplifications were performed with the same cDNA library and sequenced; reads were subsequently pooled together for data processing.

***In vitro* synthesis of full-length riboswitch RNAs with T7 RNAP and cleanup for SHAPE-MaP and DMS-MaP**

RNA for *in vitro* SHAPE/DMS-MaP was synthesized using the HiScribe T7 High Yield RNA Kit (New England Biolabs). 100 μ L reactions were assembled according to manufacturer instructions and incubated at 37 °C overnight. RNA was purified and concentrated using the RNA Clean and Concentrator kit (Zymo Research). For the *alx* riboswitch and *alx* aptamer, RNAs were gel-purified to isolate full-length transcripts away from premature transcription termination products using a denaturing PAGE (8% acrylamide, 8 M urea, 0.5x TBE (Fisher Scientific)). For gel-purification, RNAs were diluted 1:5 in buffer and mixed with an equal volume 2x formamide loading dye (95% formamide, 0.5 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol), heated at 95 °C for 2 mins, and loaded on a pre-heated gel. Gel was run at 50 W, stained with 1x SYBR Green II RNA Gel Stain (Invitrogen) in 1X TBE for 10 mins, and visualized using a

Edvotek TruBlu 2 Blue Transilluminator. Bands corresponding to full-length RNA were excised and eluted in 10 mL TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) at 4 °C overnight. The eluted solution of RNA was spun to pellet acrylamide, and RNA was concentrated using an Amicon Ultra Centrifugal filter (3 kDa molecular weight cutoff). RNAs were ethanol precipitated, stored at -20 °C, and resuspended in RNase-free H₂O. For the *mntP* riboswitch, there are no terminated transcripts, eliminating the need for gel purification. After transcription and cleanup, *mntP* RNAs were ethanol precipitated, stored at -20 °C, and resuspended in RNase free H₂O.

***In vitro* SHAPE-MaP and DMS-MaP on the full-length equilibrium refolded *alx* and *mntP* riboswitches**

5 pmol of RNA in added RNase-free H₂O to a volume of 6 µL was heat denatured for 2 mins at 95 °C, cooled at 4 °C for 2 mins, and then 3 µL buffer was added for a final volume of 9 µL. The final buffer concentration was 100 mM HEPES, 100 mM NaCl, and 5 mM MgCl₂ at either pH 7.2 or pH 8.4 and supplemented with 1 mM MnCl₂ where indicated. RNA was incubated at 37 °C for 20 mins to allow refolding. For modification with 1M7, 9 µL of RNA was mixed with 1 µL of 100 mM 1M7 (“+” modified sample) or 1 µL DMSO (“-” negative control), incubated for 75 s at 37 °C, and placed on ice. For the denatured control, 5 pmol RNA (3 µL) was mixed with 5 µL formamide and 1 µL 10x denaturing buffer (500 mM HEPES, 40 mM EDTA) at pH 7.2 or 8.4. RNA was denatured for 1 min at 95 °C, mixed with 1 µL of 100 mM 1M7, incubated at 95 °C for 1 min, and placed on ice. All RNAs were purified and eluted in 50 µL of RNase-free H₂O using MicroSpin G-25 columns (Cytiva) and stored at -20 °C. For modification with DMS, 9 µL of RNA was mixed with 1 µL of 6.5% v/v DMS (“+” modified sample) or 1 µL ethanol (“-” negative control), incubated for 5 minutes at 37 °C, quenched with 2.4 µL of 30% v/v 2-mercaptoethanol solution at 37 °C for 1 min before placing on ice. For the denatured control, 5 pmol RNA (3 µL)

was mixed with 5 μ L formamide and 1 μ L 10x denaturing buffer (500 mM HEPES, 40 mM EDTA) at pH 7.2 or 8.4. Then RNA was denatured for 1 min at 95 °C, mixed with 1 μ L of 6.5% v/v DMS, incubated at 95 °C for 1 min, quenched with 2.4 μ L of 30% 2-mercaptoethanol solution at 37 °C for 1 min before placing on ice. All RNAs were purified and eluted in 50 μ L of RNase-free H₂O using MicroSpin G-25 columns (Cytiva) and stored at -20 °C.

Reverse transcription (RT) of 10 μ L of RNA was performed by annealing 1 μ L RT primer (0277) for 5 min at 65 °C. Then, 8 μ L RT buffer was added (50 mM Tris-Cl pH 8, 75 mM KCl, 10 mM DTT, 0.5 mM dNTPs, and MnCl₂ added to 15 mM before use). Samples were incubated for 2 min at 42 °C before introducing Superscript II reverse transcriptase (Thermo Fisher Scientific). RT reactions were incubated for 3 h at 42 °C. The reverse transcriptase was inactivated for 15 min at 70 °C before storing the reactions at 4 °C. cDNA was purified and eluted in 50 μ L of RNase-free H₂O using MicroSpin G-25 columns (Cytiva) and stored at -20 °C. The first PCR (step 1) amplified cDNA through 20 amplification cycles using primers 0278 and 0279. Excess primer was removed using the Monarch PCR & DNA Cleanup Kit (New England Biolabs). The second PCR (step 2) further amplified the first PCR amplicon through an additional 10 cycles. The step 2 PCR used primers 0149 and an RPI indexing primer (0163-0178 and 0235-0248). Step 2 PCR was cleaned up using Agencourt AMPure XP beads (Beckman Coulter), and final DNA concentration was measured using the Invitrogen Quant-IT dsDNA High-Sensitivity HS Assay kit quantified with a BioTek Synergy H1 Multimode Microplate Reader before pooling libraries for sequencing. Sequencing of these pooled libraries was performed by Novogene Co. on a NovaSeq 6000 system with 100M 150 PE reads. Sequencing data for SHAPE-MaP and DMS-MaP experiments is publicly available in the Sequencing Read Archive (SRA) under project number PRJNA1197522 and BioSamples are tabulated in Supplementary Table 8.

RNA structure prediction and visualization

RNA structure prediction for co-transcriptionally probed riboswitch folding intermediates was performed using the RNAstructure V6.4³⁰ Fold command with default settings, adding the DMS outputs as a constraint, and forcing the 3'-most 11 to 14 nt to be single-stranded to account for the *E. coli* RNAP footprint. The set of minimum free-energy structures were visualized in VARNA³¹ to assess agreement with the DMS probing data, and structure diagrams were colored in accordance with DMS reactivity. Additionally, existing X-ray crystal structures for Mn-sensing aptamer domains were visualized with UCSF Chimera³² and used to guide modeling of conserved secondary and tertiary structure motifs within the *alx* and *mntP* aptamers. RNA structure prediction for equilibrium refolded riboswitch folding intermediates was performed as described above; however, the 3' most nt were not kept single-stranded since the intermediate RNA transcripts were extracted from stalled transcription elongation complexes before DMS probing.

The denatured and equilibrium-refolded full-length *mntP* riboswitch, *alx* riboswitch, and *alx* aptamer RNA structures were predicted using the RNAstructure V6.4³⁰ Fold command with default settings, adding the SHAPE or DMS outputs as a constraint. The minimum free energy structures were visualized in VARNA³¹ and colored in accordance with nucleotide reactivity.

β -galactosidase assays with *in vivo* gene reporters

Overnight culture of each strain (Supplementary Tables 4 and 5) was diluted 1:100 into LBK with appropriate antibiotic at either pH 6.8 or pH 8.4, with or without 1 mM supplemented MnCl₂. The cultures were grown to mid-log phase and supplemented with 1 mM IPTG where necessary for expression of T7 RNAP. β -galactosidase assays were performed using the Miller method³³. Briefly, bacterial growth was measured using OD₆₀₀ before cultures were placed on ice. 100 μ L cells were lysed via vortexing in 900 μ L sodium phosphate buffer (100 mM sodium

phosphate pH 7, 10 mM KCl, 2 mM MgSO₄, 38 mM 2-mercaptoethanol), 20 μ L 0.1% SDS, and 20 μ L chloroform. Substrate *O*-nitrophenyl-b-D-galactopyranoside (ONPG) was added and the reaction was stopped with 1 M Na₂CO₃ when a yellow color appeared, or after 35 min. After centrifuging 5 min at 16,000 g, absorbance was measured at 420 nm (A_{420}) to calculate activity using Miller units. Each culture was tested in duplicate and A_{420} values were averaged. Each final reported value is an average of three replicates with standard deviation.

Sequence analysis for *yybP-ykoY* riboswitches

The sequences for *yybP-ykoY* riboswitches were downloaded from the Rfam database (Rfam reference number RF00080) and aligned using the Kalign multi sequence alignment program (<https://www.ebi.ac.uk/jdispatcher/msa>). The sequence alignment and consensus L3 sequence was visualized using Jalview³⁴. The *yybP-ykoY* riboswitch sequences found within the Enterobacteriales order were accessed from both the Rfam database and from *yybP-ykoY* regulog collection (https://regprecise.lbl.gov/collection_rfam.jsp?riboswitch_id=13). These sequences were aligned with the Muscle multi sequence alignment program (<https://www.ebi.ac.uk/jdispatcher/msa>). The sequence alignment and consensus L3 sequence was visualized using Jalview³⁴.

Results

The Mn-sensing aptamers of the *alx* and *mntP* riboswitches differ structurally

In Fig. 1A, we present secondary structure models of the *alx* and *mntP* riboswitch aptamer domains based on prior experimental data for the full-length riboswitches^{10,18}, which we updated to reflect the overall aptamer architecture and conserved base-pairs identified by X-ray crystal structures^{6,12,21}. The model of the *mntP* aptamer in Fig. 1A is supported by an X-ray crystal structure of the aptamer in the apo state (PDB ID: 4Y1M)⁶. Since no detailed structural information exists for the *alx* aptamer, we mapped its secondary structure with DMS/SHAPE-MaP using the

traditional approach of *in vitro* synthesis by T7 RNA polymerase (RNAP) followed by denaturation and refolding.

The aptamer RNA was refolded at either neutral (7.2) or alkaline pH (8.5), consistent with pH values used in previously published studies that performed DMS probing, *in vitro* transcription, and gene reporter fusion experiments with the *alx* and *mntP* riboswitches^{18,19,22}. The aptamer RNAs were probed with either 1-methyl-7-nitroisatoic anhydride (1M7) or DMS. The resulting secondary structure models of the *alx* aptamer agree with the published 2D predictions¹⁸ and 3D structure of an *L. lactis* chimera with the *alx* L3 sequence²¹ (Fig. 1C and SI Fig. 1A-C). Comparison of the nucleotide (nt) reactivities as a function of refolding pH revealed clear flexibility differences in L3 and L1, with multiple nt (e.g., A37 and A11) showing decreased reactivity towards 1M7 or DMS at alkaline pH (Fig. 1D).

We next refolded the *alx* aptamer RNA at either neutral (7.2) or alkaline pH (8.5) in the presence of 1 mM MnCl₂ and probed with either DMS or 1M7. While the total physiological [Mn] (free and bound) in *E. coli* is estimated to be 15-21 μ M³⁵⁻³⁷, we chose a saturating concentration of ligand to maximize the ligand-induced shift in the RNA conformational ensemble, consistent with prior chemical probing experiments on other riboswitches^{28,38}. MgCl₂ was present at 5 mM in the RNA refolding buffer, thus Mg remained in excess of Mn, as is the case *in vivo*³⁹. We observed L3 generally becomes less reactive towards 1M7 in the presence of Mn, consistent with reduced flexibility upon Mn binding (SI Fig. 1D). Interestingly, in the presence of Mn, pH-dependent reactivity changes for multiple L1/L3 nt are abolished (e.g., A37 and A11, Fig. 1D), supporting that these nt are buried in the metal-sensing core with Mn and consistent with published Mn-bound aptamer structures^{6,12,21} (see Supplementary Note 1 and SI Fig. 1E for additional analysis).

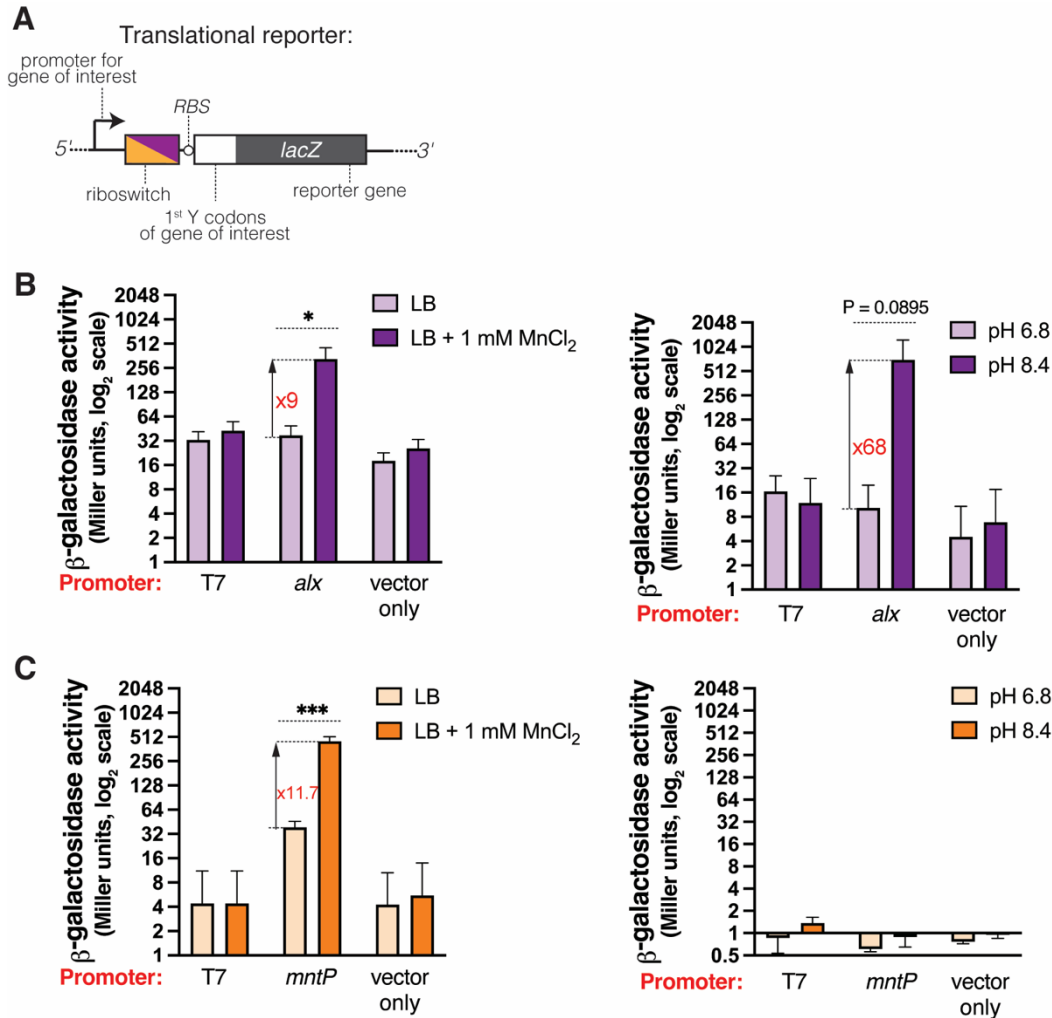


Figure 2.2. Aptamer architectures and riboswitch-controlled translational response *in vivo*.

A. Translational reporter construct design for *alx* and *mntP* translational reporters with the native or T7 promoter. **B.** Translational response of *alx* to supplemented Mn (left) or alkaline pH (right) as a function of promoter. Statistical analysis done using a parametric unpaired t-test to assess significance. **C.** Translational response of *mntP* to supplemented Mn (left) or alkaline pH (right) as a function of promoter. Statistical analysis done using a parametric unpaired t-test to assess significance.

Transcription by native RNA polymerase enables *alx* and *mntP* riboswitch-mediated translational regulation *in vivo*

We next wondered whether *alx* and *mntP* riboregulation is kinetically controlled. To test this, we constructed *alx* and *mntP* translational *lacZ* reporters cloned with either the T7 promoter

or native *alx/mntP* promoter into single copy plasmids (Fig. 2A). The effect of extracellular alkaline pH or elevated [Mn] on gene expression was measured by b-galactosidase assays in either an *E. coli* strain (MC4100) lacking *alx* (*Dalx*), to preclude negative autoregulation¹⁹, or the *Dalx* strain with T7 RNAP expressed from the *lac* promoter (chromosomally encoded, *DalxT7*). The plasmids containing the native promoter translational reporters were transformed into the *Dalx* strain and the plasmids containing the T7 promoter translational reporters were transformed into the *DalxT7* strain. The strains were cultivated in i) neutral pH (LBK pH 6.8) or alkaline pH (LBK pH 8.4) media, and ii) neutral pH (LBK pH 6.8) media with supplemented MnCl₂.

We observed the expected changes in *alx* and *mntP* translation in response to pH and supplemented MnCl₂ when reporter fusions were transcribed from their native promoters¹⁹ (Fig. 2B-C). While only *alx* translation was induced by alkaline pH, translation of both genes increased with supplemented MnCl₂ (Fig. 2B-C). Strikingly, transcription by T7 RNAP abolished the response to alkaline pH and/or Mn (Fig. 2B-C), demonstrating that faster transcription of the *alx* and *mntP* riboswitches (~220 nt/s; T7 RNAP⁴⁰ vs. ~20-90 nt/s; *E. coli* RNAP⁴¹) prevents riboswitching in response to alkalinization and/or elevated [Mn] in the cytosol. In sum, these data suggest that transcription by the native *E. coli* RNAP enables ligand-coupled structural rearrangements in the *alx* and *mntP* expression platforms.

***In vitro* analysis of the *mntP* aptamer folding during transcription using co-transcriptional chemical probing**

To investigate both pH and Mn-mediated structural changes in the *alx* and *mntP* riboswitch folding intermediates, we performed co-transcriptional chemical probing *in vitro* (Variable Length Transcription Elongation Complex RNA structure probing TECprobe-VL method, see SI Fig. 2 for construct designs)²⁸. Co-transcriptional chemical probing experiments were performed at both

neutral (7.2) and alkaline pH (8.5) $\pm$ 1 mM MnCl₂ (referred to as “+Mn” or “-Mn” condition, respectively) with 10 mM MgCl₂ present to prevent compromised transcription fidelity from Mn-coordination by RNAP, as observed for DNA polymerases⁴² and reverse transcriptases⁴³. We first present data for *alx* and *mntP* DMS-probed folding intermediates at pH 8.5 to capture broader nucleobase reactivity since published work established that alkaline pH (pH $\geq$ 8) renders all 4 nucleobases accessible to DMS modification whereas only A and C nucleobases are reactive towards DMS at neutral pH⁴⁴. Specifically, at neutral pH only N1 of adenine and N3 of cytosine are methylated, since the N1 of guanosine and N3 of uridine are protonated at neutral pH⁴⁴.

We initially mapped *mntP* aptamer folding transitions and determined the specific transcript lengths where helices in the aptamer become stably folded (Fig. 3A-B and SI Fig. 3). P3.1, P3.2, and P4 in the left leg of the aptamer fold first, as detected with coordinated drops in reactivity of nucleotides that are base-paired in these helices (Fig. 3C). In stark contrast, the right leg of the aptamer folds into transient, low-melting temperature (T_m) stem-loops that form and resolve until the synthesis of the aptamer is completed ($\sim$ nt 124, assuming a 14-nt RNAP footprint, Fig. 3B). While the 5' RNA stretch for these aptamer elements (L1, P1.1, and P1.2) is transcribed early on, their 3' pairing partner does not emerge from RNAP until late in transcription. Thus, the weak stem-loop intermediates may prevent RNA from being trapped in stable but not biologically relevant folds, consistent with other riboswitches^{16,28}. P2 in the right leg folds towards the end of aptamer transcription, even though the sequence encoding P2 emerges at transcript length $\sim$ 50 nt, indicating that folding of the entire right leg occurs at the end of aptamer synthesis (Fig. 3C).

Surprisingly, we observed Mn-dependent differences in reactivity of several nucleotides before complete *mntP* aptamer has been synthesized (Fig. 3D). For example, A48, the first nucleotide in the L3 loop, demonstrates markedly high reactivity $\pm$ Mn, with its reactivity trending

higher +Mn starting at transcript length ~115 nt – before the aptamer synthesis finished ~124 nt (Fig. 3D). We hypothesize that this is indicative of an RNA folding intermediate sampling divalent metal ions in solution in search for Mn, with its L3 and partially formed L1/P1.1. Such partial binding of Mn was observed in a crystal structure and molecular dynamics (MD) simulations of the full-length *X. oryzae* aptamer, referred to as “conformer 2”, which lacks the ordered stacking pattern in L3 and contains a partially dehydrated Mn in the M_B site¹² (Fig. 3D). Additionally, we observed multiple RNAP pauses preceding *mntP* aptamer completion that are enhanced by the presence of Mn and may mediate partial Mn binding/ sampling of divalents in the environment by the RNA (see Supplementary Note 2 and SI Figs. 4-5 for further analysis).

To identify the intermediates whose folding is truly controlled by transcription kinetics vs. thermodynamic stability of the folds, we applied a modified *in vitro* TECprobe-VL workflow wherein intermediate RNA transcripts are extracted from the stalled transcription elongation complexes and equilibrium refolded before DMS probing. These experiments identified clear structural differences between co-transcriptionally folded and equilibrium refolded *mntP* intermediates and provided additional evidence of divalent sampling by aptamer folding intermediates (see Supplementary Note 3 and SI Fig. 6 for further analysis).

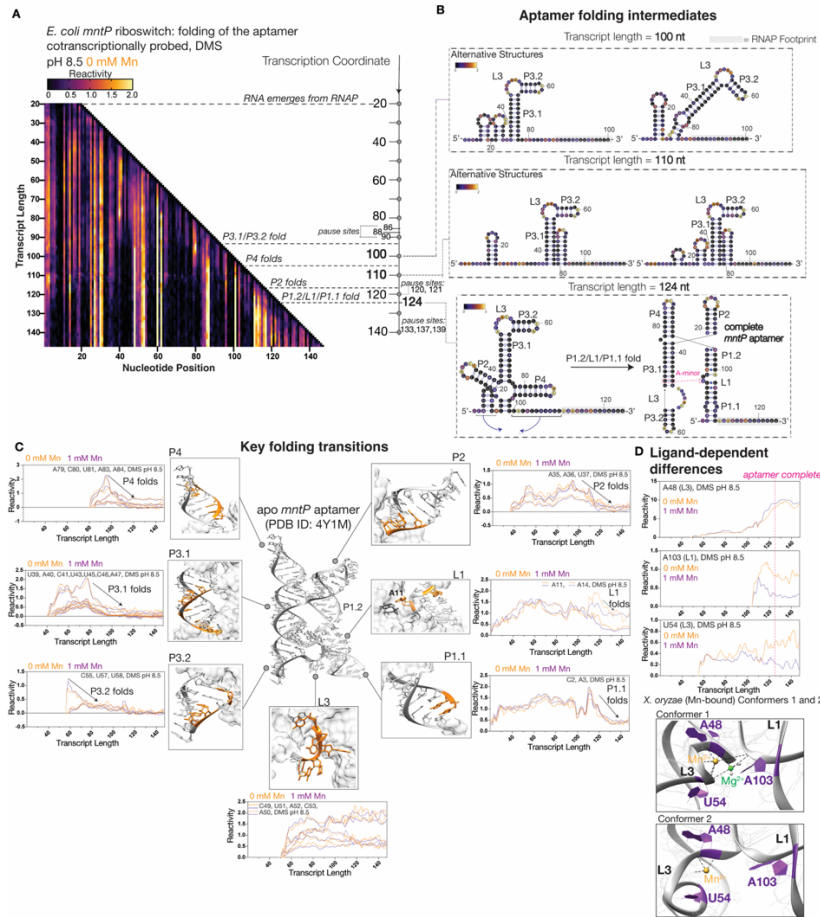


Figure 2.3. Co-transcriptional DMS probing of the *mntP* riboswitch aptamer at alkaline pH.

A. TECprobe-VL DMS reactivity matrix for the *mntP* aptamer at pH 8.5. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **B.** Secondary structures of *mntP* aptamer folding intermediates colored by DMS reactivity of the indicated transcript at pH 8.5. **C.** Transcript length-dependent reactivity changes showing folding transitions of the *mntP* aptamer at pH 8.5 ± 1 mM Mn. DMS data for 0 mM Mn (orange) and 1 mM Mn (purple) conditions from Fig. 3A and SI Fig. 3, respectively. *E. coli mntP* apo aptamer (PDB ID: 4Y1M) shown with labeled loops and helical domains. Nt highlighted in orange correspond to the nt positions with reactivities plotted in the adjacent graph. **D.** Plots show transcript length- and Mn-dependent reactivity changes in *mntP* aptamer folding intermediates at pH 8.5 ± 1 mM Mn. DMS data for 0 mM Mn (orange) and 1 mM Mn (purple) conditions from Fig. 3A and SI Fig. 3, respectively. Vertical dotted line marks completion of aptamer folding. *X. oryzae* aptamer metal-sensing core (PDB ID: 6N2V, conformer 1 = chain B, conformer 2 = chain A) with Mg²⁺ (green sphere) and Mn²⁺ (yellow sphere) highlighted. Conformer 1 shows Mn²⁺ fully bound and Conformer 2 shows Mn²⁺ partially bound. Nt highlighted in purple correspond to nt reactivity plots above. Sequence was renumbered to match the *mntP* riboswitch sequence.

***In vitro* analysis of the *alx* aptamer folding during transcription using co-transcriptional chemical probing**

We next investigated the *alx* aptamer folding transitions *in vitro* at pH 8.5 (Fig. 4A-B and SI Fig. 7). Here, P3.1, P3.2, and P4 in the left leg fold first as the aptamer is extended from ~80 nt to ~125 nt (Fig. 4B-4C). Like in the *mntP* aptamer, the 5' end of the right leg folds into transient, low- T_m stem-loops that resolve once the full-length aptamer RNA has emerged from RNAP (Fig. 4B), highlighting a potentially conserved mechanism of the *yybP-ykoY* aptamer folding.

Like with *mntP*, we captured Mn-dependent reactivity differences for L3 in *alx* aptamer folding intermediates (Fig. 4D). For example, the reactivity of U35, the first nt in the *alx* L3, trends higher +Mn starting at transcript lengths as short as ~50 nt (Fig. 4D). This trend mimics that seen with the first nt in *mntP* L3, A48, with a few key differences. First, the absolute reactivity values $\pm$ Mn are greater for A48 in *mntP* L3 (Fig. 3D). Second, the increased reactivity +Mn occurs much earlier for *alx* aptamer intermediate transcript lengths (~50 nt for *alx* vs. ~115 nt for *mntP*). The absolutely conserved adenosine in *alx* L3 that directly coordinates Mn (A37) is more reactive – Mn for aptamer folding intermediates starting at transcript length ~70 nt (Fig. 4D). The higher A37 reactivity –Mn is consistent with greater L3 flexibility in this condition and further supports that the L3 loop can begin sampling for divalents before the aptamer domain is fully folded. Curiously, the absolutely conserved adenosine in *mntP* L3 (A50) does not demonstrate this Mn-dependent reactivity difference (Fig. 3C), pointing to a difference in L3 dynamics between *alx* and *mntP* aptamer folding intermediates. Similar to *mntP*, we observed clear structural differences between co-transcriptionally folded and equilibrium refolded *alx* intermediates (see Supplementary Note 4 and SI Fig. 8 for further analysis).

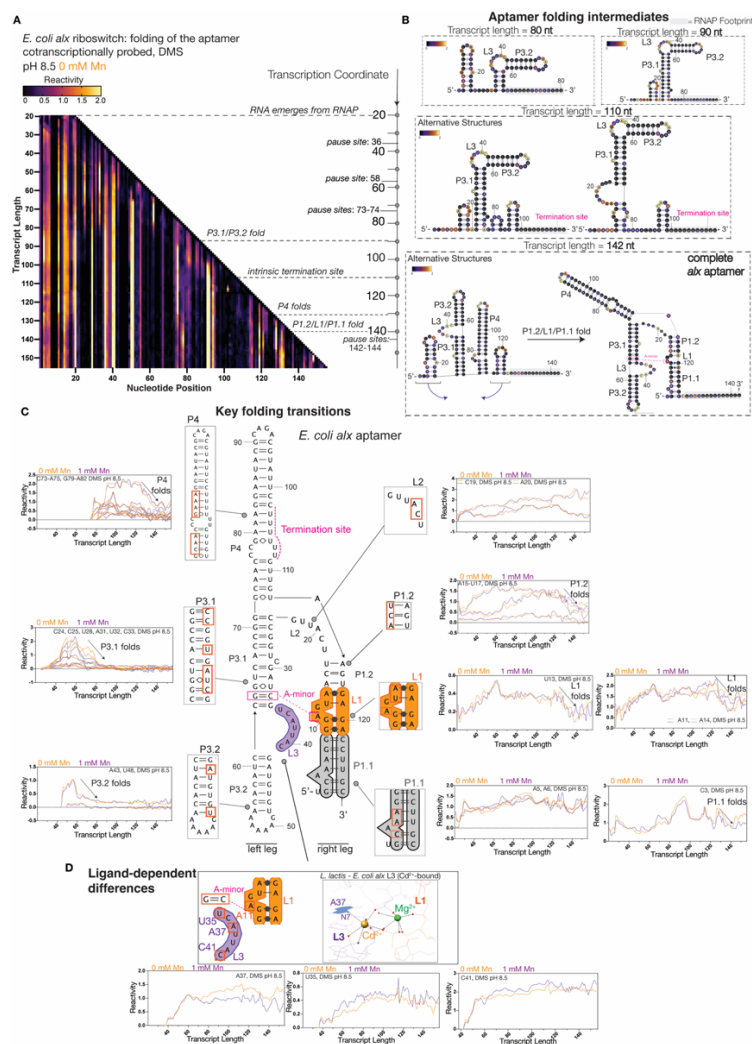


Figure 2.4. Co-transcriptional DMS probing of the *alx* riboswitch aptamer at alkaline pH.

A. TECprobe-VL DMS reactivity matrix for the *alx* aptamer at pH 8.5. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from seven independent replicates that were concatenated and analyzed together. **B.** Secondary structures of *alx* aptamer folding intermediates, which are colored by the DMS reactivity of the indicated transcript at pH 8.5. **C.** Transcript length-dependent reactivity changes showing folding transitions of the *alx* aptamer at pH 8.5 ± 1 mM Mn. DMS data for 0 mM Mn (orange) and 1 mM Mn (purple) conditions from Fig. 4A and SI Fig. 7, respectively. Secondary structure model of *alx* aptamer with labeled structural domains. L1 (orange), L3 (purple), P1.1 (gray), and intrinsic termination site (pink) are highlighted. Nt boxed in red correspond to adjacent nt reactivity plots. **D.** *L. lactis* Mn-sensing aptamer with *E. coli alx* L3 (CpD-bound). Conserved A37 (blue), Mg^{2+} (green sphere), and Cd^{2+} (yellow sphere) are highlighted. Sequence was renumbered to match the *alx* riboswitch sequence. Plots show transcript length- and Mn-dependent reactivity changes in *alx* aptamer folding intermediates at pH 8.5 ± 1 mM Mn. DMS data for 0 mM (orange) and 1 mM (purple) conditions from Fig. 4A and SI Fig. 7, respectively.

Mn binding is a prerequisite for *alx* riboswitch pH response *in vivo*

Early work characterized the *alx* riboswitch as a pH sensor¹⁸; however, recent studies have called this into question¹⁹. Since the intracellular [Mn] increases in *E. coli* at alkaline pH¹⁹, we wondered whether the *alx* riboswitch is actually sensing the increase in [Mn] as a consequence of cytosol alkalinization. To test this, we performed translational *lacZ* reporter assays in *E. coli* cells on the A37U *alx* mutant, where the absolutely conserved adenosine that directly coordinates Mn in L3 is replaced with a uridine. This point mutation was previously shown to ablate Mn-sensing in the *X. oryzae* aptamer¹² making it the best candidate mutation to decouple the effect of Mn and alkaline pH on *alx* riboregulation *in vivo*. Strikingly, the 68-fold increase in *alx* reporter translation observed upon a shift from pH 6.8 to 8.4 for the wild-type riboswitch was abolished by the A37U mutation (Fig. 5A). These cell-based data provide the first evidence that the *alx* riboswitch is not a true pH sensor; instead, the riboswitch senses increased cytosolic [Mn], which is an indirect effect of cytosol alkalinization. Published *alx* translational reporter data showed an additive effect of alkaline pH and Mn¹⁹. We thus hypothesize that alkaline pH enhances the affinity of the *alx* aptamer for Mn, rather than alters the *alx* riboswitch folding, unifying observations from early studies on this system^{18,45} with a recently published genetic study¹⁹. A combination of alkaline pH and high [Mn] produced higher *mntP* reporter translation compared to the neutral pH condition with added Mn, suggesting that alkaline pH may also enhance Mn affinity for the *mntP* aptamer, but to a lesser degree than for the *alx* aptamer¹⁹. To explore this intriguing point further, we DMS-probed the *alx* and *mntP* folding intermediates at pH 7.2 $\pm$ Mn with the TECprobe-VL method, as described in the following section.

pH effects on the *alx* and *mntP* aptamer folding *in vitro*: L3

We largely observed the same co-transcriptional folding transitions *in vitro* for the two legs in the *alx* and *mntP* aptamers at pH 7.2 as described for pH 8.5 (SI Fig. 9A-B). Although the *alx* and *mntP* aptamers exhibit the same global folding transitions at pH 7.2, key pH-dependent reactivity differences in their metal-sensing cores emerged. Here, we focused on A and C nucleotide reactivities since G and U nucleotides are protonated at the respective N1 and N3 positions at neutral pH and are thus unreactive towards DMS ($pK_a \sim 9.2^{46}$). First, the absolutely conserved adenosine in the *alx* L3 (A37) exhibits a general Mn-dependent reactivity decrease during aptamer folding at both pH (Fig. 5B). However, the magnitude of this reactivity decrease is clearly pH-dependent, with A37 consistently less reactive at pH 8.5 +Mn compared to other conditions tested (Fig. 5B), suggesting a potentially more stable A37-Mn coordination at alkaline pH. In stark contrast, the absolutely conserved adenosine in *mntP* L3 (A50) shows no drop in reactivity at pH 8.5 +Mn; instead, A50 lacks clear pH-dependent reactivity trends during aptamer folding (Fig. 5C). The first L3 nucleotide in the *mntP* aptamer (A48) is more reactive +Mn vs. –Mn regardless of pH (Fig. 5C), supporting that A48 stacked with A11 in an Mn-bound, ordered metal-sensing core increases its DMS reactivity. Interestingly, the absolute reactivity values for A48 are markedly lower at pH 7.2 $\pm$ Mn (Fig. 5C), suggesting that A48 has an upwardly shifted pK_a close to physiological intracellular pH ($\sim$ pH 7.4-7.8 for *E. coli*²⁴). We thus hypothesize that deprotonation of A48 at alkaline pH promotes its stacking with A11 in the cross-helix A-minor interaction, favoring docking of the helical legs in the aptamer (see Supplementary Note 5 and SI Figs. 9C-D,10, and 11 for further analysis of pH-dependent structural changes in *alx/mntP* L1 and L3 in co-transcriptionally folded vs. equilibrium refolded aptamer intermediates).

We wondered whether L3 sequences in *Enterobacteriales* *yybP-ykoY* family members show a unique trend that could explain the pH-dependent reactivity differences in the *alx* and *mntP*

aptamers. We aligned sequences of *yybP-ykoY* riboswitch family members within the *Enterobacteriales* order, revealing two distinctive groups: an *alx*-like group (consensus: UCAUUAC, Fig. 5D) and an *mntP*-like group (consensus: ACAUACU, Fig. 5D). We then compared the above sequences to the *yybP-ykoY* riboswitch family L3 consensus sequence generated from 1,063 aligned sequences (consensus: ACAUACU, Fig. 5D). Generally, the first nucleotide in L3 is an A – an important position since this nucleotide stacks with the conserved A11 in L1 that forms the cross-helix A-minor interaction. Therefore, this position being strictly a U in *alx*-like members suggests a functional role. Since the N3 of U has a close-to-physiological pKa of $\sim 9.2^{46}$, it is possible that a shift from neutral to alkaline pH alters its stacking with the conserved A11 and consequently Mn-coupled structural rearrangements in the expression platform.

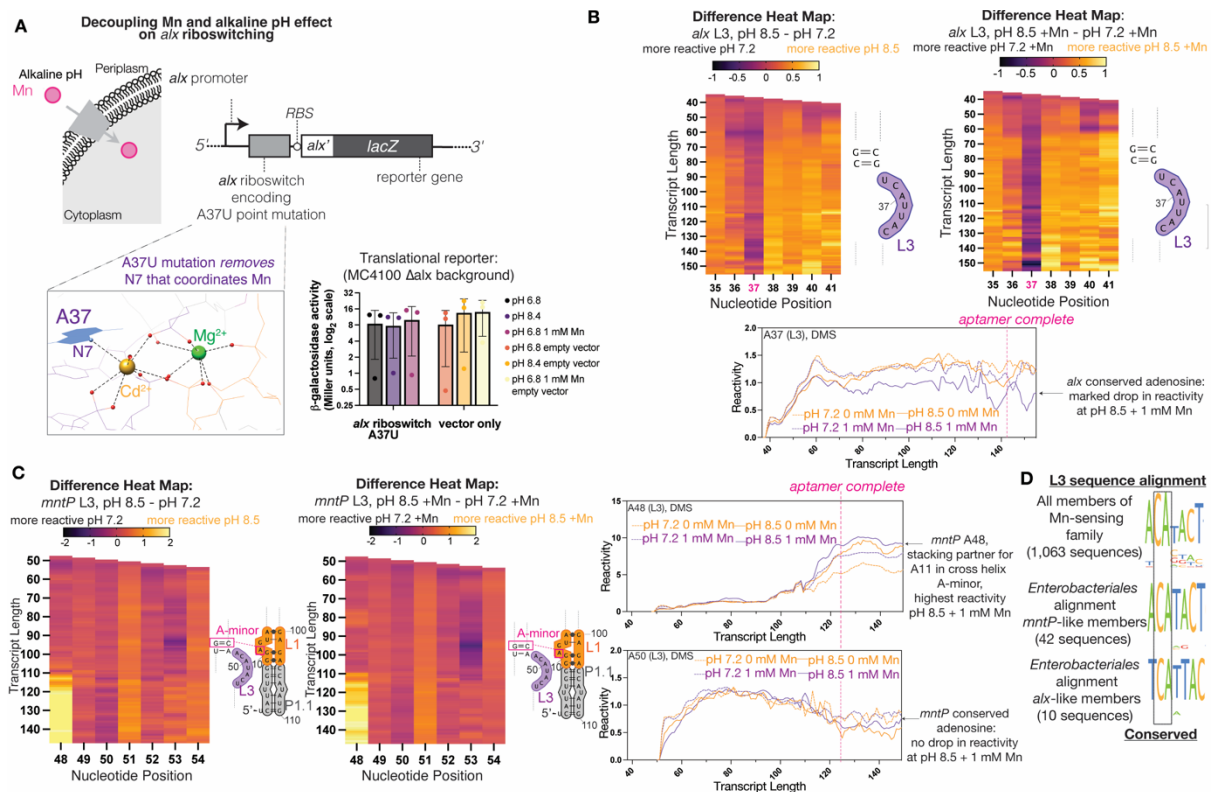


Figure 2.5. Decoupling the effects of alkaline pH and Mn on *alx* riboswitching *in vivo* and pH-dependent reactivity changes in the divergent *alx* and *mntP* L3 sequences.

A. (Top panel) *E. coli* cell with increased cytosolic [Mn] resulting from an alkaline pH challenge (left) and construct design for *alx* translational reporter (right). (Bottom panel) *L. lactis* Mn-sensing aptamer with *E. coli* *alx* L3 sequence (PDB ID: 6CC1, chain B, left). Conserved A37 (blue), Mg^{2+} (green sphere), and Cd^{2+} (yellow sphere) are highlighted. Sequence was renumbered to match the *alx* riboswitch sequence. (Bar graph) Loss of *alx* translational response to either Mn or pH upon mutation of the Mn-coordinating adenosine. **B-C.** Reactivity difference heatmaps and reactivity changes in aptamer intermediates for *alx* L3 nt (**B**) and *mntP* L3 nt (**C**) during aptamer folding at pH 7.2 vs. 8.5 ± 1 mM Mn. *alx* L3 and *mntP* L3 (purple), L1 (orange), L3 (purple), P1.1 (gray), and cross-helix A-minor (pink) are highlighted. DMS data for 0 mM Mn (orange) and 1 mM Mn (purple) conditions from Fig. 4A, SI Fig. 7, and SI Fig. 9B (**B**, *alx*) and Fig. 3A, SI Fig. 3, and SI Fig. 9A (**C**, *mntP*). Vertical dotted line marks completion of aptamer folding. **D.** L3 consensus sequences from *yybP-ykoY* family members in the Rfam database (top) and *mntP*-like (middle)/*alx*-like (bottom) members in the *Enterobacteriales* order.

In vitro analysis of *mntP* expression platform folding during transcription

The expression platform for the *mntP* riboswitch contains a single helix that begins folding at transcript length ~175 nt and was detected by coordinated drops in reactivity for its constituent nucleotides (Fig. 6A and Fig. S12A). In the absence of Mn, the aptamer remains stably folded for most of *in vitro* expression platform synthesis until the transcript is ~205 nt long (Fig. 6B). At this critical decision point – the riboswitching point – the L1 and P1.1 elements of the aptamer are disrupted if no Mn is available, allowing for alternative pairing of P1.1 switch helix in the expression platform (Fig. 6C). The disruption of P1.1 in the absence of Mn is consistent with previously published single-molecule fluorescence experiments with an isolated aptamer that show that this helix is less stable without Mn¹². Analysis of single-nucleotide trajectories revealed that A11 reactivity transiently spikes at ~205 nt, whether or not Mn is included in the transcription reaction, but the amplitude of the spike is Mn-dependent (Fig. 6D). The spike in A11 reactivity coincides with P1.1 strand invasion and its reduced amplitude +Mn indicates A11 remains trapped in the long-range A-minor interaction in Mn-bound aptamer. The L1/P1.1 reactivity difference heatmap $\pm$ Mn highlights A11 as the nucleotide most sensitive to the presence of the ligand, serving as a marker for the riboswitching point (Fig. 6D). We also assessed reactivity changes for A48 in L3 during expression platform folding, since A48 stacks with A11 and helps anchor the cross-helix A-minor interaction. A48 reactivity drops $\pm$ Mn starting at transcript length ~185 nt, with a larger decrease –Mn (Fig. 6E). A48 reactivity is consistently lower –Mn for the remainder of expression platform folding (Fig. 6E), likely due to broken A48-A11 stacking.

We observed similar reactivity trends in *mntP* expression platform folding *in vitro* at pH 7.2 as pH 8.5 (e.g., A11 reactivity spike, SI Fig. 12B-C) and uncovered additional evidence of P1.1/L1 disruption in the vicinity of the riboswitching point (see Supplementary Note 6 and SI Fig. 12D). Further, *in vitro* equilibrium refolded *mntP* intermediate transcripts did not undergo

Mn-dependent structural changes, supporting that co-transcriptionally folded RNA is required to observe Mn-coupled structural rearrangements in the expression platform (see Supplementary Notes 7-8 and SI Figs. 13-14 for reactivity analysis of equilibrium refolded *mntP* RNA).

***In vitro* analysis of *alx* expression platform folding during transcription**

The expression platform for the *alx* riboswitch contains multiple helices that begin folding at transcript length ~155 nt, as detected by coordinated drops in reactivity for the constituent nucleotides in each helix (Fig. 7A and SI Fig. 15A). The *alx* expression platform contains two helices (termed here P5 and P6) in its translationally inactive conformation that are predicted to fold $-Mn$, resulting in the ribosome binding sequence (RBS) base-pairing with a disrupted P1.1/L1 (Fig. 7B-D). On the other hand, when P1.1/L1 remain base-paired in the Mn-bound *alx* aptamer, an alternative helix in the expression platform (termed here P5/P6) is predicted to fold, and the downstream RBS-containing strand is single-stranded (Fig. 7E-G).

Unlike the *mntP* riboswitch, the *alx* P1.1/L1 rising stem is highly dynamic during the entirety of *alx* expression platform folding *in vitro* both $\pm Mn$, which was detected as consistent reactivity towards DMS for multiple nt (Fig. 7A). The strand predicted to invade P1.1/L1 in the *alx* aptamer emerges at transcript length 155 nt, resulting in formation of the P5 helix (Fig. 7A). Once the expression platform is extended to transcript length 190 nt, the P6 intrinsic terminator helix folds (Fig. 7A). Interestingly, the DMS reactivities for nucleotides in the *alx* expression platform were largely similar $\pm Mn$, resulting in similar secondary structure predictions for expression platform folding intermediates (SI Fig. 15B-D).

Despite the global structural similarities in the *alx* expression platform secondary structural models $\pm Mn$, we detected multiple Mn-dependent reactivity differences for specific nucleotides that indicate a modest ligand-dependent shift in the conformational ensemble *in vitro*. P5 stem-loop nucleotides are more reactive $-Mn$ (Fig. 7C), consistent with a population bias towards the inactive conformer. A137 (bulged in active conformer vs. base-paired in inactive conformer)

demonstrates markedly high reactivity in general and exhibits higher reactivity +Mn (Fig. 7F), indicating a shift in the conformational ensemble towards the *alx* active conformer.

We observed similar DMS reactivity patterns in the *alx* expression platform at neutral vs. alkaline pH, such as folding of P5 and P6 (SI Fig. 16A-B), indicating that a shift to alkaline pH impacts metal-sensing rather than directly affecting folding of the expression platform and thus riboswitching. However, we observed interesting pH-dependent reactivity trends for A137, which was more reactive +Mn at both pH and most reactive overall at pH 8.5 +Mn (SI Fig. 16B), supporting that the active conformer is sampled more frequently under this condition. We next assessed pH- and Mn-dependent reactivity trends in the *alx* aptamer during *in vitro* expression platform folding (Fig. 8A-C). Contrasting *mntP*, A11 remains accessible for DMS methylation across all conditions tested with no significant pH and Mn-dependent differences (Fig 8B and SI Fig. 16C). Lastly, we observed interesting pH- and Mn-dependent reactivity trends for *alx* expression platform nt in equilibrium refolded intermediates (see Supplementary Note 9 and SI Fig. 17).

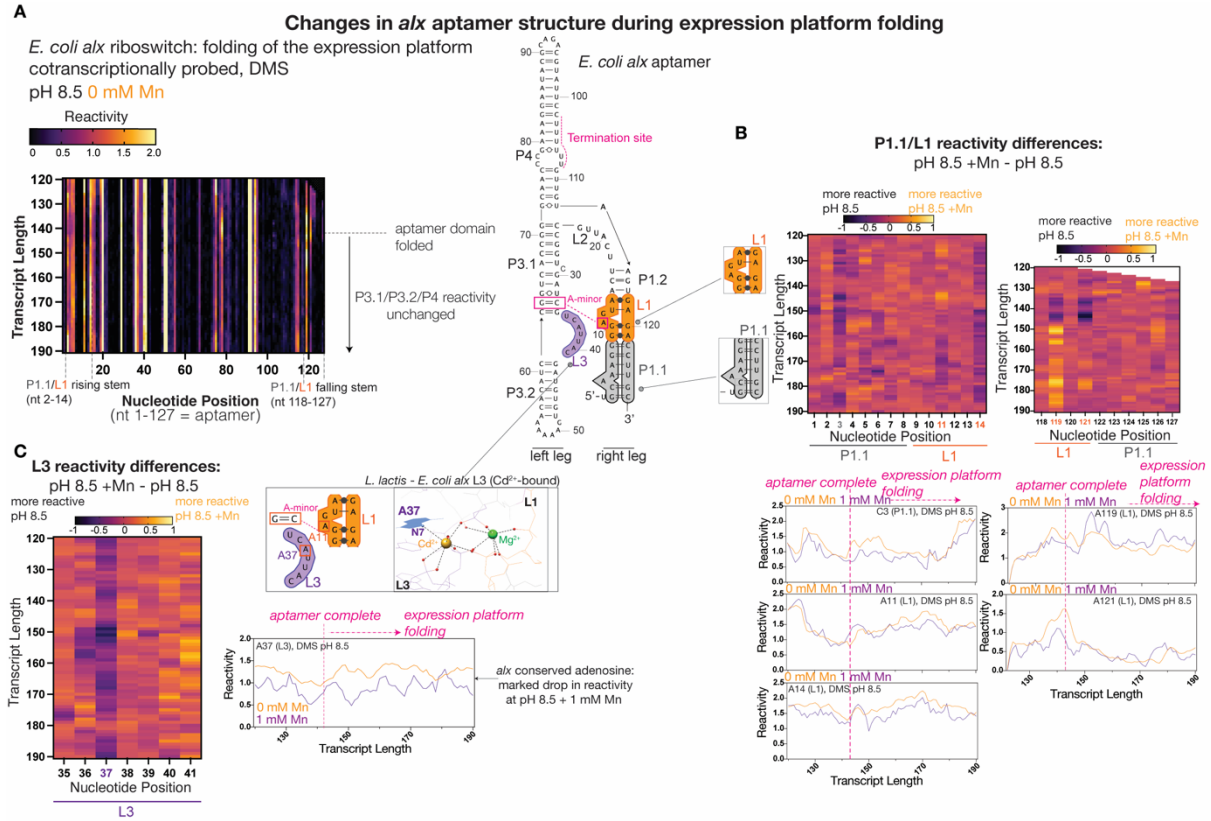


Figure 2.8. Changes in the *alx* aptamer during expression platform folding.

A. (Left panel) TECprobe-VL DMS reactivity matrix of the *alx* riboswitch aptamer during expression platform folding at pH 8.5. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from seven independent replicates that were concatenated and analyzed together. (Right panel) Secondary structure model of *alx* aptamer with labeled structural domains. L1 (orange), L3 (purple), P1.1 (gray), and intrinsic termination site (pink) are highlighted. **B-C.** Reactivity difference heatmaps for L1/P1.1 (**B**) and L3 (**C**) at pH 8.5 $\pm$ 1 mM Mn. Adjacent to heatmaps are transcript length- and Mn-dependent reactivity changes in L1/P1.1 nt (**B**) and L3 nt (**C**). DMS data for 0 mM and 1 mM Mn conditions from Fig. 8A and SI Fig. 15A, respectively. Vertical or horizontal dotted lines/arrows mark indicated RNA folding transitions. *L. lactis* Mn-sensing aptamer with *E. coli alx* L3 sequence (PDB ID: 6CC1, chain B) shown in panel B with A37 (blue), Mg^{2+} (green sphere), and Cd^{2+} (yellow sphere) highlighted. Sequence was renumbered to match the *alx* riboswitch sequence.

The *mntP* and *alx* RBS regions are dynamic in co-transcriptionally displayed RNA *in vitro*

The RNA surrounding the *mntP* RBS did not demonstrate a clear Mn-dependent reactivity trend *in vitro*, with DMS-constrained structure models predicting low- T_m hairpins interspersed

with single-stranded regions (SI Fig. 18A). We thus conclude that the RNA around the RBS is dynamic, and any Mn-dependent structural differences are likely averaged out. Prior smFRET study observed such RBS dynamics for the preQ₁ translational riboswitch with ligand-dependent accessibility bursts in the RBS region⁴⁷. Therefore, characterization of the *mntP* RBS dynamics requires complementary, e.g., single-molecule methods. Likewise, heatmap visualization of the *mntP* RBS nucleotides at pH 7.2 or 8.5 $\pm$ Mn or in equilibrium refolded intermediates at both pH showed no clear reactivity difference that would indicate increased RBS accessibility +Mn (SI Fig. 18B-C).

For *alx*, the presence of the P6 intrinsic terminator stem resulted in a stark decrease in representation for longer transcripts containing the RBS (SI Fig. 19A), precluding accurate modeling of the RNA structure in this region. To boost representation of full-length *alx*, we performed a modified *in vitro* co-transcriptional chemical probing experiment with a terminally biotinylated DNA template and the fast-acting SHAPE reagent benzoyl cyanide (BzCN, reaction time $\sim$ 1s) to better capture the short-lived co-transcriptional, Mn-dependent structural changes in the RBS. Consistent with *mntP*, the single-length BzCN probing experiment revealed no clear Mn-dependent differences in the reactivities of RBS nucleotides (SI Fig. 19B), supporting that the *alx* RBS is dynamic. These dynamics may serve a functional role; for instance, the RBS may be rapidly docking/undocking with another tertiary structural element in *alx*, with the docking rate fine-tuning RBS accessibility. Underscoring the importance of co-transcriptional chemical probing, we found that T7-synthesized, equilibrium refolded *alx* riboswitch adopts a translationally inactive conformation $\pm$ Mn (DMS/1M7-probed, described further in Supplementary Note 10 and SI Fig. 20). Taken together, while *alx* and *mntP* differ structurally, they may share common dynamic features that gate RBS accessibility.

Discussion

In this work, we performed a suite of *in vitro* chemical probing experiments on the Mn-sensing translational riboswitches in *E. coli* that precede the *mntP* and *alx* genes encoding Mn exporters. While static structural snapshots are available for Mn-sensing aptamers, these structures do not answer the question of how binding of Mn to the aptamer is “communicated” to the expression platform – the question at the heart of riboswitch function. Because the *mntP* and *alx* aptamers display unique architectures, they are an interesting test case of how structurally distinct aptamers in the same riboswitch family “talk” to the expression platform. We demonstrated that co-transcriptional folding with the native *E. coli* RNAP is essential for Mn-coupled structural rearrangements in the *mntP* and *alx* riboswitches *in vivo*, which prompted us to perform co-transcriptional chemical probing experiments *in vitro*.

The *in vitro* co-transcriptional chemical probing revealed that 5'-proximal halves in both *alx* and *mntP* aptamers traverse low- T_m RNA hairpin intermediates during folding, presumably as a safeguard against premature folding of RNA into stable but non-functional folds (Fig. 9B). Surprisingly, we found that both riboswitches show Mn-dependent reactivity changes *before* the full aptamer domain has folded. The Mn-dependent structural changes in aptamer folding intermediates map to nucleotides in the *alx* and *mntP* metal-sensing cores, indicating that a partially formed metal-sensing core can interrogate the environment for Mn. Curiously, these Mn-dependent differences emerge at much earlier transcript lengths for *alx* aptamer folding, which may permit a rapid response to the subtle increase in intracellular [Mn] prompted by cytosol alkalinization¹⁹.

Notably, other riboswitches that have been investigated with *in vitro* co-transcriptional SHAPE-seq or SHAPE-MaP (ZTP, ppGpp, and fluoride) did not show ligand-dependent structural changes for folding intermediates²⁸. Ligands such as ZTP and ppGpp are larger than an Mn ion

and require a network of interactions to bind their cognate aptamers^{48,49}. The resulting structural complexity of these aptamer-ligand interactions likely prevents their stable interaction with an aptamer folding intermediate. In the case of fluoride riboswitch, binding of fluoride anion to the aptamer is mediated by a network of Mg^{2+} ions to overcome electrostatic repulsion by the phosphate backbone⁵⁰, making fluoride less likely to bind an aptamer folding intermediate. Additionally, it will be interesting to see whether other divalent-sensing riboswitches (e.g., Mg^{2+} - and Ni^{2+}/Co^{2+} -sensing riboswitches) can begin sampling for ligand before the complete aptamer has folded. Lastly, riboswitch classes that sense monovalent alkali metals, Na^+ and Li^+ , have been discovered^{51,52}; however no 3D structural information for these riboswitch aptamers has been published to our knowledge. Since the RNA folds responsible for sensing Na^+ and Li^+ , as well as the binding stoichiometry, remain unknown, it will be intriguing to explore potential shared features in co-transcriptional folding and ligand sensing between the divalent- and monovalent-sensing riboswitch classes.

To get at the molecular basis of the divergent pH response of the *alx* and *mntP* riboswitches, we performed *in vitro* co-transcriptional DMS probing of RNA at both neutral and alkaline pH $\pm Mn$. We found that pH uniquely affects the structure of the *alx* and *mntP* metal-sensing cores, providing the first clues to how pH differentially affects Mn sensing. The *alx* and *mntP* aptamers differ in their global architecture, forming a 3WJ and a 4WJ, respectively. Future study should investigate how nucleobase protonation states in the *alx* and *mntP* metal-sensing cores are coupled to junction dynamics, which controls the docking of the aptamer's helical legs. Prior smFRET study on the *X. oryzae* aptamer, a 4WJ aptamer like *mntP*, illustrated how Mn binding promotes docking of the two helical legs that in turn stabilizes the P1.1 switch helix¹². The dynamics of *alx*-

like aptamers has not yet been studied but may play a role in the differential pH effect on *alx* vs. *mntP* Mn sensing and consequent riboswitching.

The stark differences in the flexibility, architecture, and pH/Mn response of the *mntP* vs *alx* riboswitches may be coupled to the cellular function of their respective Mn exporters. We showed that alkaline pH alone does not rearrange the *alx* riboswitch into a translationally active conformer; rather, the *alx* riboswitch senses the increased cytosolic [Mn] brought about by cytosol alkalization. We hypothesize that alkaline pH lowers the K_D of the *alx* aptamer for Mn, explaining why an alkaline pH shift and consequent increase of [Mn] from 28 to 42 μ M (free and bound) activates expression of *alx* but not *mntP*¹⁹ (Fig. 9). The physiological functions of such pH-enhanced expression of Mn exporters could be multi-fold. For example, Mn(II) is oxidized at alkaline pH^{53,54}, deeming it unusable by Mn-dependent enzymes. Further, oxidized Mn(IV) is an extremely reactive agent able to oxidize carbon-carbon double bonds in nucleobases, the capability exploited in permanganate footprinting of protein-nucleic acid complexes^{55,56}. Removal of Mn by exporters to minimize the pool of oxidized Mn could thus be a protective measure for the cell experiencing alkaline stress.

Limitations of this study. There are multiple limitations to the experimental approaches used here. First, *in vitro* co-transcriptional chemical probing experiments are an endpoint measurement and do not capture the true structural intermediates traversed by an RNA during transcription. Further, the DMS probing reaction is 5 min, meaning that the co-transcriptionally displayed RNA can equilibrate to some degree. As a control, we performed DMS probing experiments on equilibrium refolded intermediates, which showed structural differences. However, we cannot rule out the possibility that the RNA folds observed with co-transcriptional DMS probing are not always representative of a true folding intermediate. Lastly, since the DMS

probing reactions were performed *in vitro*, the absence of ribosomal proteins may have altered the observed structural ensemble, which should be addressed in future work.

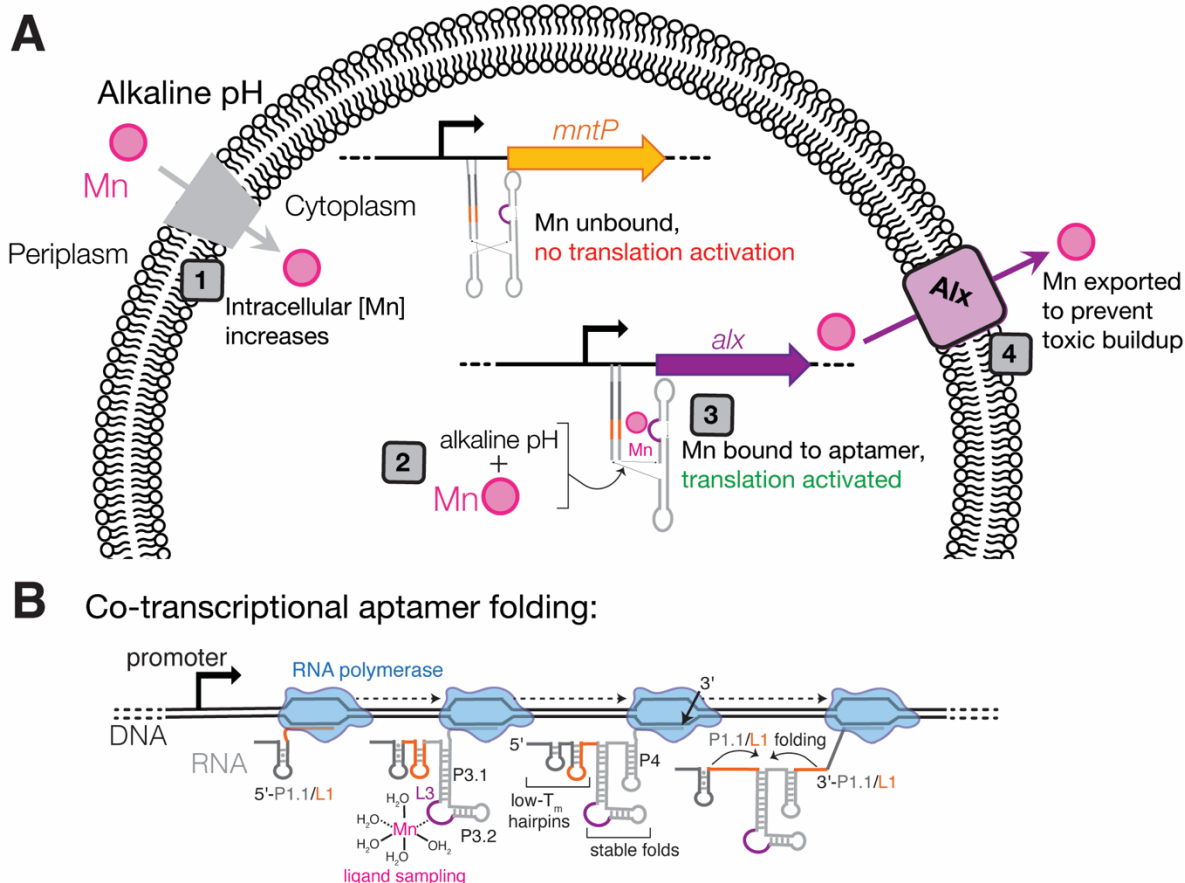


Figure 2.9. Model for *alx* and *mntP* riboregulation in *E. coli*.

A. Cartoon model of *alx* and *mntP* riboregulation of their respective genes in *E. coli*. At alkaline pH, the cytosolic [Mn] increases, resulting in the *alx* riboswitch turning “on” expression of Alx to prevent toxic accumulation of Mn in the cytosol. The modest increase in cytosolic [Mn] brought about by alkaline pH is insufficient to shift the *mntP* riboswitch towards its translationally active conformation, leaving *mntP* expression “off”. **B.** Key concepts learned from co-transcriptional chemical probing of the *alx* and *mntP* Mn-sensing aptamers. Ligand sampling begins with intermediate RNA transcripts. The 5’ and 3’ halves of the aptamer fold differently: 3’-proximal motifs fold stably, whereas 5’-proximal motifs traverse “weak” RNA hairpins until the base-pairing partners emerge late in aptamer synthesis.

Data Availability

The raw sequencing data generated in this study is publicly available in the Sequencing Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) with the BioProject accession code

PRJNA1197522. Information for individual BioSamples is provided in Supplementary Tables 7-8. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon request.

Supplementary Data

Supplementary Data are available at NAR online.

Author contributions

C.S. and T.V.M, conceptualization; C.S. and D.P., methodology; C.S., D.P., and C.B., investigation; C.S. and D.P., formal analysis; C.S. and D.P., visualization; C.S. and D.P., writing-initial draft; C.S., D.P., and T.V.M, writing-review and editing; T.V.M, supervision; T.V.M., funding acquisition.

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Conflict of Interest

The authors declare no competing interests.

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Supplementary Information for: Structurally distinct manganese-sensing riboswitch aptamers regulate diverse expression platform architectures

Authors

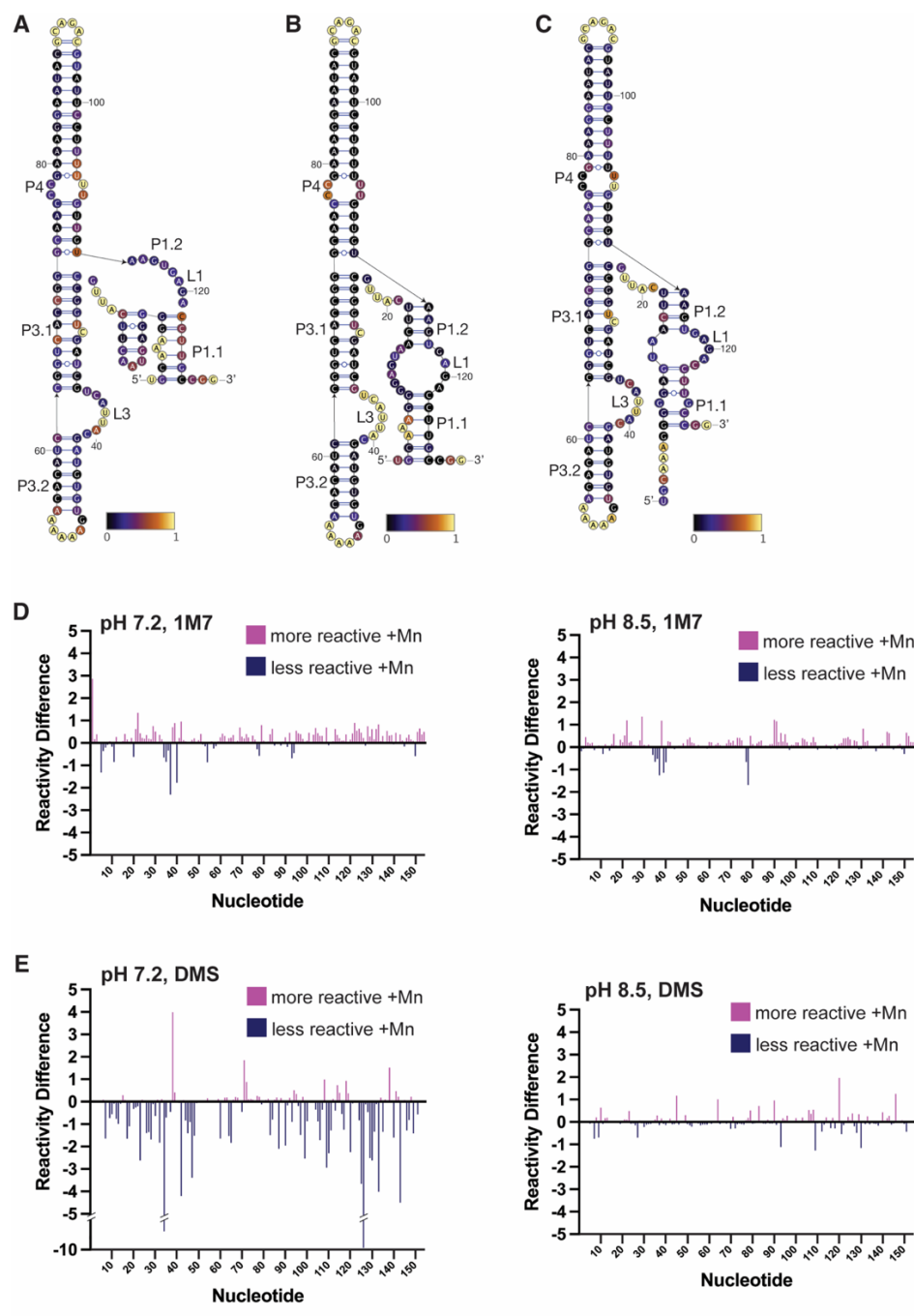
Christine N. Stephen¹, Danae E. Palmer¹, Clarisa Bautista, and Tatiana V. Mishanina^{1*}

¹Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Dr, La Jolla, CA 92093

* To whom correspondence should be addressed. Tel: +1 858 246-5883; Email: tmishanina@ucsd.edu

This pdf file includes: Supplementary Figures 1-32, Supplementary Notes 1-10, and Supplemental Methods.

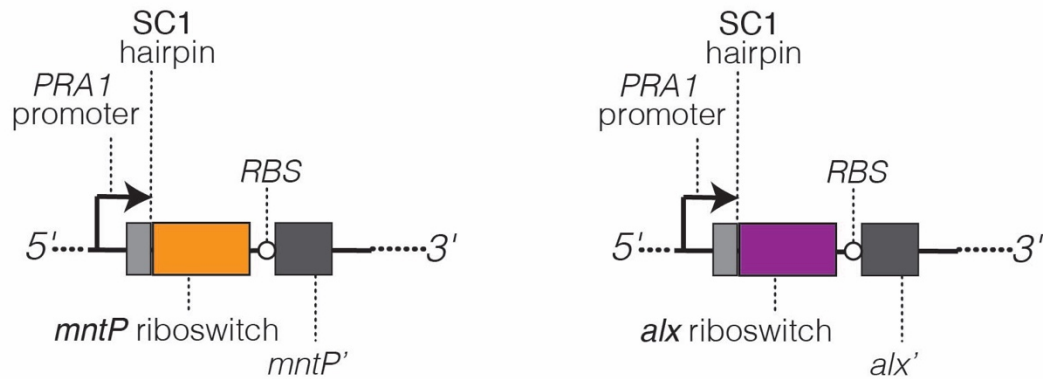
Supplementary Figures



Supplemental Figure 2.1. Structure probing of equilibrium-refolded *alx* aptamer RNA.

Secondary structure of equilibrium-refolded *alx* aptamer RNA modeled based on reactivity with 1M7 at **A**. pH 7.2 +Mn. **B**. pH 8.5 -Mn. **C**. pH 8.5 +Mn. **D**. Individual nucleotide reactivity differences when modified with 1M7 to compare +Mn vs. -Mn, measured at both pH 7.2 and 8.5. **E**. Individual nucleotide reactivity differences when modified with DMS to compare +Mn vs. -Mn, measured at both pH 7.2 and 8.5.

TECprobe-VL construct designs, *alx* and *mntP* riboswitches

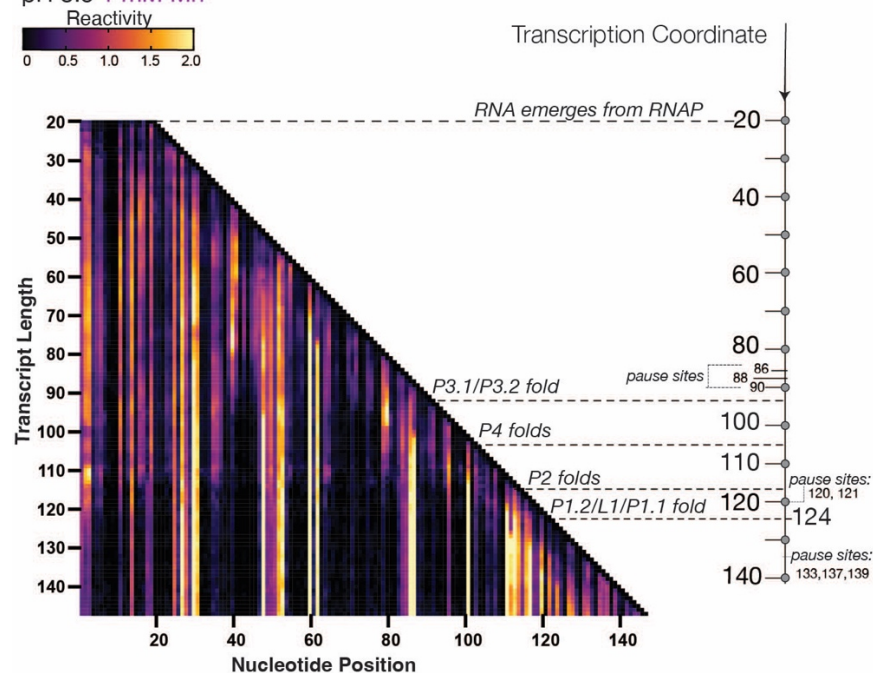


Supplemental Figure 2.2. TECprobe-VL construct designs for the *alx* and *mntP* riboswitches.

Transcription template schematics annotated with key sequence elements. All TECprobe-VL transcription templates contained the PRA1 promoter, 5' SC1 hairpin, full-length *alx* or *mntP* riboswitch sequence, the RBS, and a portion of the downstream gene sequence (termed *alx'* - nt 1-100 *alx* coding sequence, and *mntP'* - nt 1-45 *mntP* coding sequence).

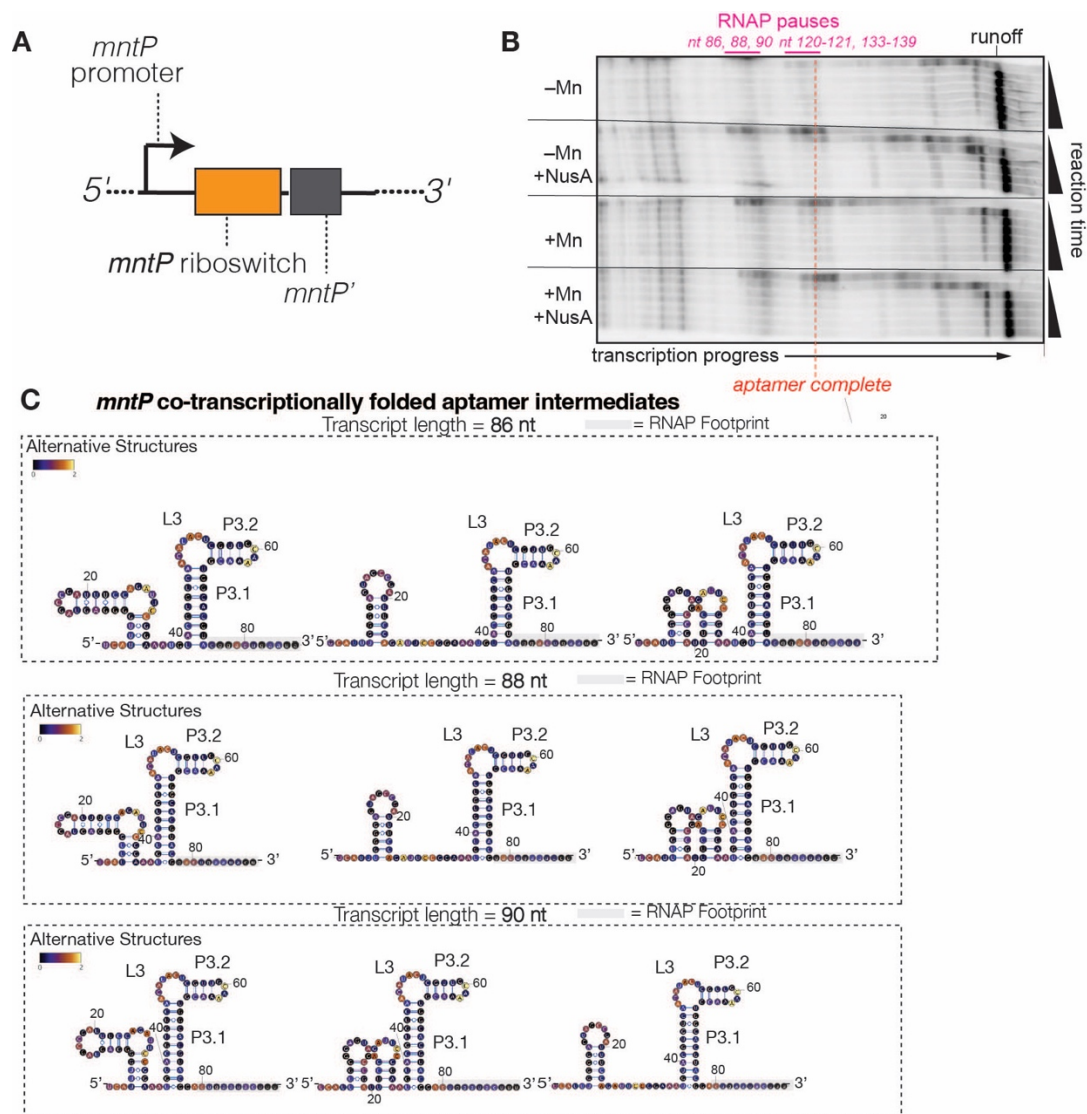
E. coli mntP riboswitch: folding of the aptamer
cotranscriptionally probed, DMS

pH 8.5 1 mM Mn



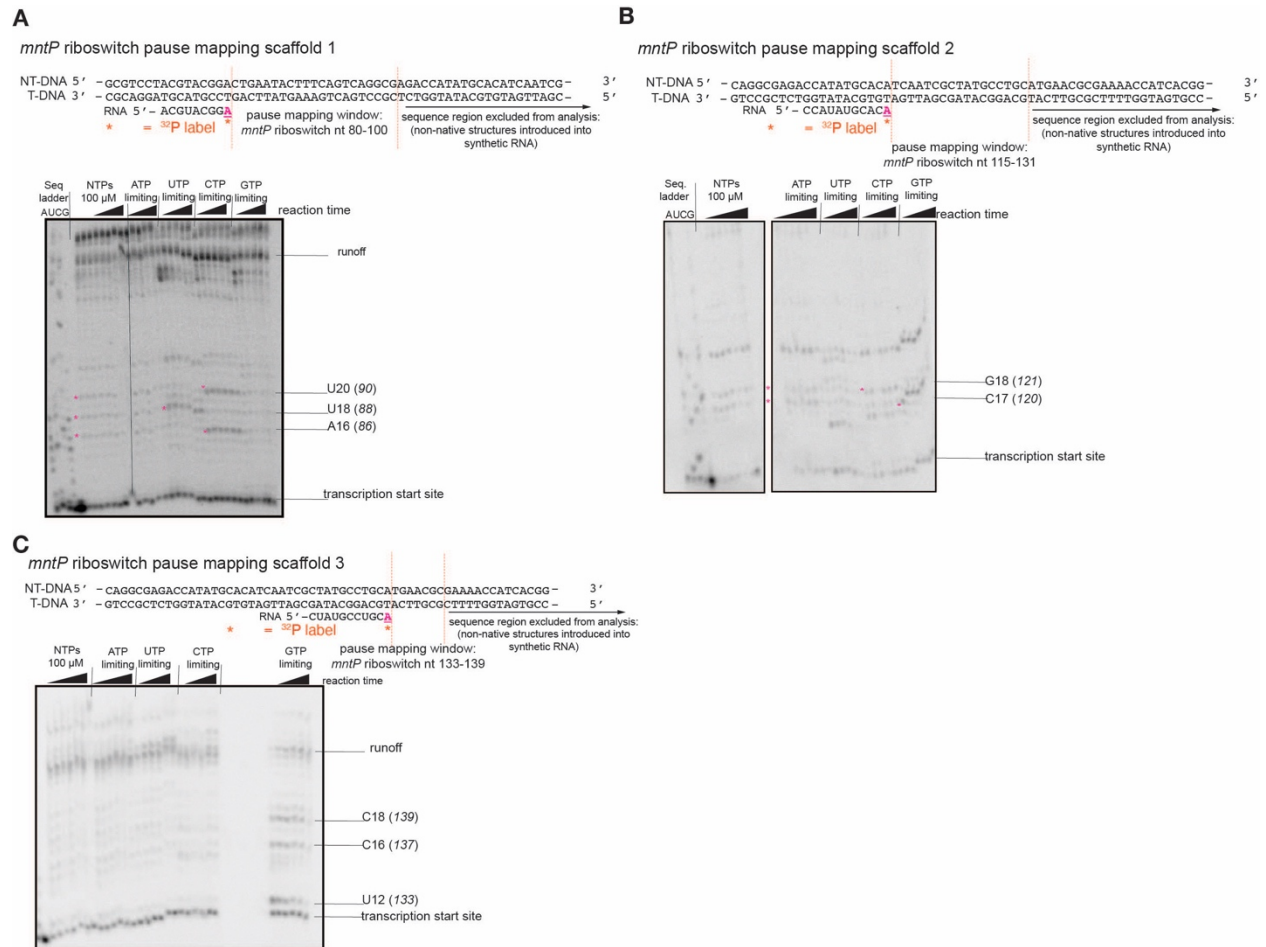
Supplemental Figure 2.3. Co-transcriptional DMS probing of the *mntP* riboswitch aptamer at alkaline pH +Mn.

TECprobe-VL DMS reactivity matrix for the *mntP* aptamer at pH 8.5 +1 mM Mn. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together.



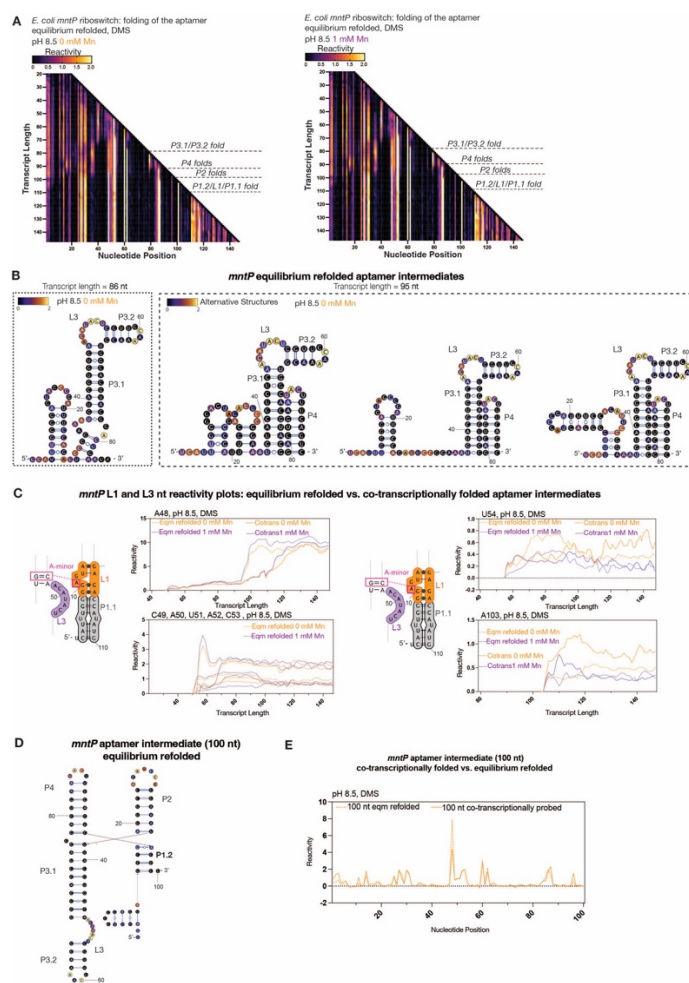
Supplemental Figure 2.4. Synchronized, single-round *in vitro* transcription initiated from the *mntP* promoter and RNA structure models for pause-adjacent aptamer folding intermediates.

A. Transcription template schematic with key sequence features highlighted, including the native *mntP* promoter, full-length *mntP* riboswitch, and a portion of the downstream *mntP* gene (termed *mntP'*). **B.** *mntP* riboswitch transcripts were radiolabeled via incorporation of [α - 32 P]ATP and visualized on a 6% denaturing polyacrylamide gel. RNA species in the vicinity of pause sites are labeled with transcript lengths. Transcription elongation assays were performed $\pm$ Mn and $\pm$ NusA. **C.** Secondary structures of *mntP* aptamer folding intermediates, which were colored by the DMS reactivity of the indicated transcript at pH 8.5 -Mn.



Supplemental Figure 2.5. Mapping *mntP* pause locations during aptamer and early expression platform folding.

A-C. Experiments performed on synthetic nucleic acid scaffolds to map pause sites spanning transcript lengths 80-139 nt encoded by the *mntP* riboswitch sequence. Elongation complexes were positioned upstream of the designated pause mapping window and radiolabeled by incorporation of [α -³²P]ATP. Transcription was initiated by addition of all four NTPs with one NTP present at a limiting concentration to increase pause duration. Radiolabeled RNAs were visualized on a 15% denaturing polyacrylamide gel. All pause sites are labeled next to gel images by the 3' RNA nucleotide in the RNAP active site and the length of radiolabeled RNA, with the corresponding transcript length in full-length *mntP* riboswitch denoted in parentheses. Pause sites were located based on the sequencing ladder and by comparing results from limiting NTP time courses.

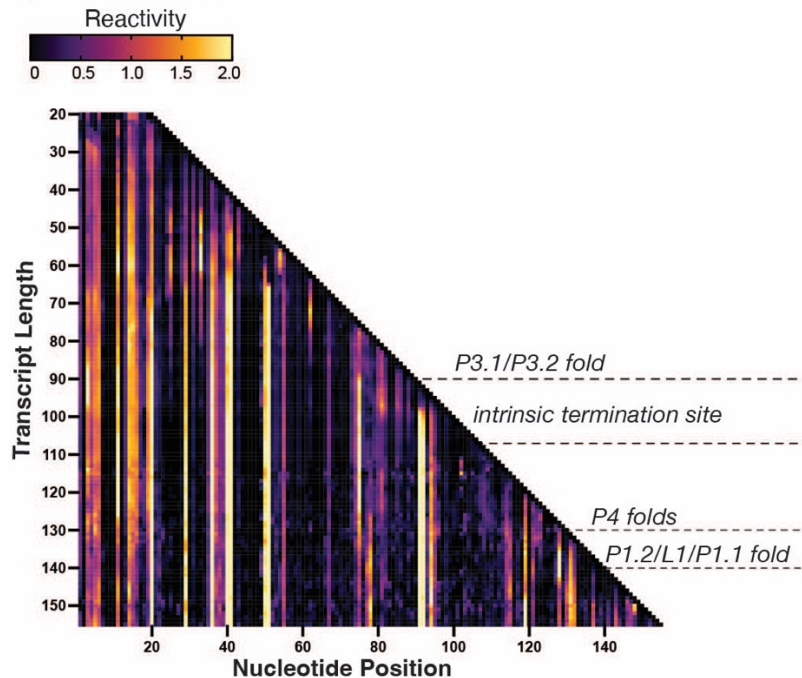


Supplemental Figure 2.6. Equilibrium DMS probing of *mntP* aptamer folding intermediates at alkaline pH $\pm$ Mn.

A. TECprobe-VL DMS reactivity matrices for *mntP* aptamer folding intermediates, which were purified and equilibrium refolded at pH 8.5 $\pm$ 1 mM Mn. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **B.** Secondary structures of *mntP* aptamer folding intermediates, which were purified and equilibrium refolded prior to DMS probing. Secondary structure models are colored by the DMS reactivity of the indicated transcript at pH 8.5 -Mn. **C.** Plots showing *mntP* L3 and L1 nt transcript length- and Mn- dependent reactivity changes in equilibrium refolded vs. co-transcriptionally folded aptamer intermediates. DMS data from 0 mM Mn (orange) and 1 mM Mn (purple) conditions from Fig. 3A, SI Fig. 3, and SI Fig. 6A. **D.** Secondary structure model for the 100 nt *mntP* aptamer folding intermediate, which was purified and equilibrium refolded prior to DMS probing. The secondary structure model is colored by the DMS reactivity at pH 8.5 -Mn. **E.** Plot of nt reactivities in the equilibrium refolded vs. co-transcriptionally folded 100 nt *mntP* aptamer intermediate, probed with DMS at pH 8.5 -Mn. The DMS reactivities for the co-transcriptionally folded RNA are from transcript length 115nt, which has 100 nt of structured RNA exposed for chemical probing due to the RNAP footprint. Data from SI Fig. 6A and Fig. 3A.

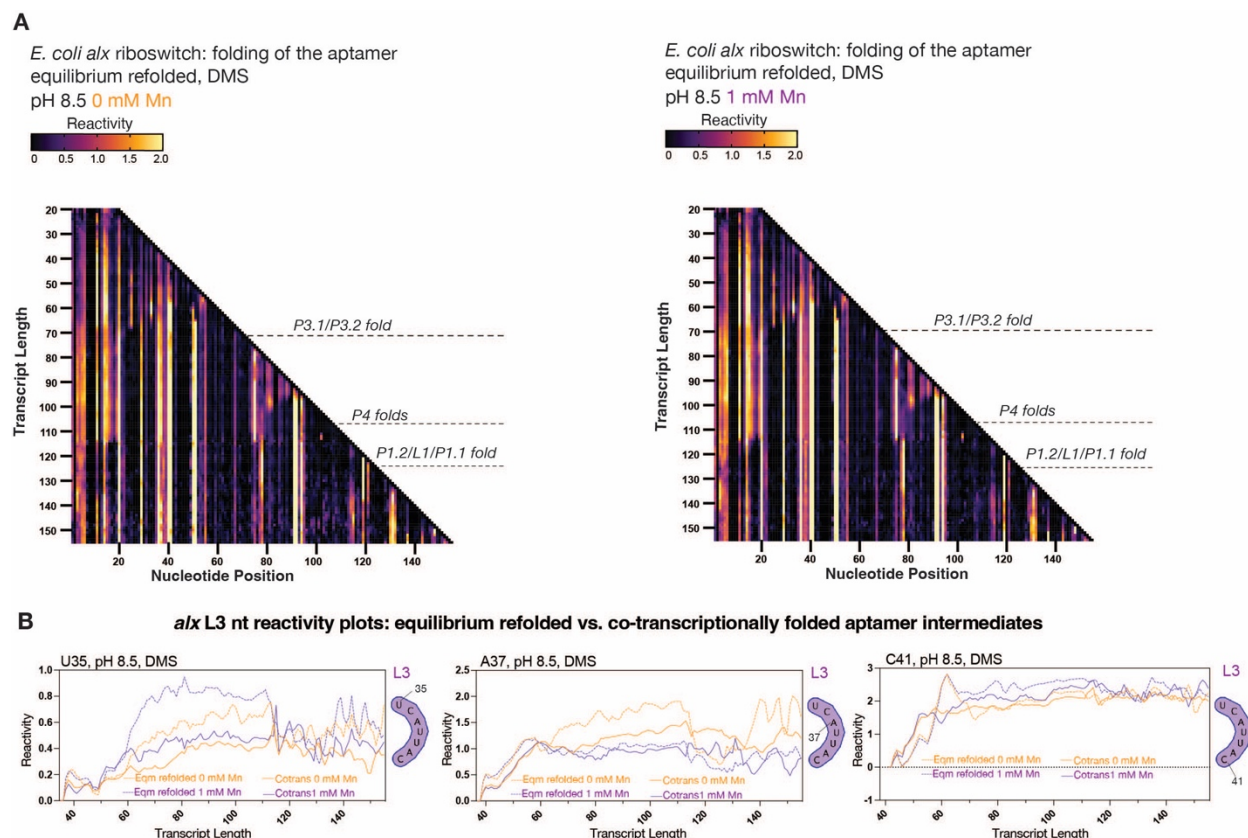
E. coli alx riboswitch: folding of the aptamer
cotranscriptionally probed, DMS

pH 8.5 1 mM Mn



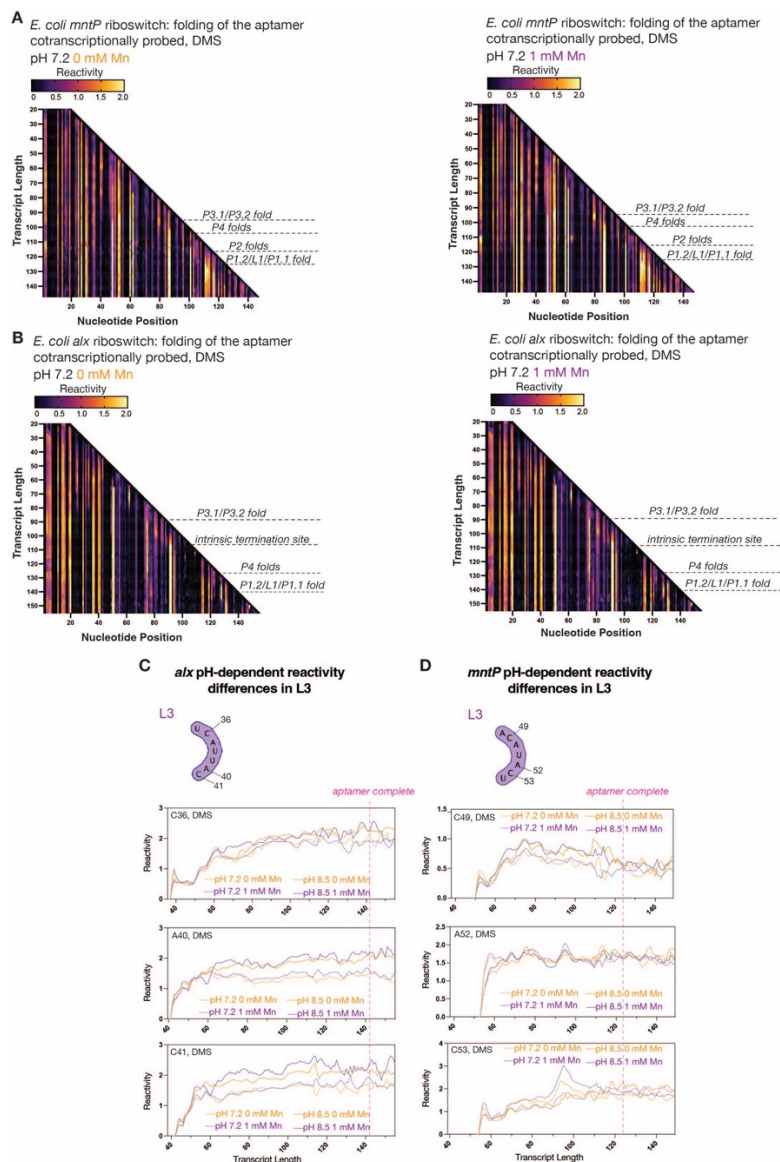
Supplemental Figure 2.7. Co-transcriptional DMS probing of the *alx* riboswitch aptamer at alkaline pH +Mn.

TECprobe-VL reactivity matrix for the DMS-probed *alx* aptamer at pH 8.5 + 1 mM Mn. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from five independent replicates that were concatenated and analyzed together.



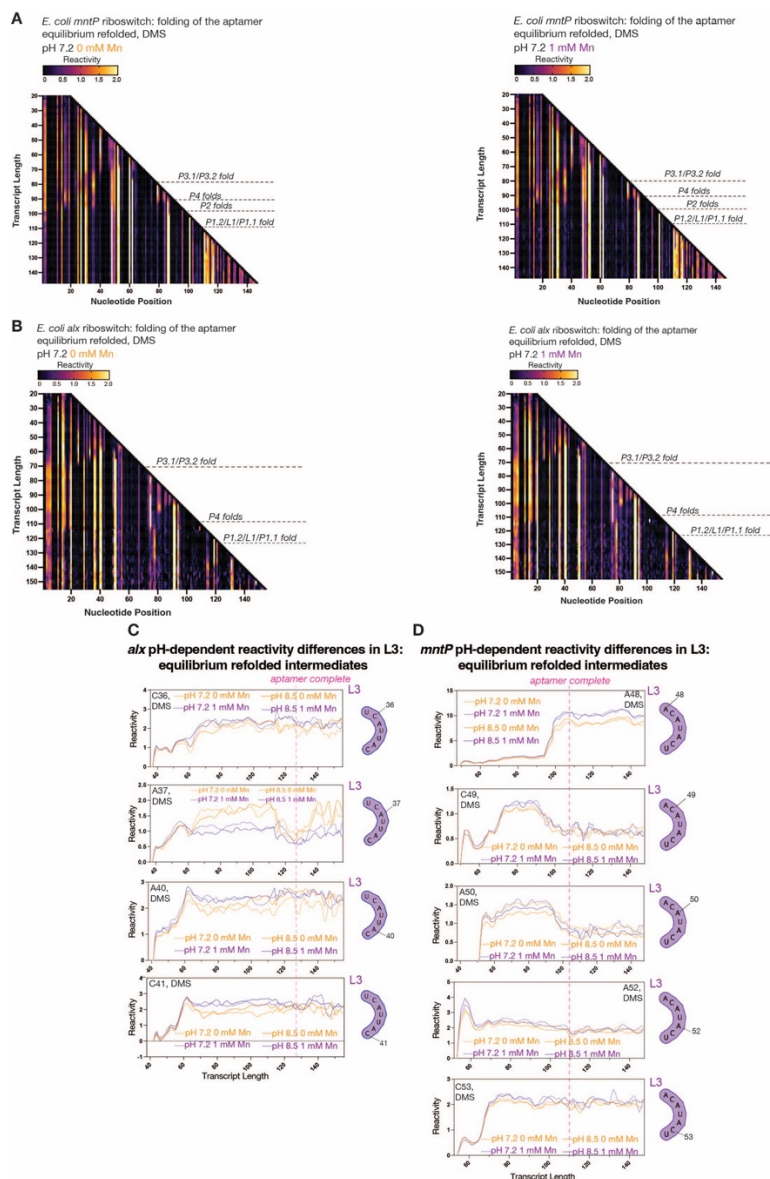
Supplemental Figure 2.8. Equilibrium DMS probing of *alx* aptamer folding intermediates at alkaline pH $\pm$ Mn.

A. TECprobe-VL DMS reactivity matrices for *alx* aptamer folding intermediates, which were purified and equilibrium refolded prior to DMS probing at pH 8.5 $\pm$ 1 mM Mn. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **B.** Plots showing *alx* L3 nt transcript length- and Mn-dependent reactivity changes in equilibrium refolded vs. co-transcriptionally folded aptamer intermediates at pH 8.5 $\pm$ 1 mM Mn. DMS data from 0 mM Mn (orange) and 1 mM Mn (purple) conditions are from Fig. 4A, Fig. 7, and SI Fig. 8A.



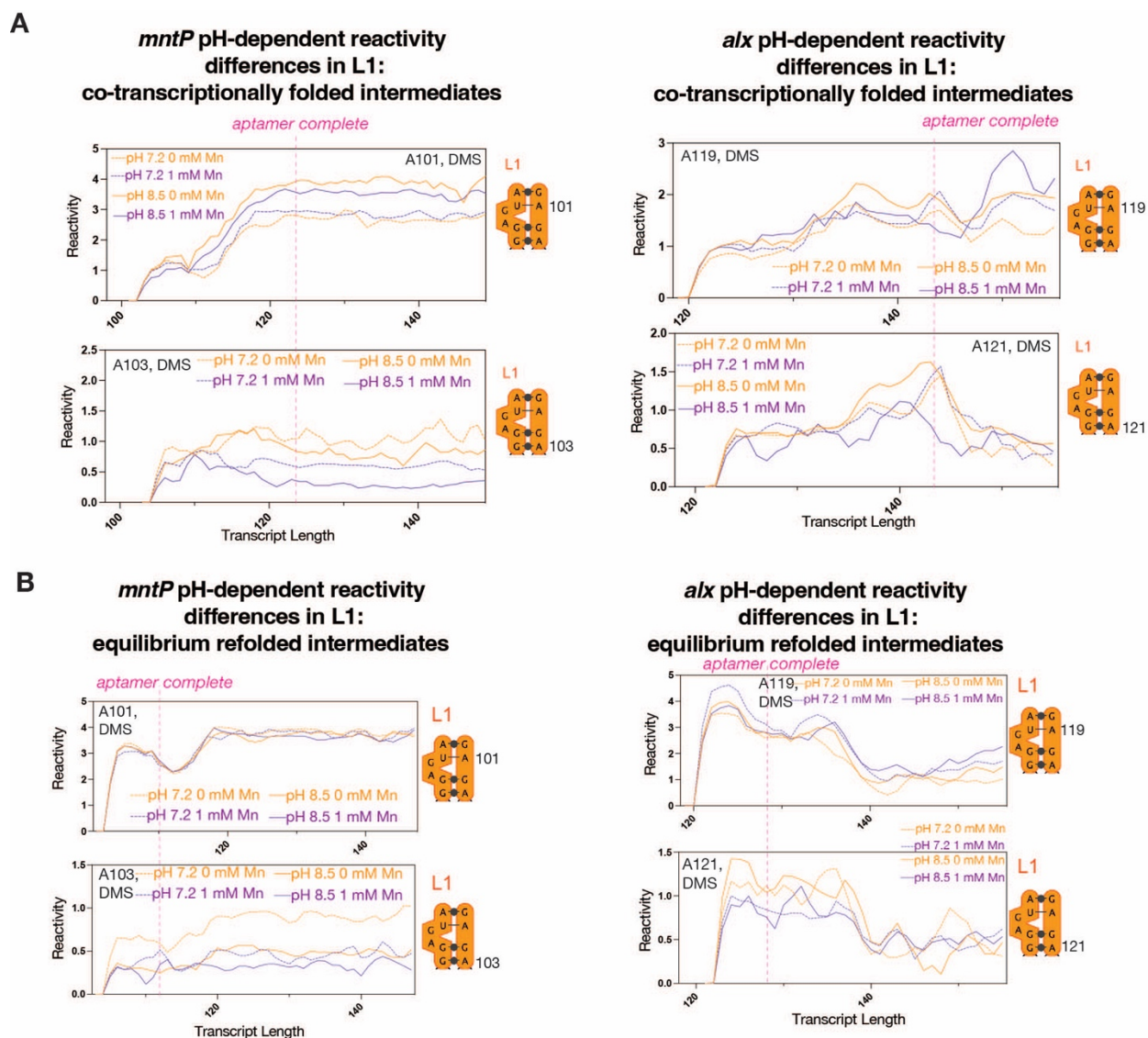
Supplemental Figure 2.9. Co-transcriptional DMS probing of the *alx* and *mntP* aptamers at neutral pH $\pm$ Mn and pH-dependent reactivity changes in L3.

A-B. TECprobe-VL DMS reactivity matrices for the *mntP* (**A**) and *alx* (**B**) aptamers at pH 7.2 $\pm$ 1 mM Mn. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two (*mntP*, **A**) or five (*alx*, **B**) independent replicates that were concatenated and analyzed together. **C-D.** Plots showing *alx* (**C**) and *mntP* (**D**) L3 nt transcript length-, pH-, and Mn-dependent reactivity changes in co-transcriptional aptamer folding intermediates at pH 7.2 or 8.5 $\pm$ 1 mM Mn. DMS data from 0 mM Mn (orange) and 1 mM Mn (purple) are from Fig. 4A, Fig. 7, and SI Fig. 9B for *alx* and Fig. 3A, SI Fig. 3, and SI Fig. 9A for *mntP*. Vertical dotted line marks completion of aptamer folding.



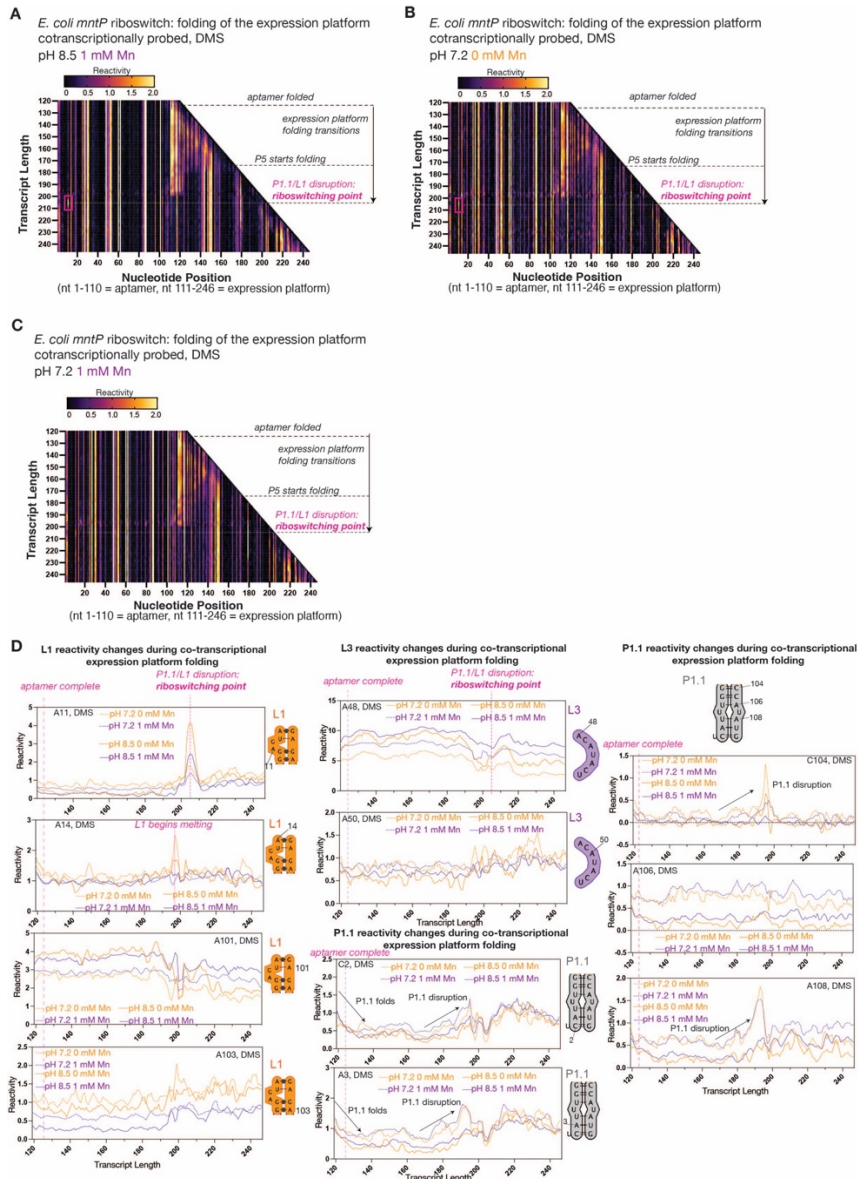
Supplemental Figure 2.10. Equilibrium DMS probing of *alx* and *mntP* aptamer folding intermediates at neutral pH $\pm$ Mn and pH-dependent reactivity changes in L3.

A-B. TECprobe-VL DMS reactivity matrices for the *mntP* (A) and *alx* (B) aptamer intermediates, which were purified and equilibrium refolded at pH 7.2 $\pm$ 1 mM Mn. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **C-D.** Plots showing *alx* (C) and *mntP* (D) L3 transcript length-, pH-, and Mn-dependent reactivity changes in equilibrium refolded aptamer intermediates at pH 7.2 or 8.5 $\pm$ 1 mM Mn. DMS data from 0 mM Mn (orange) and 1 mM Mn (purple) are from SI Fig. 8A and SI Fig. 10B for *alx* and SI Fig. 6A and SI Fig. 10A for *mntP*. Vertical dotted line marks completion of aptamer folding.



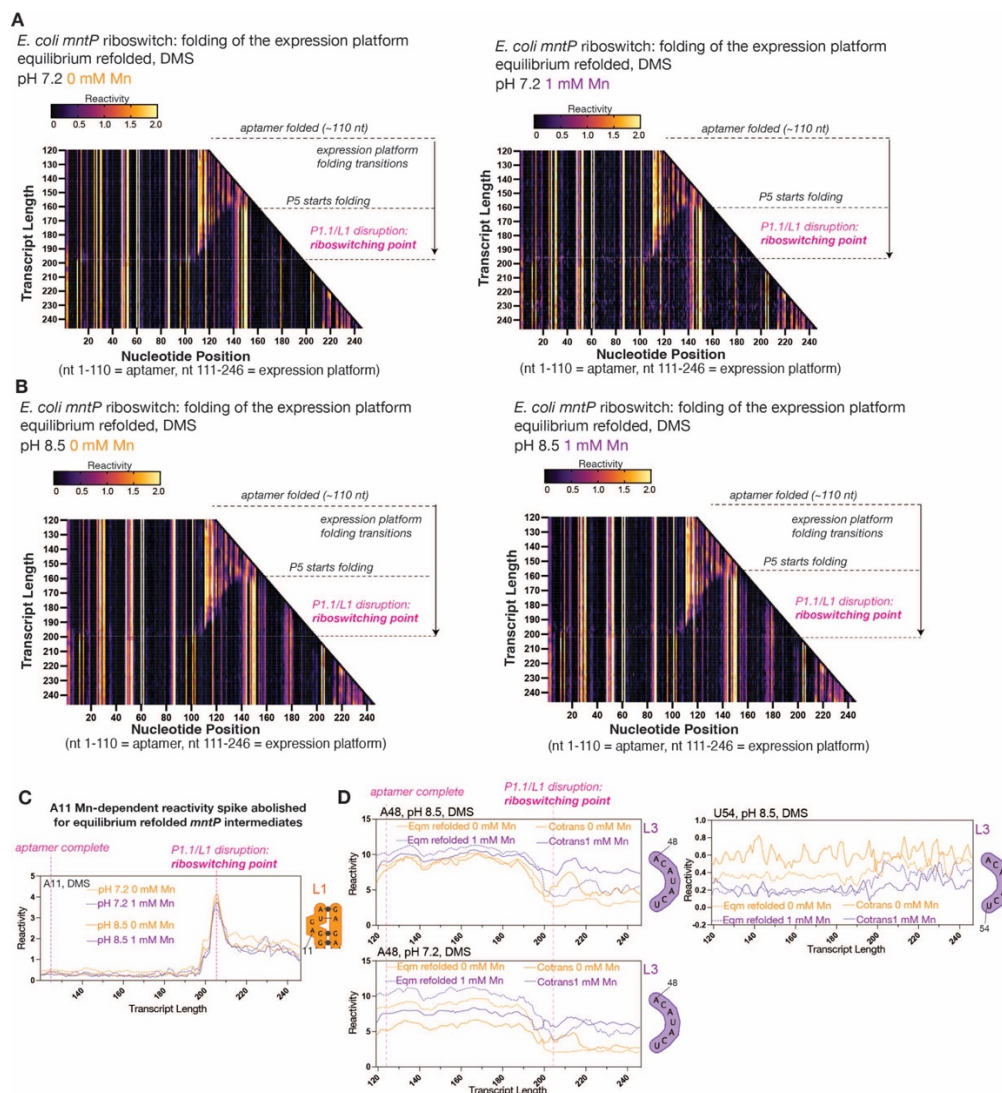
Supplemental Figure 2.11. pH-dependent reactivity changes in *alx* and *mntP* L1 in co-transcriptionally folded and equilibrium refolded aptamer intermediates

A. Plots showing *alx* and *mntP* L1 nt transcript length-, pH-, and Mn-dependent reactivity changes in co-transcriptionally folded aptamer intermediates at pH 7.2 or 8.5 $\pm$ 1 mM Mn. DMS data from 0 mM (orange) and 1 mM Mn (purple) conditions are from Fig. 4A, SI Fig. 7, and SI Fig. 9B for *alx* and Fig. 3A, SI Fig. 3, and SI Fig. 9A for *mntP*. Vertical dotted line marks completion of aptamer folding. **B.** Plots showing *alx* and *mntP* L1 nt transcript length-, pH-, and Mn-dependent reactivity changes in equilibrium refolded aptamer intermediates at pH 7.2 or 8.5 $\pm$ 1 mM Mn. DMS data from 0 mM (orange) and 1 mM Mn (purple) conditions from SI Fig. 8A and SI Fig. 10B for *alx* and SI Fig. 6A and SI Fig. 10A for *mntP*. Vertical dotted line marks completion of aptamer folding.



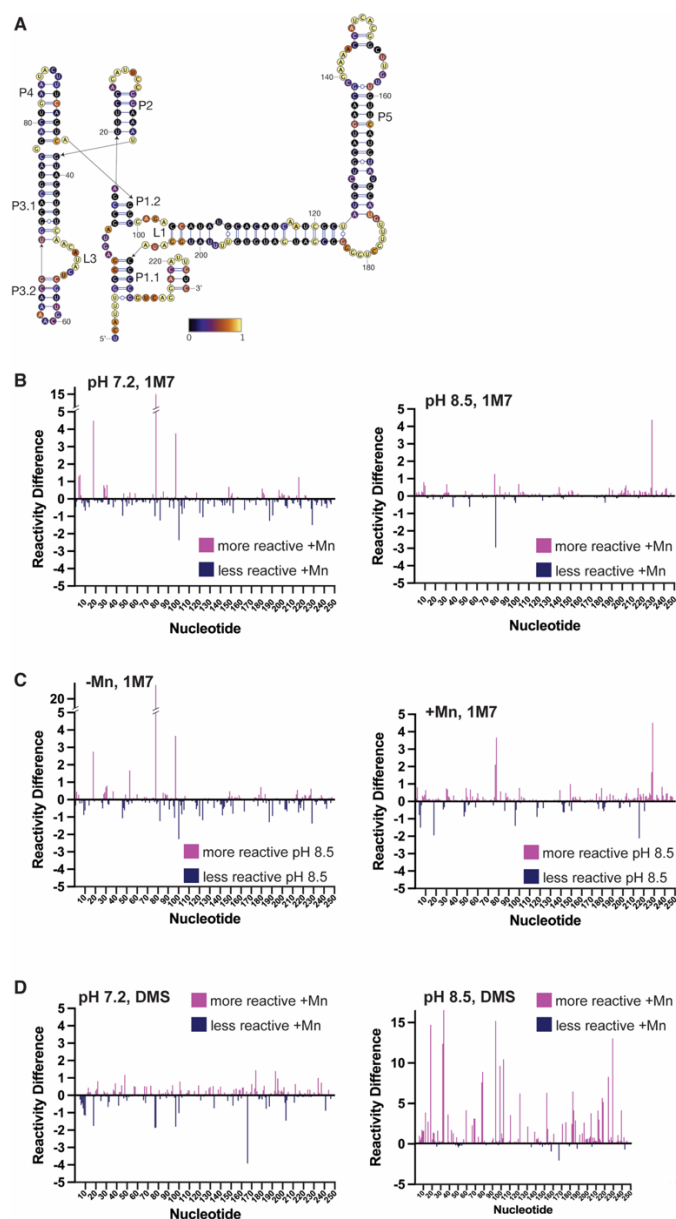
Supplemental Figure 2.12. Co-transcriptional DMS probing of *mntP* riboswitch during expression platform synthesis at neutral and alkaline pH.

A-C. TECprobe-VL DMS reactivity matrices for *mntP* folding intermediates at pH 8.5 +1 mM Mn (**A**), pH 7.2 (**B**), and pH 7.2 + 1 mM Mn (**C**). Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **D.** Plots showing *mntP* L1, L3, and P1.1 nt transcript length-, pH-, and Mn-dependent reactivity changes in co-transcriptionally folded intermediates at pH 7.2 or 8.5 $\pm$ 1 mM Mn. DMS data from 0 mM Mn (orange) and 1 mM Mn (purple) conditions from Fig. 6A-B, and SI Fig. 12A-C. Vertical pink dotted lines mark key co-transcriptional RNA folding transitions.



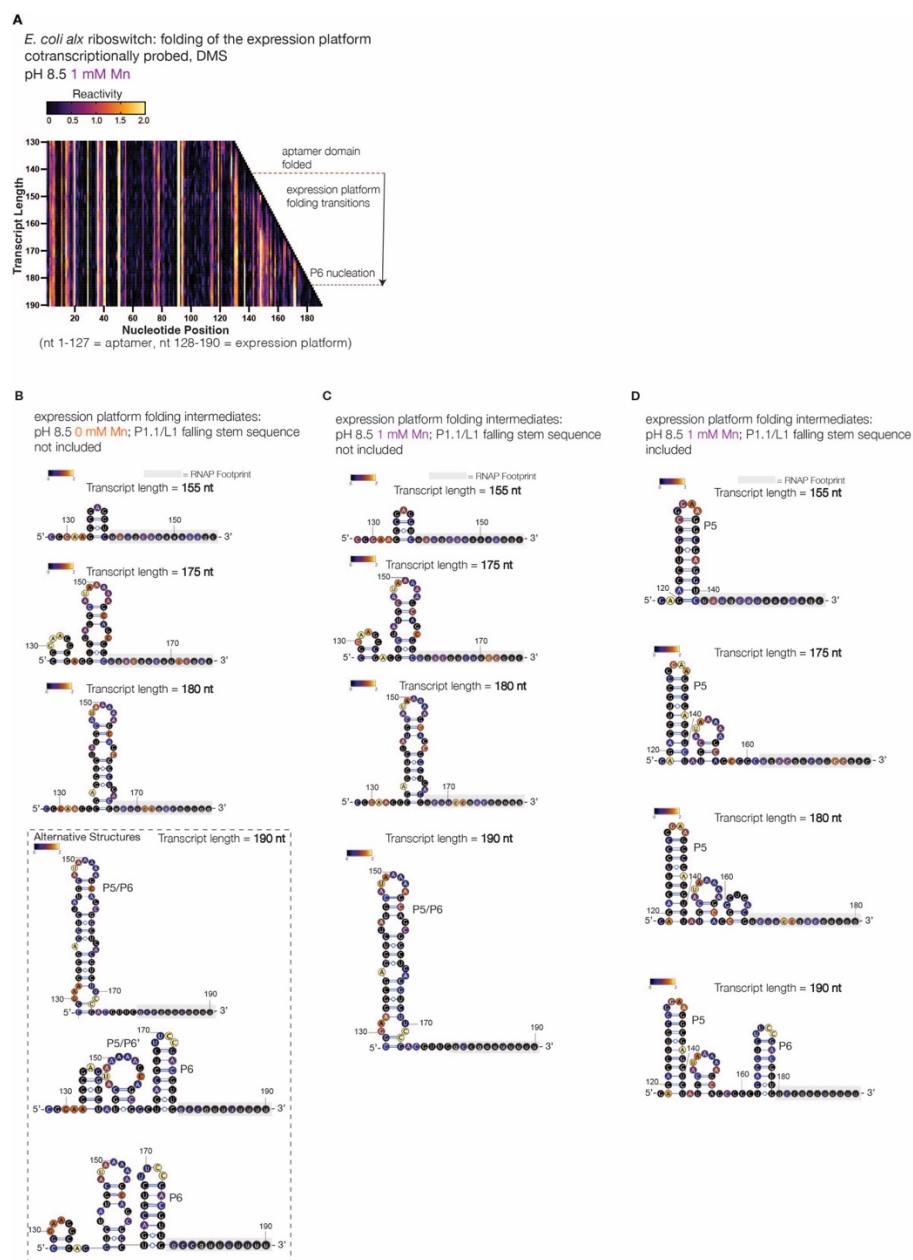
Supplemental Figure 2.13. Equilibrium DMS probing on *mntP* folding intermediates during expression platform synthesis.

A-C. TECprobe-VL DMS reactivity matrices for *mntP* folding intermediates, which were purified and equilibrium refolded at pH 7.2 ± 1 mM Mn (**A**) or pH 8.5 ± 1 mM Mn (**B**). Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **C.** Plot showing *mntP* A11 transcript length-, pH-, and Mn-dependent reactivity changes in equilibrium refolded intermediates at pH 7.2 or 8.5 ± 1 mM Mn. Data for 0 mM Mn (orange) and 1 mM Mn (purple) from SI Figs. 13A-B. Vertical dotted lines mark indicated RNA structural transitions. **D.** Plots showing *mntP* L3 nt transcript length- and Mn-dependent reactivity changes in co-transcriptionally folded vs. equilibrium refolded intermediates, which were DMS-probed at the designated pH value. DMS data for 0 mM Mn (orange) and 1 mM Mn (purple) conditions from Fig. 6A-B, SI Fig. 12A-C, and SI Figs. 13A-B. Vertical dotted lines mark indicated co-transcriptional RNA folding transitions.



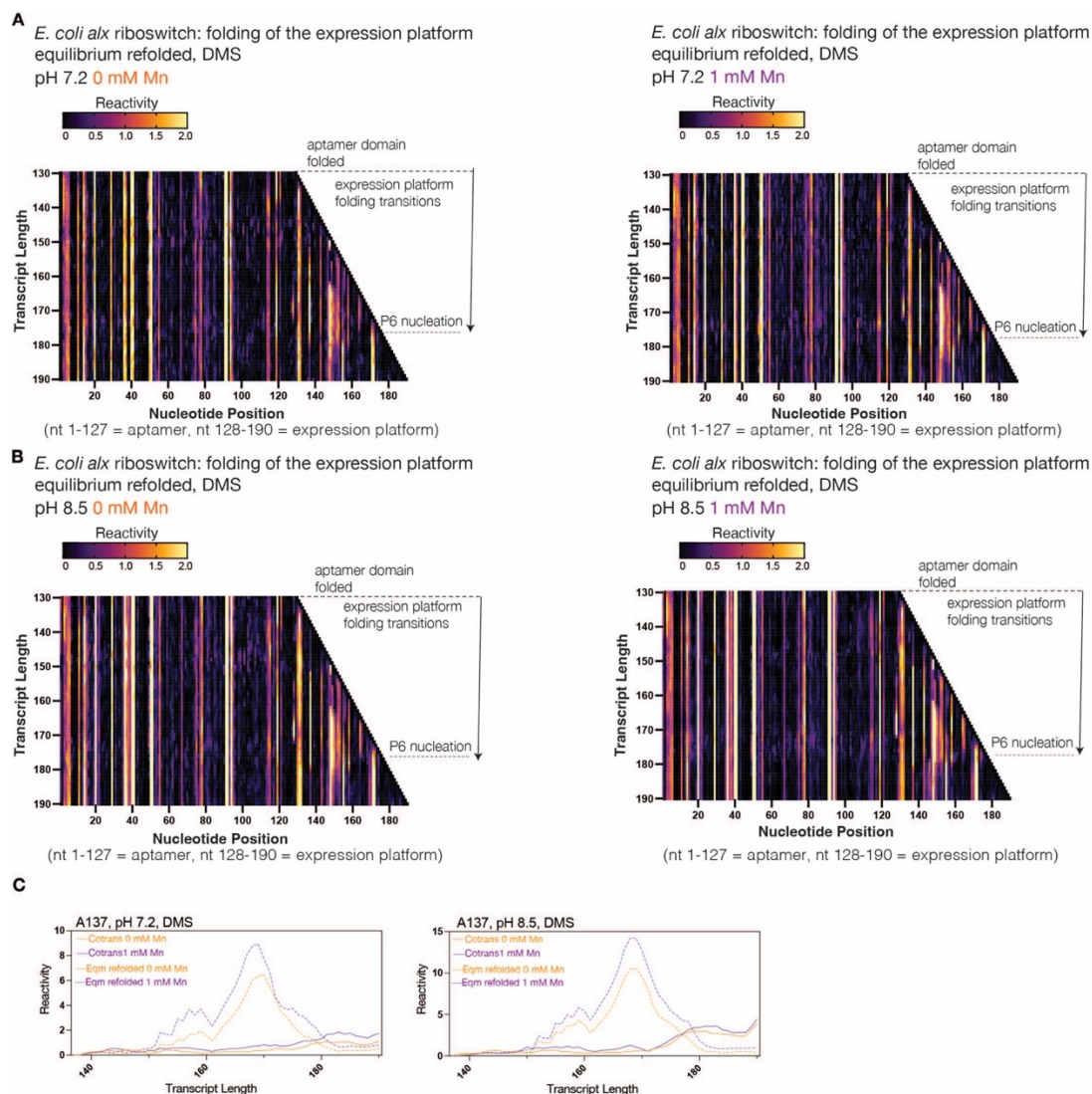
Supplemental Figure 2.14. Structure probing of equilibrium-refolded *mntP* full-length riboswitch RNA.

A. Secondary structure of equilibrium-refolded *mntP* full-length RNA modeled based on reactivity with 1M7 at pH 7.2 -Mn. **B.** Individual nucleotide reactivity differences when modified with 1M7 to compare +Mn vs. -Mn, measured at both pH 7.2 and 8.5. **C.** Individual nucleotide reactivity differences at neutral pH (7.2) vs. alkaline pH (8.5), measured both in the presence and absence of Mn. **D.** Individual nucleotide reactivity differences when modified with DMS to compare +Mn vs. -Mn, measured at both pH 7.2 and 8.5.



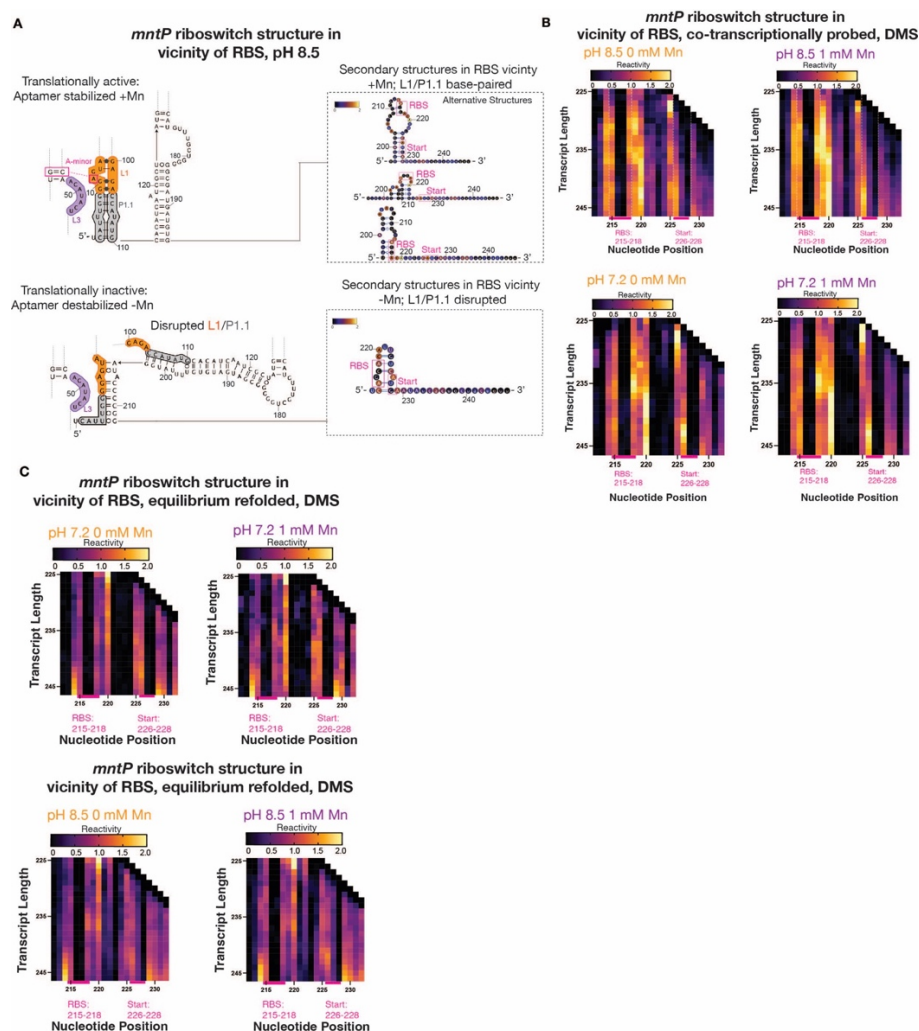
Supplemental Figure 2.15. Co-transcriptional DMS probing of the *alx* riboswitch during expression platform synthesis at alkaline pH.

A. TECprobe-VL DMS reactivity matrix for *alx* folding intermediates at pH 8.5 +1 mM Mn. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from five independent replicates that were concatenated and analyzed together. **B-D.** Secondary structure models for *alx* expression platform folding intermediates, which were co-transcriptionally folded prior to DMS probing. The secondary structure models are colored by DMS reactivity at pH 8.5 at the designated Mn concentration. To simplify the structure modeling, we executed the RNAstructure “Fold” command constrained by DMS reactivities for the expression platform sequence only, with the L1/P1.1 falling stem either included or excluded.



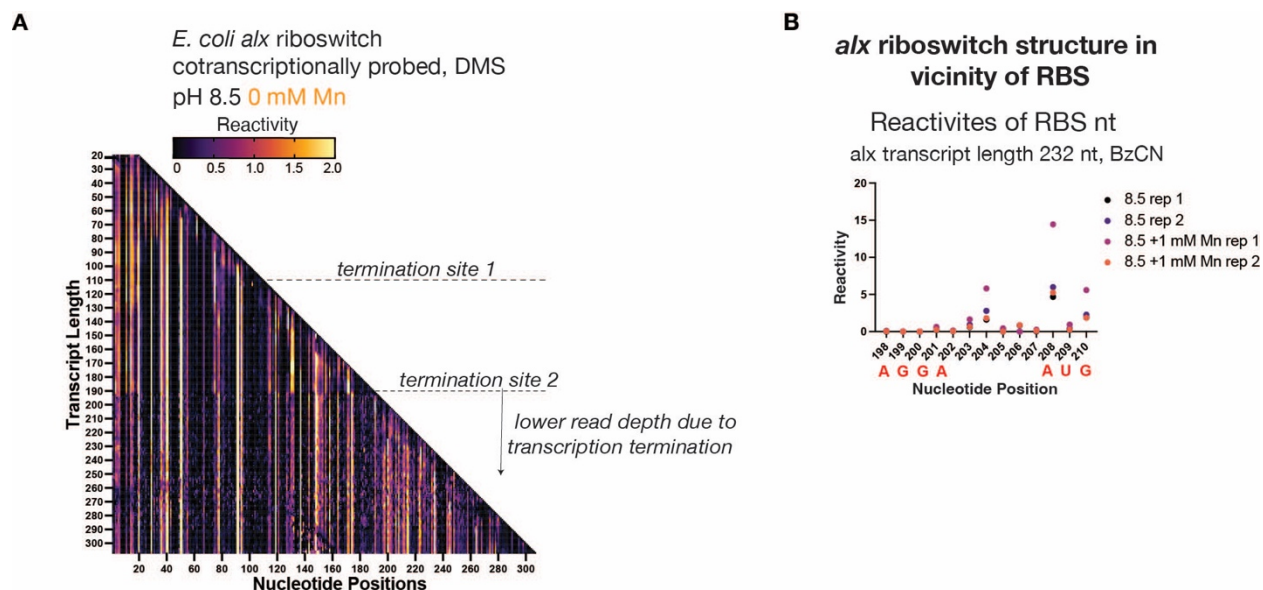
Supplemental Figure 2.17. Equilibrium DMS probing of the *alx* riboswitch during expression platform synthesis at neutral vs. alkaline pH and Mn-dependent reactivity differences in the expression platform.

A-B. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates, which were purified and equilibrium refolded at pH 7.2 ± 1 mM Mn (**A**) or pH 8.5 ± 1 mM Mn (**B**). Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **C.** Plots showing comparison of A137 transcript length- and Mn-dependent reactivity changes in co-transcriptionally folded vs. equilibrium refolded intermediates at the designated pH ± 1 mM Mn. DMS data for 0 mM Mn (orange) and 1 mM Mn (purple) from Fig. 7A, SI Fig. 15A, SI Fig. 16A, and SI Fig. 17A-B.



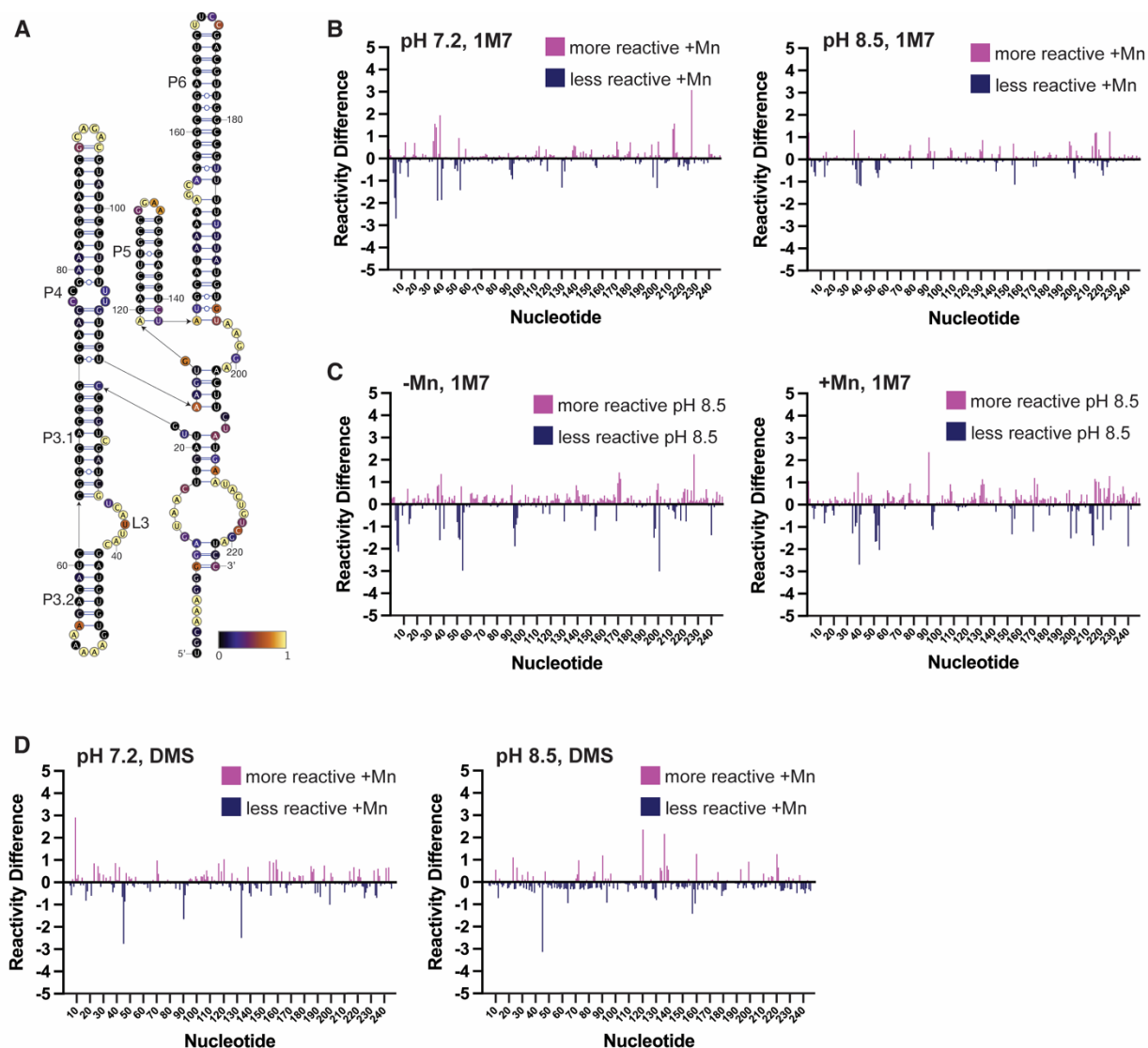
Supplemental Figure 2.18. DMS reactivities of *mntP* RBS nt in co-transcriptionally folded and equilibrium refolded intermediates.

A. (Top panel) Truncated secondary structure prediction of full-length, Mn-bound *mntP* riboswitch, with L1 and P1.1 stabilized. Adjacent are secondary structure models for *mntP* expression platform structure at transcript length 246 nt, which are colored by DMS reactivity at pH 8.5 +Mn. (Bottom panel) Truncated secondary structure prediction of full-length, Mn-unbound *mntP* riboswitch, with L1 and P1.1 disrupted. Adjacent is a secondary structure model for *mntP* expression platform structure at transcript length 246 nt, which is colored by DMS reactivity at pH 8.5 -Mn. **B.** TECprobe-VL DMS reactivity matrix for co-transcriptionally folded *mntP* intermediates at pH 7.2 and 8.5 $\pm$ 1 mM Mn., windowing over transcript lengths containing the RBS and start codon. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together. **C.** TECprobe-VL DMS reactivity matrix for equilibrium refolded *mntP* intermediates at pH 7.2 and 8.5 $\pm$ 1 mM Mn., windowing over transcripts lengths containing the RBS and start codon nt. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from two independent replicates that were concatenated and analyzed together.



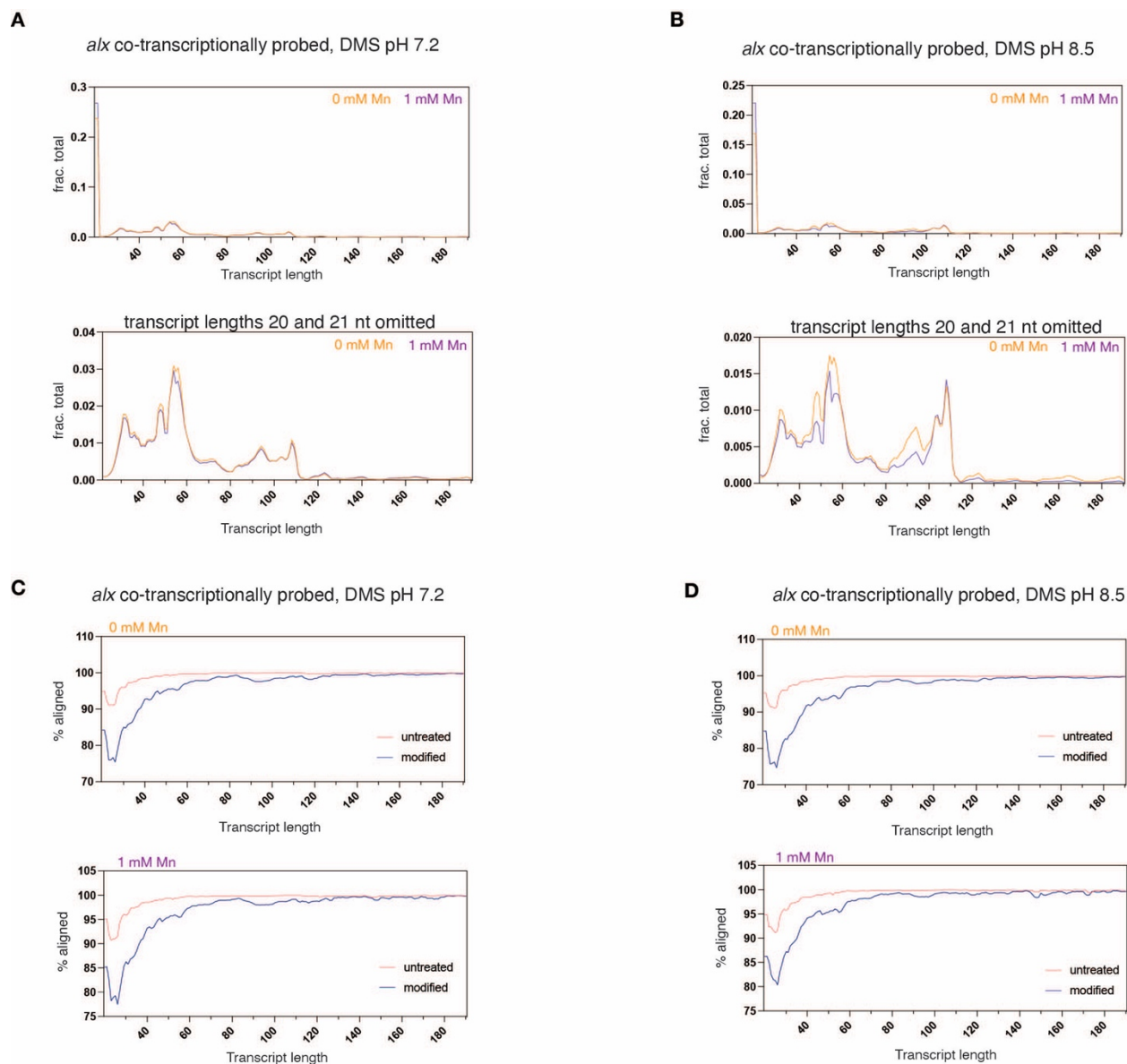
Supplemental Figure 2.19. Dropoff in sequencing depth following second *alx* intrinsic termination site and co-transcriptional SHAPE probing of full-length *alx* to capture RBS reactivities

A. TECprobe-VL DMS reactivity matrix for *alx* co-transcriptionally folded intermediates at pH 8.5. Intrinsic transcription termination sites are annotated adjacent to the heatmap, showing a clear drop in reactivity data quality following the second intrinsic termination site. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. Data are from seven independent replicates that were concatenated and analyzed together. **B.** Plot showing BzCN reactivities of *alx* RBS and start codon nt in the full-length, co-transcriptionally folded riboswitch at pH 8.5 $\pm$ 1 mM Mn, corresponding to transcript length 232 nt. Two independent replicates were performed for the $\pm$ Mn conditions.



Supplemental Figure 2.20. Structure probing of equilibrium-refolded *alx* full-length riboswitch RNA.

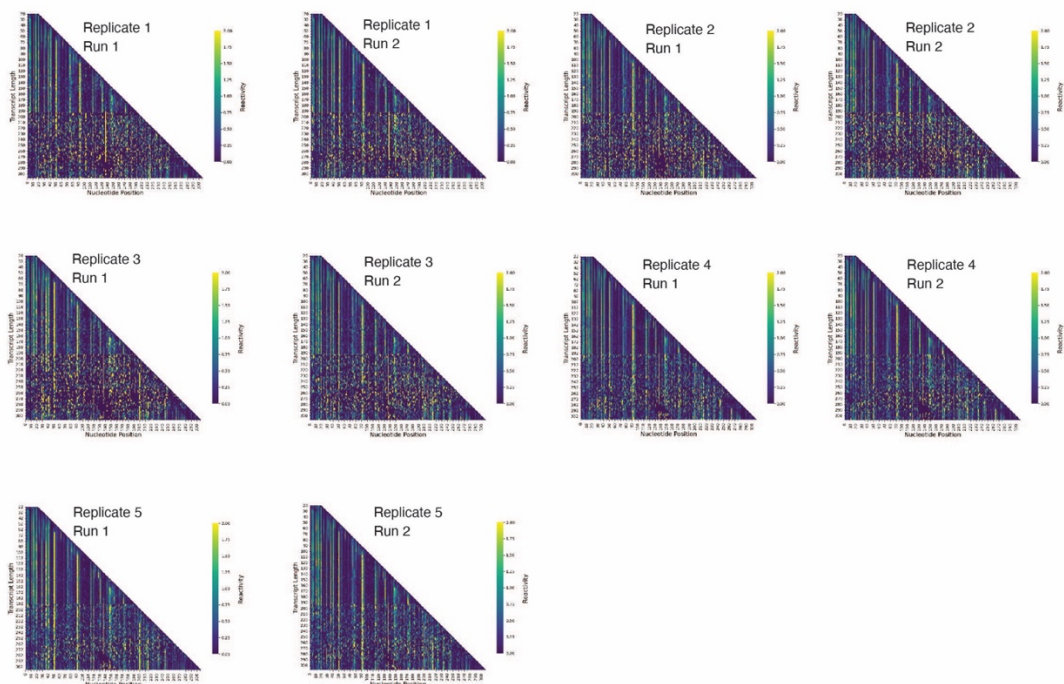
A. Secondary structure of equilibrium-refolded *alx* full-length RNA modeled based on reactivity with 1M7 at pH 7.2 -Mn. **B.** Individual nucleotide reactivity differences when modified with 1M7 to compare +Mn vs. -Mn, measured at both pH 7.2 and 8.5. **C.** Individual nucleotide reactivity differences at neutral pH (7.2) vs. alkaline pH (8.5), measured both in the presence and absence of Mn. **D.** Individual nucleotide reactivity differences when modified with DMS to compare +Mn vs. -Mn, measured at both pH 7.2 and 8.5.



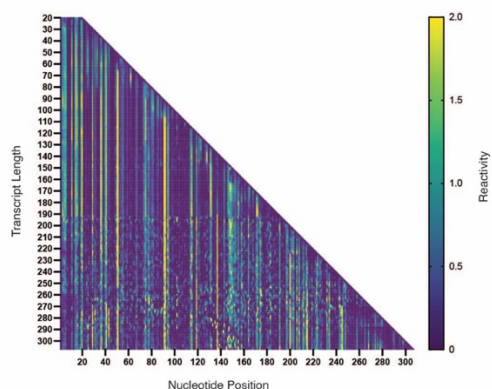
Supplemental Figure 2.21. Aligned reads and alignment rates for co-transcriptional DMS probing of the *alx* riboswitch.

A-B. (Top panel) Plot showing fraction of aligned reads mapped to each transcript length. (Bottom panel) Plot showing fraction of aligned reads mapped to each transcript length, with transcript lengths 20 and 21 nt omitted to better visualize the fraction of aligned reads for transcript lengths 22-190 nt. **C-D.** Percentage of reads retained after splitting those that aligned to each intermediate transcript length.

A *alx* co-transcriptionally probed, DMS pH 7.2 individual replicate heat maps



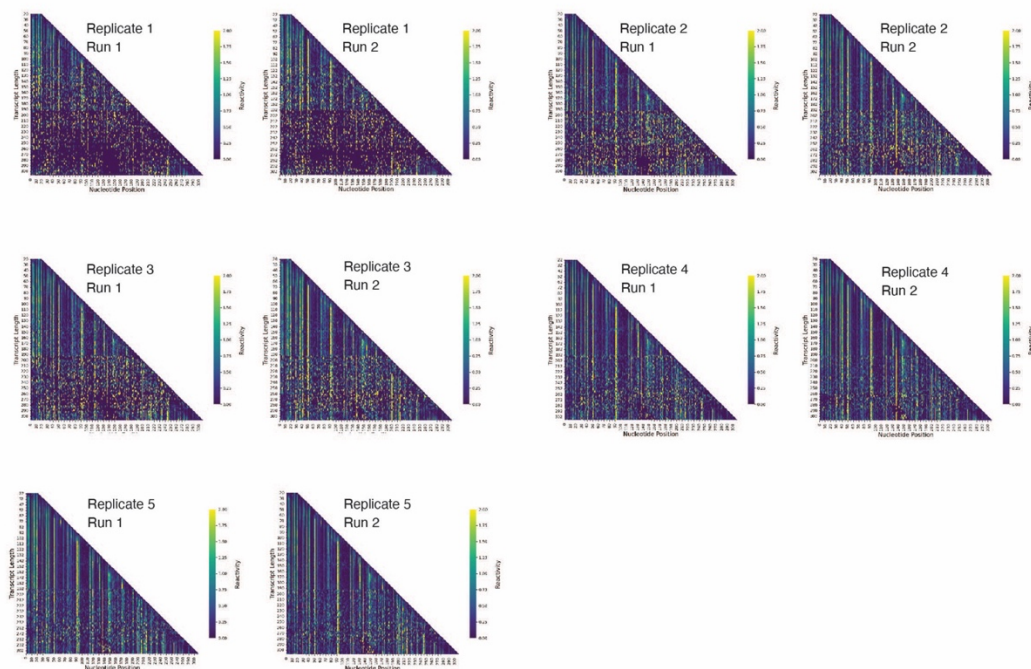
B *alx* co-transcriptionally probed, DMS pH 7.2 full concatenated heat map



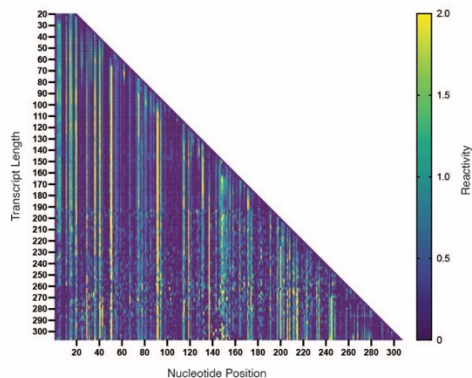
Supplemental Figure 2.22. Reactivity matrices for individual and concatenated replicates for co-transcriptional DMS probing of the *alx* riboswitch at pH 7.2.

A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from five independent replicates at pH 7.2. “Run 1” and “Run 2” denote sequencing runs on independent PCR amplifications of the same cDNA library. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* co-transcriptionally folded intermediates at pH 7.2. Reads from the five independent replicates shown in panel A, which were concatenated and analyzed together.

A *alx* co-transcriptionally probed, DMS pH 7.2 1mM Mn individual heat maps



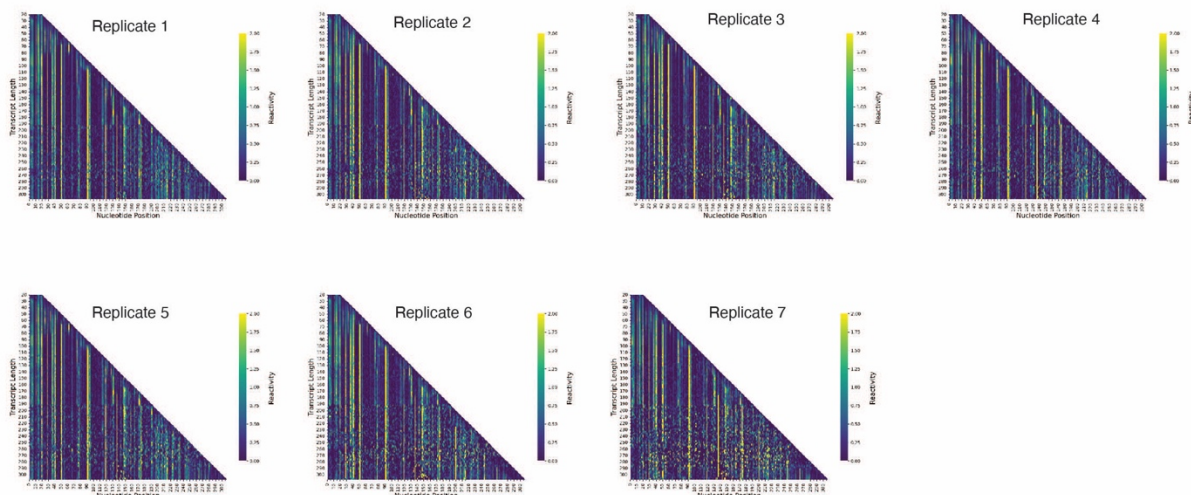
B *alx* co-transcriptionally probed, DMS pH 7.2 1 mM Mn full concatenated heat map



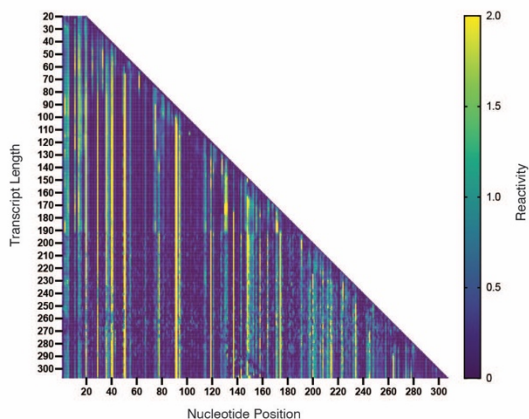
Supplemental Figure 2.23. Reactivity matrices for individual and concatenated replicates for co-transcriptional DMS probing of the *alx* riboswitch at pH 7.2 +Mn.

A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from five independent replicates at pH 7.2 + 1 mM Mn. “Run 1” and “Run 2” denote sequencing runs on independent PCR amplifications of the same cDNA library. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* co-transcriptionally folded intermediates at pH 7.2 + 1 mM Mn. Reads from the five independent replicates shown in panel A, which were concatenated and analyzed together.

A *alx* co-transcriptionally probed, DMS pH 8.5 individual heat maps



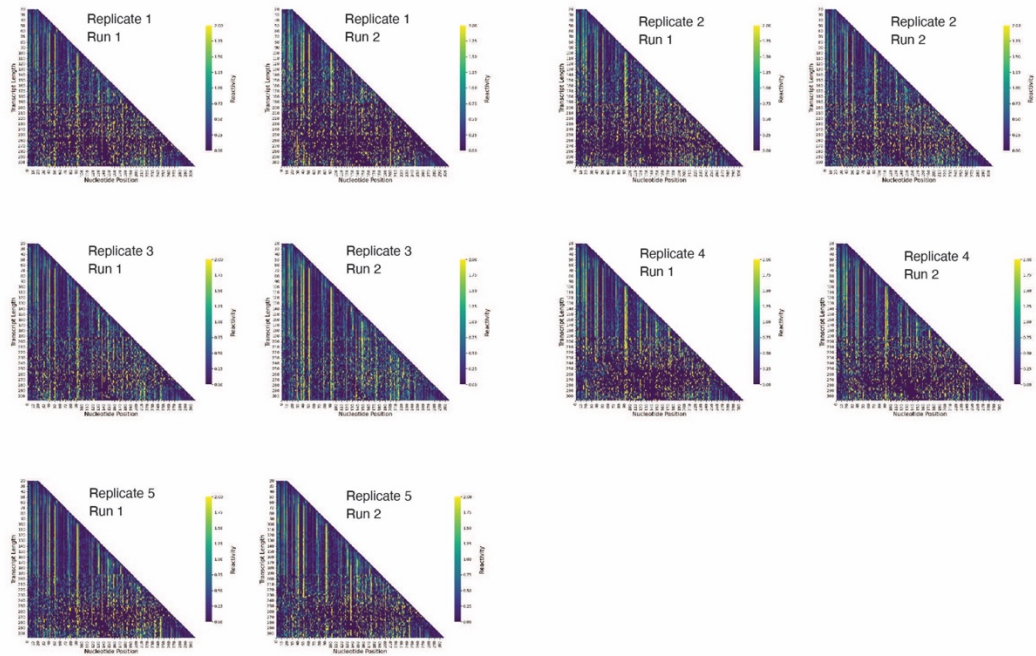
B *alx* co-transcriptionally probed, DMS pH 8.5 full concatenated heat map



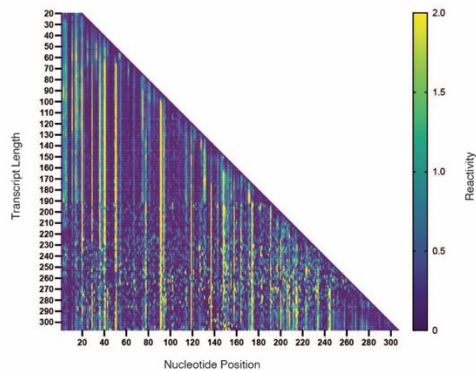
Supplemental Figure 2.24. Reactivity matrices for individual and concatenated replicates for co-transcriptional DMS probing of the *alx* riboswitch at pH 8.5.

A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from seven independent replicates at pH 8.5. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* co-transcriptionally folded intermediates at pH 8.5. Reads from the seven independent replicates shown in panel A, which were concatenated and analyzed together.

A *alx* co-transcriptionally probed, DMS pH 8.5 1 mM Mn individual heat maps

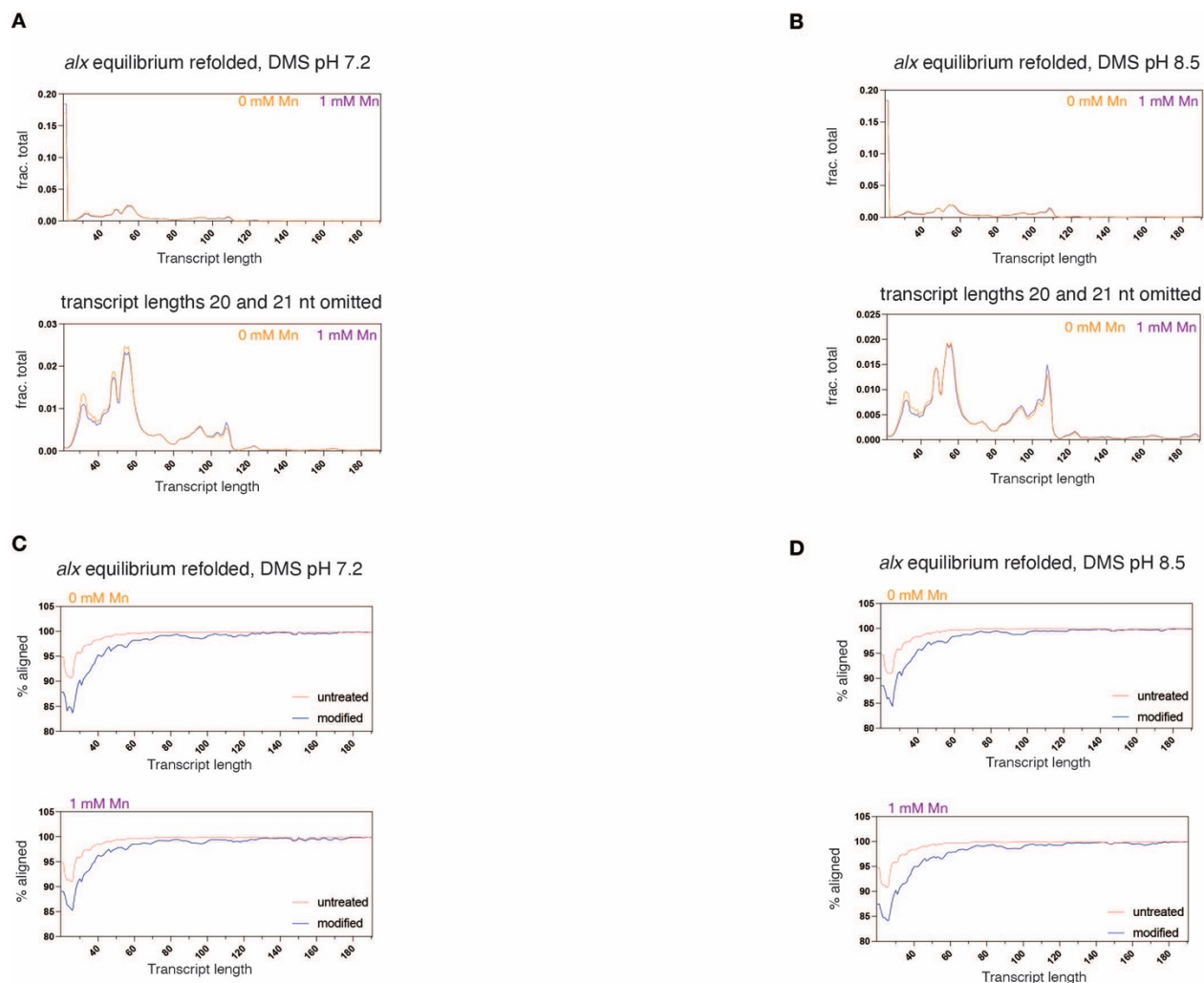


B *alx* co-transcriptionally probed, DMS pH 8.5 1 mM Mn full concatenated heat map



Supplemental Figure 2.25. Reactivity matrices for individual and concatenated replicates for co-transcriptional DMS probing of the *alx* riboswitch at pH 8.5 +Mn.

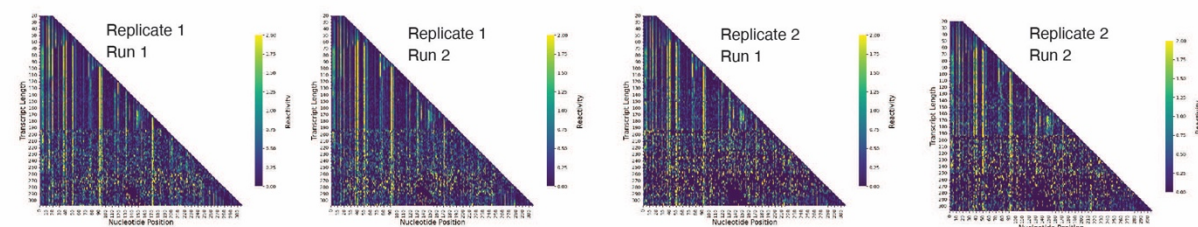
A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from five independent replicates at pH 8.5 + 1 mM Mn. “Run 1” and “Run 2” denote sequencing runs on independent PCR amplifications of the same cDNA library. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* co-transcriptionally folded intermediates at pH 8.5 + 1 mM Mn. Reads from the five independent replicates shown in panel A, which were concatenated and analyzed together.



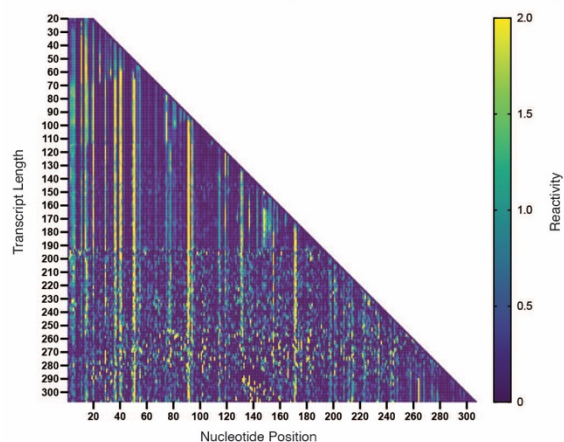
Supplemental Figure 2.26. Aligned reads and alignment rates for DMS probing of *alx* equilibrium refolded intermediates.

A-B. (Top panel) Plot showing fraction of aligned reads mapped to each transcript length. (Bottom panel) Plot showing fraction of aligned reads mapped to each transcript length, with transcript lengths 20 and 21 nt omitted to better visualize the fraction of aligned reads for transcript lengths 22-190 nt. **C-D.** Percentage of reads retained after splitting those that aligned to each intermediate transcript length.

A *alx* equilibrium refolded, DMS pH 7.2 individual heat maps



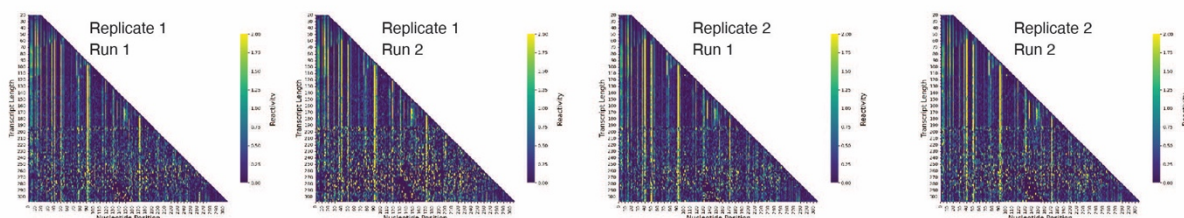
B *alx* equilibrium refolded, DMS pH 7.2 full concatenated heat map



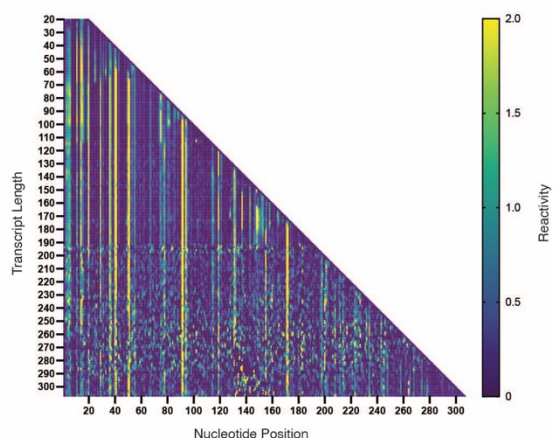
Supplemental Figure 2.27. Reactivity matrices for individual and concatenated replicates for equilibrium DMS probing of the *alx* riboswitch at pH 7.2.

A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from two independent replicates at pH 7.2. “Run 1” and “Run 2” denote sequencing runs on independent PCR amplifications of the same cDNA library. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* equilibrium refolded intermediates at pH 7.2. Reads from the two independent replicates shown in panel A, which were concatenated and analyzed together.

A *alx* equilibrium refolded, DMS pH 7.2 1mM Mn individual heat maps



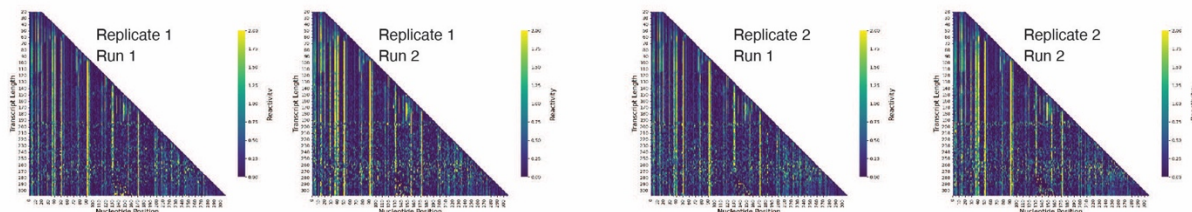
B *alx* equilibrium refolded, DMS pH 7.2 1mM Mn full concatenated heat map



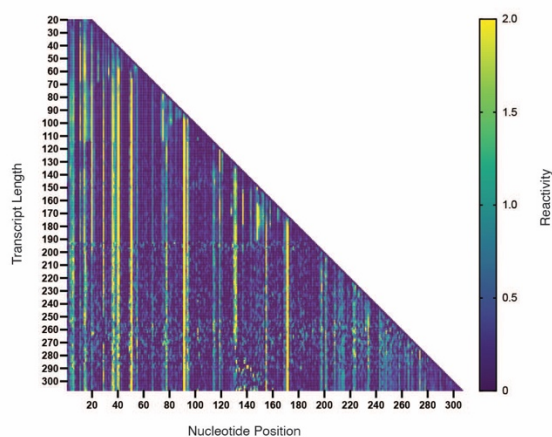
Supplemental Figure 2.28. Reactivity matrices for individual and concatenated replicates for equilibrium DMS probing of the *alx* riboswitch at pH 7.2 +Mn.

A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from two independent replicates at pH 7.2 + 1 mM Mn. “Run 1” and “Run 2” denote sequencing runs on independent PCR amplifications of the same cDNA library. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* equilibrium refolded intermediates at pH 7.2 + 1 mM Mn. Reads from the two independent replicates shown in panel A, which were concatenated and analyzed together.

A
alx equilibrium refolded, DMS pH 8.5 individual heat maps



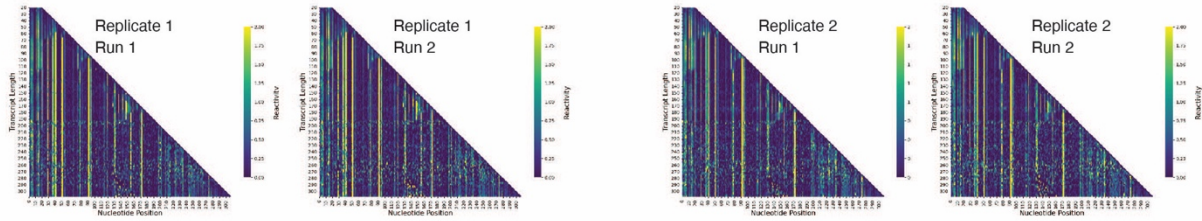
B
alx equilibrium refolded, DMS pH 8.5 full concatenated heat map



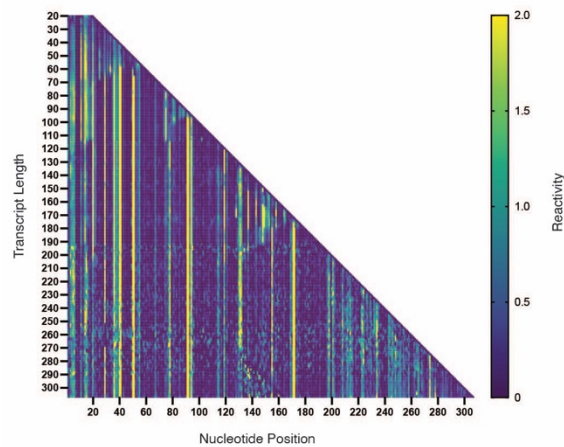
Supplemental Figure 2.29. Reactivity matrices for individual and concatenated replicates for equilibrium DMS probing of the *alx* riboswitch at pH 8.5.

A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from two independent replicates at pH 8.5. “Run 1” and “Run 2” denote sequencing runs on independent PCR amplifications of the same cDNA library. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* equilibrium refolded intermediates at pH 8.5. Reads from the two independent replicates shown in panel A, which were concatenated and analyzed together.

A *alx* equilibrium refolded, DMS pH 8.5 1 mM Mn individual heat maps

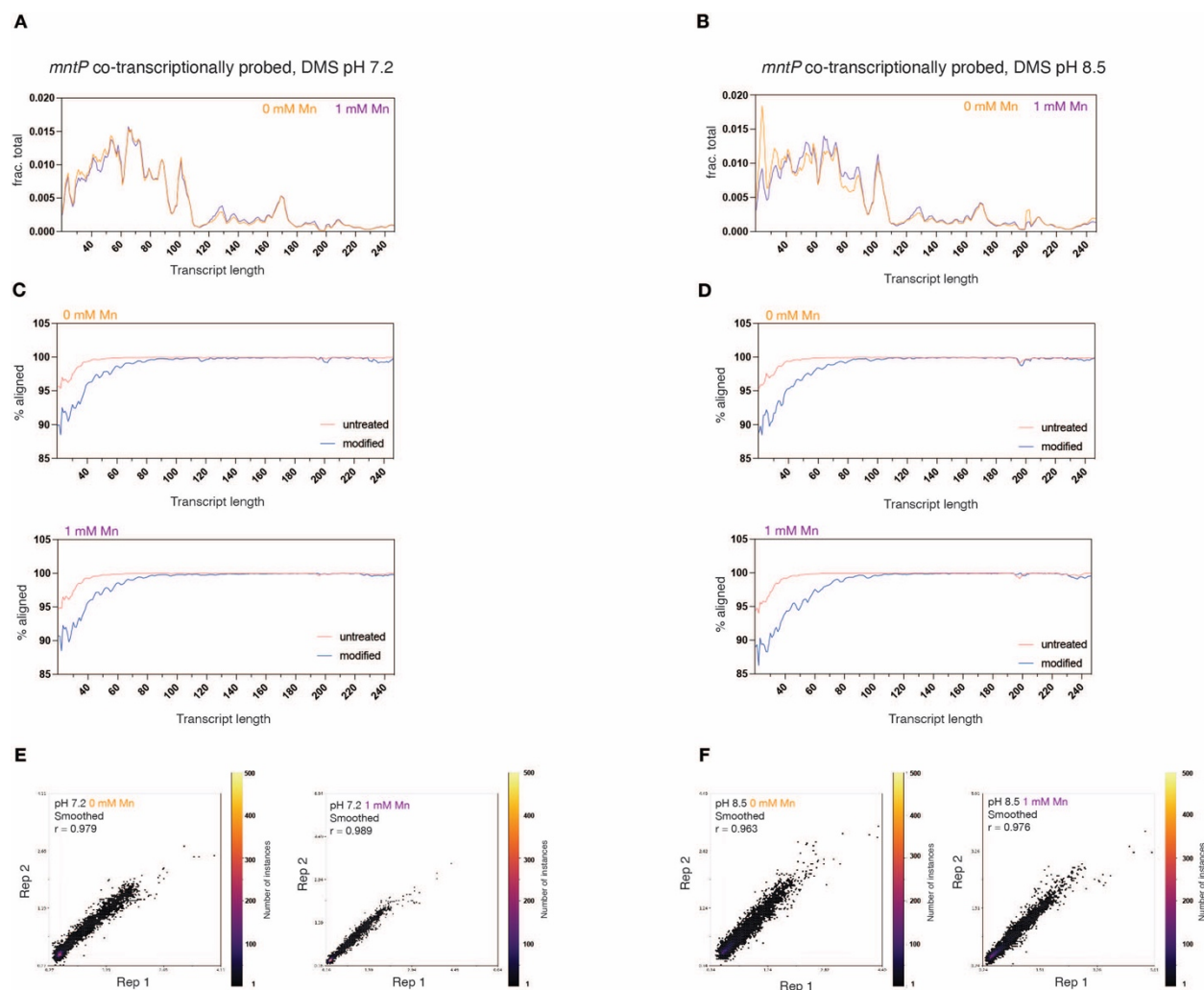


B *alx* equilibrium refolded, DMS pH 8.5 1 mM Mn full concatenated heat map



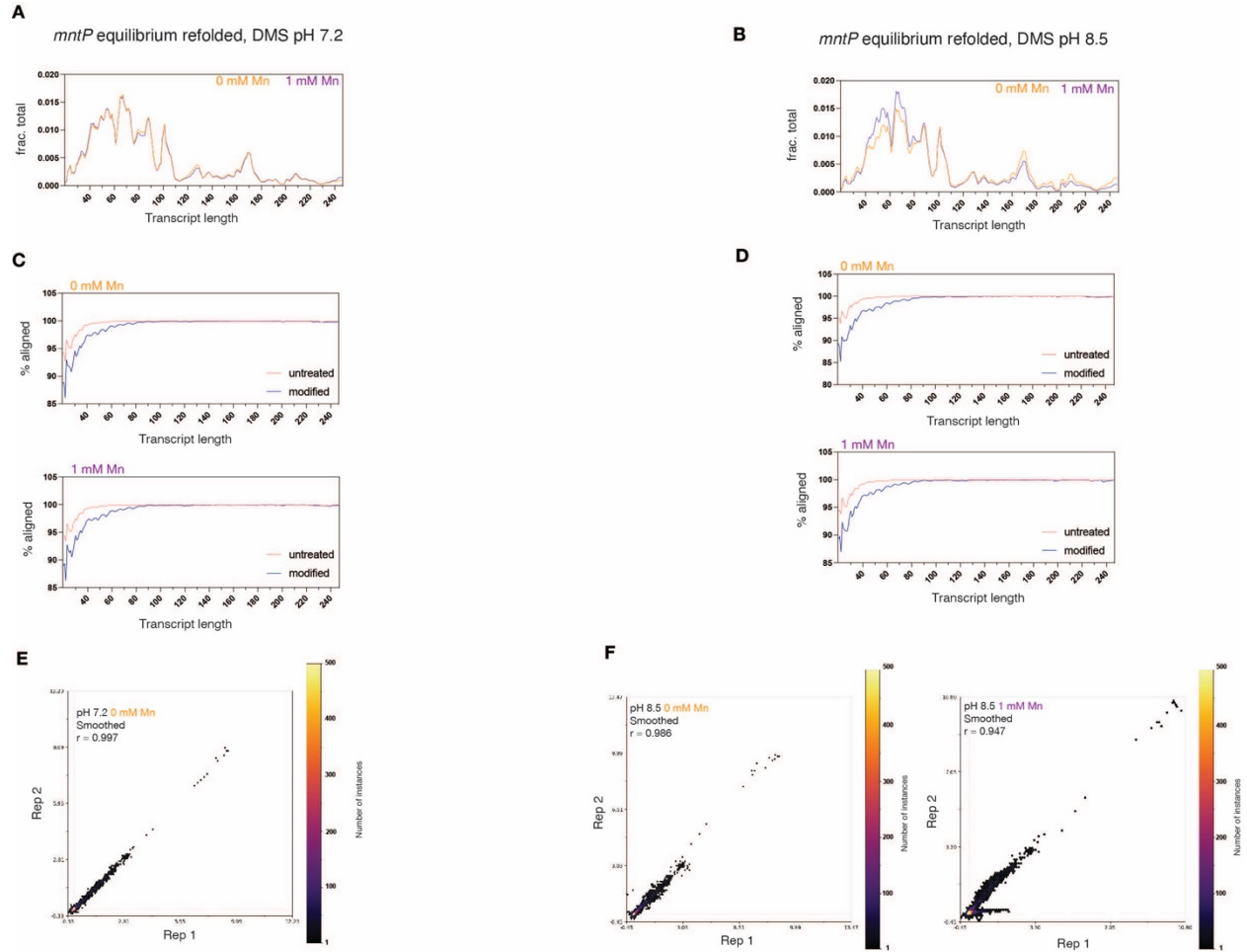
Supplemental Figure 2.30. Reactivity matrices for individual and concatenated replicates for equilibrium DMS probing of the *alx* riboswitch at pH 8.5 +Mn.

A. TECprobe-VL DMS reactivity matrices for *alx* folding intermediates from two independent replicates at pH 8.5 + 1 mM Mn. “Run 1” and “Run 2” denote sequencing runs on independent PCR amplifications of the same cDNA library. Reactivities shown were normalized with a single, whole-dataset calculated normalization factor. **B.** TECprobe-VL DMS reactivity matrix for *alx* equilibrium refolded intermediates at pH 8.5 + 1 mM Mn. Reads from the two independent replicates shown in panel A, which were concatenated and analyzed together.



Supplemental Figure 2.31. Aligned reads and alignment rates for co-transcriptional DMS probing of the *mntP* riboswitch.

A-B. Plot showing fraction of aligned reads mapped to each transcript length. **C-D.** Percentage of reads retained after splitting those that aligned to each intermediate transcript length. **E-F.** Hexbin plots comparing the DMS reactivity of replicates.



Supplemental Figure 2.32. Aligned reads and alignment rates for equilibrium DMS probing of the *mntP* riboswitch.

A-B. Plot showing fraction of aligned reads mapped to each transcript length. **C-D.** Percentage of reads retained after splitting those that aligned to each intermediate transcript length. **E-F.** Hexbin plots comparing the DMS reactivity of replicates. No hexbin plot is shown for the pH 7.2 + 1 mM Mn condition because only one replicate was analyzed for this condition.

Supplemental Notes

Supplementary Note 1

*pH effect on the refolded *alx* aptamer structure.* Comparison of the nucleotide (nt) reactivities as a function of refolding pH revealed clear flexibility differences in L3 (Fig. 2C). The strictly conserved adenosine (A37) that coordinates Mn is less accessible for modification by both DMS and 1M7 at alkaline pH, despite the absence of Mn (Fig. 2C and SI Fig. 1E). Similarly, most L3 nt exhibit lower reactivities at alkaline vs neutral pH when modified with 1M7; however, the C36 and U38 that neighbor A37 are both more reactive at alkaline pH (Fig. 2C). Also, the L1 conserved adenosine (A11) that forms the cross-helix A-minor interaction becomes less reactive towards 1M7 at alkaline pH -Mn (Fig. 2C). Interestingly, this A11 pH-dependent reactivity difference is abolished when RNA is modified with DMS (SI Fig. 1E). Together, these data indicate that alkaline pH alters the reactivity of key nucleotides in the *alx* metal-sensing core, which could be due to a local pH effect on L1/L3 structure or a global pH-dependent trend in aptamer dynamics.

*Mn effect on the refolded *alx* aptamer structure.* Reactivity analysis of 1M7-modified RNA revealed that L3 generally becomes less flexible +Mn, consistent with Mn binding, except for U38, which remains more flexible +Mn regardless of pH (SI Fig. 1D). U39 is also more reactive towards 1M7 +Mn at neutral pH (SI Fig. 1D). For DMS-modified aptamer RNA, most L3 nt do not show significant Mn-dependent reactivity differences; however, consistent with 1M7 reactivity analysis, U38 and U39 are both more flexible at neutral pH +Mn (SI Fig. 1E). The crystal structure of a Cd²⁺-bound *L. lactis* Mn-sensing aptamer mutated to have the *alx* L3 sequence shows that metal

ion binding flips U38 and U39 out of the nucleobase stack formed by the A-minor motif, explaining the observed reactivity increase in reactivity when Mn is present¹.

Consistent with the role of A37 in directly coordinating Mn, no pH-dependent change in reactivity is observed +Mn when RNA is modified with either 1M7 or DMS (SI Fig. 1D-E). Further, in the presence of Mn, the pH-dependent change in reactivity towards 1M7 for A11, which forms the cross-helix A-minor interaction, is abolished (Fig. 2C). This finding agrees with published smFRET studies on the related *X. oryzae* Mn-sensing aptamer, which demonstrated that Mn increases docking of the two helical legs². Since A11 is a key player in this docking via the A-minor interaction, it is expected to be more frequently buried in the metal-sensing core, and thus less reactive towards a chemical probe +Mn.

Besides A11, L1 nt in 1M7-modified RNA are either more reactive or show minor reactivity differences +Mn at neutral pH; however, upon shifting to alkaline pH, L1 nt become less reactive +Mn (SI Fig. 1D). When aptamer RNA was DMS-probed at neutral pH, most L1 nucleotides are less reactive +Mn; however, upon a shift to alkaline pH, Mn-dependent reactivity differences are lessened, with G10 and G120 showing increased reactivity (SI Fig. 1E). Taken together, these data show that Mn alone reduces the flexibility of critical Mn-sensing nucleotides in L1 and L3 of the *alx* aptamer and are consistent with prior structural study of Mn-bound aptamers¹⁻³.

Supplementary Note 2

Mn-enhanced RNA polymerase (RNAP) pause longevity during mntP aptamer synthesis.

As a further support for Mn interacting with RNA folding intermediates, we mapped multiple

RNAP pauses during *mntP* aptamer synthesis (nt 86-90) that are enhanced by Mn (SI Fig. 4A-B). Similar enhancement of pauses within the *mntP* aptamer was achieved by addition of NusA in the absence of Mn (SI Fig. 4B), pointing to RNA hairpin stabilization of paused RNAP conformation at these sites. Thus, these pauses are likely prolonged via allosteric effect of Mn-RNA on the RNAP conformation akin to the allosteric effects of nascent RNA hairpins^{4,5}. The presence of these hairpin-stabilized pauses is further supported by RNA structure models constrained by DMS reactivities (stabilized by the P3.1 hairpin based on pause locations, SI Fig. 4C). All pause sites described above were mapped by scaffold-based transcription assays, which provided single-nt precision for mapping pause locations (SI Fig. 5A-C). Taken together, these data suggest that the hairpin-stabilized pauses during aptamer synthesis mediate sampling of divalents in the environment by the *mntP* RNA folding intermediates. Recent smFRET study on the *L. lactis* Mn-sensing riboswitch also identified a pause site enhanced either in the presence of Mn or NusA, with the pause in this case positioned at the end of aptamer folding to stabilize P1.1 switch helix⁶.

Supplementary Note 3

Some but not all co-transcriptional folding features are preserved in unfolded-refolded RNA intermediates. To identify the intermediates whose folding is truly controlled by the transcription kinetics vs. thermodynamic stability of the folds, we applied a modified TECprobe-VL workflow wherein intermediate RNA transcripts are extracted from the stalled transcription elongation complexes and equilibrium refolded before DMS probing (SI Fig. 6A). As with co-transcriptionally folded intermediates, we observed transient, low- T_m stem-loops that form and resolve once the full aptamer sequence is present (SI Fig. 6B). We observed similar Mn-dependent

differences in reactivity in equilibrium refolded as the co-transcriptionally folded intermediates for A48, U54, and A103 (SI Fig. 6C). Despite the L3 being single-stranded throughout the aptamer folding in both the equilibrium refolded and co-transcriptional intermediates, the reactivity of A48 shows a marked increase ~10 nt before the aptamer folds (SI Fig. 6C). This suggests that once the first 100 nt of the aptamer are available for base-pairing, a structural rearrangement occurs to reposition the L3, resulting in increased reactivity of A48 overall. A key folding transition that likely prompts this structural rearrangement is the pairing of P1.2 in the right leg, where nt 15-18 at the 5' end pair with nt 96-99. Once P1.2 folds, the DMS reactivity profile for transcript length 100 nt largely resembles that of the folded aptamer, with only L1 and P1.1 absent due to lack of their pairing partners (SI Fig. 6D-E). Thus, formation of P1.2 initiates folding of the right leg, which finishes folding once the 5' end of the aptamer transcript (nt 2-18) pairs with the nt that emerge later in transcription (nt 96-110, Fig. 3B). We hypothesize that folding of P1.2 positions nt 2-14 in closer proximity to the partially folded right leg to facilitate pairing of nt 2-14 with nt 100-110 once they emerge from RNAP, permitting efficient completion of the aptamer and metal-sensing core folding, and thus a rapid response to ligand. Further, repositioning of nt 1-14 likely brings these nucleotides into an even closer proximity to L3, resulting in nucleation of the cross-helix A-minor with A11 stacked atop A48 and explaining the Mn-dependent changes in A48 reactivity at this point.

For U54, Mn-dependent differences in reactivity emerge before the transcript is 100 nt, indicating that some positions in the L3 loop can begin sampling for metal even before the right leg in the aptamer begins folding (SI Fig. 6C). Interestingly, the magnitude of the U54 reactivity difference $\pm$ Mn is higher for the early equilibrium refolded intermediates (transcript lengths ~60-100 nt) compared to the co-transcriptionally folded intermediates (SI Fig. 6C). However, past

transcript length ~100 nt, the magnitude of the U54 reactivity difference $\pm$ Mn is higher for the co-transcriptionally folded intermediates, with U54 being more reactive in the absence of ligand (SI Fig. 6C). This indicates that the L3 is more flexible in co-transcriptionally folded intermediates once the metal-sensing core begins folding, resulting in higher reactivity at U54. Additionally, past transcript lengths ~110-120 nt, A103 demonstrates higher reactivity $-$ Mn, with the magnitude of the reactivity difference larger for the co-transcriptionally folded intermediates (SI Fig. 6C).

Supplementary Note 4

alx L3 nt show distinct Mn-dependent reactivity trends in equilibrium refolded intermediates. Similar to *mntP*, we observed Mn-dependent reactivity differences for equilibrium refolded *alx* intermediates (SI Fig. 8A). First, U35 reactivity trends higher $+$ Mn for equilibrium refolded intermediates starting at transcript length ~50 nt. Curiously, the magnitude of the reactivity difference $\pm$ Mn is greater for the early equilibrium refolded intermediates (transcript lengths ~60-115 nt, SI Fig. 8B) than for later intermediates, indicating a potential structural change (e.g., a loss of a tertiary RNA contact) induced by equilibrium refolding that affects L3 dynamics in these early intermediates. Additionally, A37 in the *alx* aptamer demonstrates higher reactivity $-$ Mn for equilibrium refolded intermediates, mimicking the trend observed for co-transcriptionally folded intermediates. However, the magnitude of the Mn-dependent reactivity difference for A37 is greater for equilibrium refolded intermediates across transcript lengths ~60-115 nt and ~135-155 nt (SI Fig. 8B). Lastly, C41 demonstrates higher reactivity $+$ Mn for equilibrium refolded intermediates, with a larger Mn-dependent reactivity difference for equilibrium refolded intermediates spanning transcript lengths ~70 nt-110 nt (SI Fig. 8B). Taken together, these data

indicate a potential structural change induced by equilibrium refolding that affects L3 dynamics, resulting in differing reactivity patterns compared to co-transcriptionally folded intermediates. Further, these data provide additional pieces of evidence that aptamer intermediates undergo Mn-dependent structural changes in L3.

Supplementary Note 5

*pH-dependent reactivity trends in *alx* and *mntP* L3 nt in equilibrium refolded intermediates.* We next assessed pH differences in L3 nucleotide reactivities for *alx* and *mntP* equilibrium refolded aptamer intermediates, after verifying that the global folding transitions in the aptamers are unaffected by pH (SI Fig. 10A-B). First, *alx* A37 shows a clear Mn-dependent difference in reactivity at both pH from transcript lengths ~60-115 nt, with lower reactivity in general +Mn (SI Fig. 10C). This trend is different from that seen for A37 in co-transcriptionally folded intermediates, where the lowest reactivity overall was observed for the pH 8.5 +Mn condition throughout aptamer folding (Fig. 5B). Additionally, positions C36, A40 and C41 in *alx* equilibrium refolded intermediates do not show the same increased reactivity at pH 8.5 ±Mn as described for co-transcriptionally folded intermediates (SI Fig.9C and SI Fig. 10C). These data support that an alkaline pH shift uniquely affects the structure of co-transcriptionally folded *alx* aptamer intermediates.

The *mntP* L3 also demonstrates different reactivity trends in equilibrium refolded aptamer intermediates. A48 shows no clear pH difference in reactivity (SI Fig. 10D); this is in stark contrast to co-transcriptionally folded aptamer intermediates, where A48 is more reactive at alkaline pH (Fig. 5C). This result indicates that equilibrium refolding of *mntP* aptamer intermediates alters the

orientation of A48, the A11 stacking partner, such that pH no longer alters the accessibility of its Watson-Crick face for DMS methylation. The absolutely conserved adenosine in *mntP* L3 (A50) is more reactive at neutral pH $\pm$ Mn for the duration of aptamer folding (SI Fig. 10D), which was not observed for co-transcriptionally folded intermediates (Fig. 5C). These data support that equilibrium refolding of *mntP* aptamer intermediates repositions the L3 loop and changes how pH affects L3 structure.

*pH-dependent reactivity trends in *alx* and *mntP* L1 nt in co-transcriptionally folded vs. equilibrium refolded intermediates.* Beyond L3, both the *alx* and *mntP* aptamers also exhibit curious pH-dependent reactivity differences in the absolutely conserved L1. A101 in the *mntP* aptamer is more reactive at alkaline pH whether or not Mn is present (SI Fig. 11A). A103 in *mntP* is generally more reactive $-$ Mn at both pH, with the absolute reactivity values greater at pH 7.2 $\pm$ Mn (SI Fig. 11A). At the analogous positions in the *alx* aptamer (A119 and A121) we observed different reactivity trends. In the absence of Mn, A119 in *alx* is more reactive at alkaline pH during aptamer folding. In the presence of Mn, however, there is a clear pH-dependent divergence in the *alx* A119 reactivity at the end of aptamer folding, with a peak in reactivity observed at pH 7.2 and a drop in reactivity at pH 8.5 (SI Fig. 11A). A121 exhibited similar trends to those described above for A119, with a stark divergence in reactivity between the pH 7.2 +Mn and pH 8.5 +Mn conditions towards the end of aptamer folding (SI Fig. 11A). These data solidify that a shift to alkaline pH exerts distinct structural effects on the *alx* and *mntP* aptamers.

Lastly, we assessed single nucleotide reactivity trajectories for *alx* and *mntP* L1 nucleotides in equilibrium refolded intermediates and compared them to their co-transcriptionally folded counterparts. For *mntP* A101, there is no clear pH-dependent reactivity trend (SI Fig. 11B), which

differs from co-transcriptionally folded intermediates where A101 was more reactive at pH 8.5 in general (SI Fig. 11A). A119 and A121 in the *alx* L1 showed no clear pH trends for equilibrium refolded aptamer intermediates. A119 reactivity in general was higher towards the end of aptamer folding in equilibrium refolded transcripts for all conditions tested compared to co-transcriptionally folded transcripts (SI Figs 11A-B), indicating that equilibrium refolding for *alx* aptamer intermediates results in a more dynamic L1. Taken together, these data support that equilibrium refolding alters the structure and dynamics of the metal-sensing core.

Supplementary Note 6

pH- and Mn-dependent changes in mntP aptamer structure during co-transcriptional expression platform folding. We observed similar reactivity trends in *mntP* expression platform folding at pH 7.2 as pH 8.5 (SI Fig. 12B-C). For example, at pH 7.2, we also observed the transient spike in A11 reactivity at transcript length ~205 nt; however, both the amplitude of this spike and the difference $\pm$ Mn is smaller compared to pH 8.5 (SI Fig. 12D). Other curious ligand- and pH-dependent trends stood out for *mntP* nucleotides in L3, L1, and the switch helix P1.1 during expression platform folding. A48 in L3 drops in reactivity at transcript length ~200 nt for all conditions followed by an increase in reactivity at pH 7.2 +Mn and pH 8.5 +Mn compared to the respective –Mn conditions (SI Fig. 12D). This indicates that a global, Mn-dependent structural rearrangement occurs in the aptamer at this point, consistent with the reactivity trend described for A11. Since A11 and A48 are stacking partners (Fig. 6C), it makes sense that their reactivity changes are coupled upon disruption of the cross-helix A-minor interaction. A50 in L3, which

coordinates Mn, also exhibits ligand-dependent reactivity differences for the duration of expression platform folding (SI Fig. 12D).

L1 nucleotides besides A11 undergo ligand-dependent structural changes at the riboswitching point. Similar to A11, A14 reactivity spikes towards the end of expression platform folding –Mn (SI Fig. 12D); however, the increase in reactivity occurs ~5-10 nt before the spike in A11 reactivity, indicating that the A14-G100 base-pair in L1 is broken before the cross-helix A-minor is disrupted. A101 in L1 remains generally more reactive at pH 8.5 ±Mn vs. pH 7.2 ±Mn from transcript lengths ~120-190 nt and demonstrates high reactivity in general despite its predicted base-pairing with U13 (SI Fig. 12D). Curiously, at transcript length ~200 nt, a drop in A101 reactivity is observed for all conditions, with the +Mn condition trending higher for the remainder of expression platform folding (SI Fig. 12D). When L1 is disrupted –Mn, A101 is predicted to be single-stranded vs. remaining base-paired with U13 in the Mn-stabilized aptamer. However, past transcript length ~220 nt, A101 is generally more reactive +Mn (SI Fig. 12D), indicating that the Watson-Crick face of A101 is transiently exposed +Mn at the end of expression platform folding, perhaps due to differences in the expression platform tertiary structure. A103 in L1 remains generally more reactive –Mn at pH 7.2 and 8.5 for the entirety of expression platform folding (SI Fig. 12D). At transcript length ~200 nt, A103 reactivity increases across all conditions, with the absolute reactivity values being higher –Mn (SI Fig. 12D). A103 is predicted to base-pair with G9 in the Mn-stabilized aptamer vs. single-stranded –Mn (Fig. 6C); thus, higher A103 reactivity –Mn is consistent with these predictions. Notably, A14, A101, and A103 are reactive towards DMS in general across all conditions tested, indicating that L1 transiently melts during expression platform folding in a pH- and Mn-dependent manner.

Lastly, we analyzed co-transcriptional Mn- and pH-dependent reactivity differences in the P1.1 switch helix, the key structural riboswitching element. C2 and A3 at the base of the P1.1 switch helix undergo a steady increase in reactivity from transcript lengths ~180-195 nt, followed by a reactivity drop ~200 nt and subsequent increase across all conditions tested (SI Fig. 12D). There is no clear Mn-dependent trend observed for C2 and A3, suggesting that a disrupted P1.1 is transiently sampled even in the presence of Mn. C104 spikes in reactivity at transcript length ~195 nt and the amplitude of this spike is higher –Mn (SI Fig. 12D), supporting that the C104-G8 base-pair in the P1.1 helix is disrupted –Mn before C104 alternatively pairs with G203 in the expression platform (Fig. 6C). A106 exhibits high reactivity at pH 7.2 in general for the entire duration of expression platform folding, despite its base-pairing U6 in P1.1 (SI Fig. 12D). Further, A106 reactivity is higher at pH 7.2 +Mn and pH 8.5 +Mn compared to their respective –Mn conditions (SI Fig. 12D). This suggests that in the presence of Mn, the Watson-crick face of A106 is more often accessible for DMS methylation. Lastly, A108 is generally more reactive at pH 7.2 during expression platform folding and demonstrates a clear pH-dependent spike in reactivity at transcript length ~190 nt, with the amplitude of the spike being higher at pH 7.2 ±Mn (SI Fig. 12D). Taken together, our observations of *mntP* L3, L1, and P1.1 reactivities during expression platform folding highlight that a global structural change occurs in the expression platform at transcript lengths ~200-205 nt, which is likely prompted by the start of strand invasion at P1.1 in the aptamer.

Supplementary Note 7

pH- and Mn-dependent reactivity changes in mntP equilibrium refolded intermediates. We expanded our analysis of equilibrium refolded *mntP* intermediates to other nucleotides in the

metal-sensing core, A48 and U54. At pH 8.5, A48, the A11 stacking partner, markedly drops in reactivity $\pm$ Mn at transcript length $\sim$ 200 nt, with its reactivity trending higher +Mn (SI Fig. 13D). While a similar trend is observed for co-transcriptionally folded intermediates, the magnitude of the Mn-dependent reactivity difference is smaller in the equilibrium refolded context. This result indicates that the *mntP* L3 in the aptamer can still perform divalent sampling even with the fully folded expression platform. A similar trend was observed at pH 7.2, illustrating that pH affects L3 structure when the expression platform is present (SI Fig. 13D). U54 in L3 also shows a clear difference in reactivity between co-transcriptionally folded vs. equilibrium refolded intermediates. Starting at transcript length $\sim$ 115 nt, U54 is more reactive +Mn at pH 8.5 and this trend is maintained for the entirety of expression platform folding (SI Fig. 13D). Although a similar trend is observed for equilibrium refolded intermediates, the magnitude of the reactivity difference is decreased, indicating that equilibrium refolding impacts L3 structure and consequently its sampling for Mn. Taken together with A48, these data support that L3 structure is altered in the equilibrium refolded context.

Supplementary Note 8

We observed that, regardless of pH or presence of Mn, the T7-synthesized, equilibrium refolded *mntP* riboswitch assumes the same translationally inactive conformation, supported by DMS- and 1M7-constrained secondary structures (SI Fig. 14A). Nevertheless, interesting trends were extracted from the refolded full-length riboswitch chemical probing.

pH- and Mn-dependent trends in L3 reactivity. We observed interesting reactivity trends in the L3 loop for *mntP*. First, the absolutely conserved A50 shows less reactivity +Mn vs. -Mn when the RNA was refolded at pH 7.2, likely due to its direct coordination of Mn (SI Fig. 14B). However, there are no significant reactivity changes for A50 when RNA is refolded at pH 8.5 ±Mn, demonstrating that the flexibility of this nucleotide is limited by alkaline pH alone (SI Fig. 14B-C). In DMS-modified *mntP* RNA, A50 is less reactive +Mn at both pH (SI Fig. 14D), further supporting its direct coordination with Mn. Lastly, A48 in *mntP* L3 is less reactive towards 1M7 in general at alkaline pH but does not show a ligand-dependent reactivity difference (SI Fig. 14B-C). In contrast to 1M7 data, we observed that A48 was more reactive towards DMS +Mn at either pH (SI Fig. 14D). This observation supports that Mn binding specifically orients the Watson-Crick face of L3 such that A48 is more susceptible to DMS methylation. Together, these data support that L3 retains its ability to sample for divalents even when the RNA is refolded into an inactive conformer with a disrupted metal-sensing core.

pH- and Mn-dependent 1M7 reactivity trends in the mntP expression platform. In the *mntP* expression platform, several pH- and ligand-dependent reactivity differences are observed when RNA is modified with 1M7. Upon a shift to alkaline pH, A117 and A118 in P5 become less reactive at both ±Mn (SI Fig. 14C). At alkaline pH, other P5 nucleotides G140-A144 are less reactive -Mn. Interestingly, this effect becomes overall less pronounced when Mn is introduced and C139 shows a marked increase in reactivity at pH 8.5 +Mn (SI Fig. 14B-C). Finally, when Mn is present, the P5 loop from G174-G183 shows a variety of reactivity difference upon a shift to alkaline pH (Fig. 14C). Notably, G174 and C179 are both more reactive at alkaline pH compared to neutral pH when Mn is present while U176, U180, and G181 are less reactive (SI Fig. 14C).

RBS nucleotides are reactive with 1M7 at all conditions, but show some interesting reactivity differences. At pH 7.2, A215 is more reactive +Mn than -Mn while G216 and G217 are less reactive +Mn (SI Fig. 14B). In contrast, A215 is also less reactive $\pm$ Mn at pH 8.5 (SI Fig 14C). Taken together, these findings show that shifts to alkaline pH $\pm$ Mn affect the reactivity of many single stranded nucleotides in the *mntP* expression platform.

Supplementary Note 9

*pH- and Mn-dependent reactivity changes in *alx* equilibrium refolded intermediates.*

Comparison between co-transcriptionally folded and equilibrium refolded *alx* intermediates revealed pH- and Mn-dependent reactivity trends for multiple nt in the *alx* expression platform that were unique to equilibrium refolded intermediates (see SI Figs. 17A-B for *alx* expression platform reactivity heatmaps, pH 7.2 and 8.5 $\pm$ Mn). A137 demonstrates a stark increase in reactivity $\pm$ Mn starting at transcript length ~150 nt, which reaches an apex at transcript length ~170 nt and then decreases until transcript length ~180 nt (SI Fig. 17C). The peak in reactivity observed for A137 $\pm$ Mn in equilibrium refolded intermediates is markedly higher than its reactivity in co-transcriptionally folded intermediates at both pH 7.2 and 8.5 (SI Fig. 17C), pointing to a distinct structural transition induced by equilibrium refolding spanning transcript lengths ~150-180 nt. Further, the amplitude of the A137 peak for equilibrium refolded intermediates is Mn-dependent with A137 being more reactive in the presence of Mn (SI Fig. 17C), indicating that Mn can influence the expression platform structure post-transcriptionally.

Supplementary Note 10

Like with *mntP*, the equilibrium refolded *alx* riboswitch assumes the same translationally inactive conformation regardless of pH or presence of Mn, as supported by DMS- and 1M7-constrained secondary structures (SI Fig. 20A). The following trends of interest were observed.

pH- and Mn-dependent trends in L3 reactivity. For *alx*, we saw that the strictly conserved A37 in L3 is always less reactive +Mn regardless of pH when modified with both 1M7 and DMS (SI Fig. 20B-D). Similar to *mntP*, this provides additional evidence that L3 can sample for divalents even when the RNA is largely refolded into the inactive conformation. When the *alx* aptamer-only was refolded, we observed that L3 becomes less flexible at pH 7.2 +Mn (SI Fig. 1D), with the exception of U38 and U39 that were shown to be flipped out of the A-minor stack in a published crystal structure¹. In contrast to the 1M7-probed aptamer at pH 7.2 +Mn, the full-length *alx* riboswitch RNA shows increased reactivity for U35 and C36 (SI Fig. 20B), likely due to a difference in L3 positioning when RNA is refolded with the expression platform.

pH- and Mn-dependent 1M7 reactivity trends in alx expression platform. In the *alx* expression platform, the loop nt G129-A132 in P5 are more reactive +Mn at alkaline vs. neutral pH (SI Fig. 20C). In P6, A153 and G154 are less reactive when refolded -Mn at pH 8.5 than at pH 7.2 (SI Fig. 20C). In the presence of Mn, A153 and C155 are less reactive when refolded at alkaline pH than at neutral pH while A156 is more reactive (SI Fig. 20C). Interestingly, U169 in the P6 loop of the inactive conformer is less reactive upon a shift to pH 8.5 ±Mn while even though U170-C172 in the same loop are more reactive upon such shift, demonstrating clear pH reactivity differences between proximal nt within the same structural element (SI Fig. 20C). C171 and C172

are also more reactive when Mn is present at neutral pH (SI Fig. 20C) We observe that RBS nucleotides are overall less reactive +Mn, where A198 and A201 are less reactive at pH 7.2 and G199 and G200 are less reactive at pH 8.5 (SI Fig. 20B-C). Surprisingly, G199 and G200 are more reactive at pH 8.5 -Mn than pH 7.2 -Mn, but adding Mn abolishes this reactivity difference (SI Fig. 20C). Taken together, these RBS reactivity differences demonstrate condition-dependent fluctuations in the flexibility of these nucleotides when the RNA has been equilibrium refolded.

Supplemental Methods

Proteins

Q5 High-Fidelity DNA Polymerase, Vent (exo-) DNA polymerase, Mth RNA Ligase (as part of the 5' DNA Adenylation kit), T4 RNA Ligase 2 truncated KQ, ET SSB, RNase H, RNase If, T4 DNA Ligase, Sall, HindIII, EcoRI, PstI, KpnI, BamHI, XbaI, DpnI, TIPP, NEBNext rRNA depletion kit, and HiScribe T7 High Yield RNA Kit were purchased from New England Biolabs. TURBO DNase, SuperaseIN RNase Inhibitor, and SuperScript II were purchased from Thermo Fisher Scientific. SAvPhire Monomeric Streptavidin was purchased from Sigma-Aldrich. Catalog numbers for all commercial proteins are tabulated in Supplementary Table 1. Preparation of *E. coli* RNAP, *E. coli* σ^{70} , and *E. coli* NusA were performed as described previously⁷.

Bacterial strains

The strains used in this study are derivatives of *E. coli* K-12 and are tabulated in Supplementary Table 4.

The $\Delta alx::Kan$ mutations were sourced from strains in the Keio collection⁸ and introduced into MC4100 *E. coli* strain via P1 transduction. Chromosomally encoded T7 RNAP was introduced to this MC4100 Δalx strain using P1 transduction for the *alx* and *mntP* reporter fusions and was expressed from a *lac* promoter.

Plasmids and cloning procedures

The plasmids used in this study are tabulated in Supplementary Table 5.

The plasmid pMIS309 carrying the template for promoter-initiated transcription on the wild-type *mntP* riboswitch sequence (Supplementary table 5) was constructed by PCR-amplifying from a plasmid template, pRA57, (Supplementary table 5) from 486 nt upstream and 42 nt downstream of the *mntP* translation start site using oligos 0257 and 0258. The PCR product was then cloned into the BamHI and EcoRI sites of pUC19. The plasmid was assembled *via* restriction-ligation using T4 DNA Ligase (New England Biolabs).

The plasmid pMIS313 carrying the template for TECprobe-VL experiments on the *alx* riboswitch (Supplementary table 5) was constructed using a two-step PCR strategy. The PCRs employed oligos with 5' overhangs that introduced the PRA1 promoter and SC1 hairpin upstream of the *alx* riboswitch and additional downstream *alx* sequence. The first PCR used pMIS301 (Supplementary table 5) as a template and oligos 0155 and 0187. The second PCR used the purified first step PCR product as a template and oligos 0156 and 0188. The second-step PCR product was cloned into the HindIII and EcoRI sites of pUC19 *via* Gibson assembly using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs).

The plasmid pMIS315 carrying the template for TECprobe-VL experiments on the *mntP* riboswitch (Supplementary table 5) was constructed using a two-step PCR strategy. The PCRs employed oligos with overhangs that introduced the PRA1 promoter and SC1 hairpin upstream of

the *mntP* riboswitch. The first PCR used pRA57 (Supplementary table 5) as a template and oligos 0258 and 0259. The second PCR used the purified first step PCR product as a template and oligos 0264 and 0265. The second step PCR product was cloned into the BamHI and EcoRI sites of pUC19 *via* restriction-ligation using T4 DNA Ligase (New England Biolabs).

The plasmid pMIS349 carrying the *alx* translational reporter with the A37T mutation in the sequence encoding the *alx* riboswitch was constructed by overlap extension PCR using published methods⁹ with oligos 0229,0232,0458, and 0459.

The plasmid pMIS508 carrying the template for the *alx* aptamer under the control of a T7 promoter was constructed by cloning the template sequence (Supplementary Table 5) as a gene block from Integrated DNA Technologies into the BamHI site of pUC57. The plasmid was assembled *via* restriction-ligation using T4 DNA Ligase (New England Biolabs).

The plasmid pMIS509 carrying the template for the *mntP* aptamer under the control of a T7 promoter was constructed by cloning the template sequence (Supplementary Table 5) as a gene block from Integrated DNA Technologies into the BamHI site of pUC57. The plasmid was assembled *via* restriction-ligation using T4 DNA Ligase (New England Biolabs).

The plasmid pMIS511 carrying the template for *in vitro* SHAPE/DMS-MaP of the *alx* riboswitch was constructed by cloning the template sequence (Supplementary Table 5) as a gene block from Integrated DNA Technologies into the PstI and XbaI sites of pUC57. The plasmid was assembled *via* restriction-ligation using T4 DNA Ligase (New England Biolabs).

The plasmid pMIS513 carrying the template for *in vitro* SHAPE/DMS-MaP of the *alx* aptamer was constructed by cloning the template sequence (Supplementary Table 5) as a gene block from Integrated DNA Technologies into the PstI and XbaI sites of pUC57. The plasmid was assembled via restriction-ligation using T4 DNA Ligase (New England Biolabs).

The plasmid pMIS518 carrying the template for *in vivo* DMS-MaP of the *alx* riboswitch was constructed by PCR amplifying the template sequence (Supplementary Table 5) from pMIS447 (BioBasic) using oligos 0005 and 0026 and cloning it into the XbaI and PstI sites of pUC19. The plasmid was assembled via restriction-ligation using T4 DNA Ligase (New England Biolabs).

The plasmids with *alx* and *mntP* reporter gene fusions under the control of a native *E. coli* promoter are pRA54 (*alx* translational reporter) and pRA57 (*mntP* translational reporter) and were constructed in prior work¹⁰ and used as controls in this work (Supplementary Table 5). Reporter gene fusions under the control of a T7 promoter (*alx*: pMIS501 and *mntP*: pMIS517, Supplementary Table 5) were constructed via sequential PCR amplification. For *alx*, the first amplification generated 208 nucleotides of the *alx* 5'-UTR under control of a T7 promoter. This was amplified from pMIS303 using oligos 0202 and 0203, adding a PstI restriction site. For the second amplification, *alx-lacZ* was amplified from pRA54 for the translational fusion using oligos 0204 and RAV23, giving a 97 nucleotide overlap with the first PCR amplification. A third PCR step using 0202 and RAV23 combined the first two PCRs into the full length *alx* translational fusion under the control of a T7 promoter. The translational fusion (pMIS501) was cloned into the

PstI and BamHI sites of a single copy plasmid pMU2386. The plasmids were assembled via restriction-ligation using T4 DNA Ligase (New England Biolabs). For *mntP*, the first amplification generated 228 nucleotides of the *mntP* 5'-UTR under the control of a T7 promoter. This was amplified from pMIS310 using oligos 0202 and 0454, adding a PstI restriction site. For the second amplification, *mntP-lacZ* was amplified from pRA57 for the translational fusion using oligos 0455 and RAV126, giving a 95 nucleotide overlap with the first PCR amplification. A third PCR step using 0202 and RAV126 combined the first two PCRs into the full length *mntP* translational fusion under the control of a T7 promoter. The translational fusion (pMIS517) was cloned into the PstI and SalI sites of a single copy plasmid pMU2386. The plasmids were assembled via restriction-ligation using T4 DNA Ligase (New England Biolabs).

***In vitro* transcription template preparation**

Supplementary Table 3 tabulates details for the linear double-stranded DNA templates prepared for this study, including oligonucleotides used for PCR amplification and cleanup method used. Supplementary Table 6 provides all DNA template sequences used in this work.

Unmodified or terminally biotinylated DNA templates for promoter-initiated *in vitro* transcription were prepared by PCR with high-fidelity Q5 DNA polymerase (New England Biolabs), treated with DpnI (New England Biolabs) if a plasmid was the PCR template, and purified by either phenol extraction followed by ethanol precipitation or Monarch PCR & DNA cleanup kit (New England Biolabs).

DNA templates for TECprobe-VL were PCR amplified from a linear DNA template containing a terminal biotin modification (*alx*: Template 2, *mntP*: Template 5) using Vent (exo-) DNA polymerase (New England Biolabs). The PCR contained 1X ThermoPol Buffer (New England Biolabs), 200 μ M dNTP Solution Mix, 250 nM forward primer, 250 nM reverse primer, 0.02 nM template DNA, 0.02 U/ μ l Vent (exo-) DNA polymerase, and a concentration of biotin-11-dNTPs (AAT BioQuest and Biotium) that promoted incorporation of ~3 biotin modifications in the transcribed region of the DNA template¹¹. The PCR was performed using the following thermal cycler protocol: 95 °C for 3 min [95 °C for 20 s, 58 °C for 30 s, 72 °C for 25 s] x 30 cycles, 72 °C for 5 min, hold at 12 °C. The PCR products were purified by phenol extraction followed by ethanol precipitation and quantified using the Quant-iT dsDNA Assay Kit (HS) (Invitrogen) with a BioTek Synergy H1 microplate reader (Agilent).

Linear DNA templates were prepared for transcription by T7 RNAP via PCR amplification of a plasmid using Q5 DNA Polymerase. For the *alx* riboswitch SHAPE/DMS-MaP template DNA, oligos 0026 and 0277 were used to amplify the template DNA from plasmid pMIS511 and DpnI was used to digest original plasmid DNA (Template 8). For the *alx* aptamer SHAPE/DMS-MaP template DNA, oligos 0026 and 0277 were used to amplify the template DNA from plasmid pMIS513 and DpnI was used to digest original plasmid DNA (Template 9). For the *mntP* riboswitch SHAPE/DMS-MaP template DNA, oligos 0297 and 0298 were used to amplify the template DNA from plasmid pMIS314 and DpnI was used to digest original plasmid DNA (Template 10).

Synchronized single-round *in vitro* transcription from a promoter

To monitor transcription on the *mntP* riboswitch sequence when initiated from its native promoter, we performed synchronized, single-round *in vitro* transcription assays. The template contained the *mntP* promoter (261 nt upstream of the transcription start site), the wild-type *mntP* riboswitch (nt 1-225), and nt 1-45 of *mntP* coding sequence (Template 4, Supplementary Table 3). To prepare the RNAP holoenzyme (holoRNAP), *E. coli* RNAP (2 μ M) and s^{70} (4 μ M) were combined in 1x transcription buffer (20 mM Tris-Cl, pH 8.5, 150 mM KCl, 10 mM MgCl₂, and 5 mM DTT) and incubated for 30 min at 37 °C. Following holoRNAP assembly, *in vitro* transcription reactions were initiated with an ApU dinucleotide and synchronized, taking advantage of the natural lack of cytosines between positions 3 and 15 of the *mntP* riboswitch. The initiation mix for *in vitro* transcription thus lacked CTP and contained 0.1 μ M of the holoRNAP, 200 nM of the DNA template, 1 μ M ATP, 2.5 μ M CTP, 2.5 μ M GTP, 150 μ M ApU (Trilink), [³²P]ATP (Revvity), and transcription buffer. After allowing initiation to proceed for 15 min at 37 °C, rifampicin (Fisher) was added to a final concentration of 10 μ g/ml, to inhibit reinitiation. Completion of this step yielded ECs stalled at the G15 position on the *mntP* riboswitch.

Next, transcription elongation reactions were performed by combining 9 μ l of ECs with 1 μ l of an NTP mix containing 1 mM each ATP, UTP, CTP, and GTP (100 μ M final, each NTP). All elongation reactions were performed at 37 °C. MnCl₂ was introduced concurrently with NTPs to a final concentration of 100 μ M to test the effect of Mn on transcription elongation and pausing. The transcription factor NusA was introduced concurrently with NTPs to a final concentration of 250 nM to test its effect on transcription elongation and pausing. After beginning elongation, the reaction was quenched at desired time points with an equal volume of 2x stop buffer (90 mM Tris-

borate buffer, 8 M urea, 50 mM EDTA, and 0.02% of both xylene cyanol and bromophenol blue). The time points collected were 10 s, 30 s, 60 s, 90 s, 2 min, 3 min, and 4 min. Additionally, a “chase” reaction was performed with each NTP present at a final concentration of 500 μ M. Radiolabeled RNAs in the quenched samples were denatured for 2 min at 95 °C and separated using denaturing PAGE (6% 19:1 acrylamide:bisacrylamide, 8 M urea). Samples were run alongside a 5'-³²P radiolabeled ladder (MspI-digested pBR322 plasmid). The gel was exposed to a PhosphorImager screen, and the screen was scanned using a Typhoon PhosphorImager.

***mntP* riboswitch pause mapping using scaffold-based transcription**

To precisely map pauses in the *mntP* riboswitch, transcription elongation assays using nucleic acid scaffolds were performed. Each scaffold initiated transcription upstream of the sequence window where pauses were expected to occur.

Multiple nucleic acid scaffolds were designed to map pauses on the *mntP* riboswitch sequence towards the end of aptamer domain folding and beginning of expression platform folding. The sequence window for scaffold-based pause mapping experiments was narrowed based on results from promoter-based assays. These experiments are specifically designed to map elemental pauses, meaning that the influence of local RNA structure on pause duration is omitted as a variable. Prior work has established that the elemental pause is an obligate precursor to longer-lived pauses stabilized by nascent RNA structure¹². Therefore, these minimalistic experiments are relevant for mapping pauses encountered by ECs when the full-length riboswitch RNA is present. A brief description of the scaffolds is presented here and are described pictorially in Supplementary

Figure 5. The nucleotide numbering corresponds to the numbering in the full-length *mntP* riboswitch sequence. Scaffold 1 employed an 8-mer starting RNA to position RNAP upstream of nt 79-100. Scaffold 2 used a 10-mer RNA to position RNAP upstream of nt 101-113. Two separate experiments with different starting RNAs were performed on scaffold 3. The first 10-mer RNA positioned RNAP upstream of nt 114-131, and the second 10-mer RNA positioned RNAP upstream of nt 132-139. Past these defined sequence windows for pause mapping on scaffolds 1-3, the RNA emerging from RNAP could form non-native secondary structures that may have influenced pause behavior. Therefore, RNA species outside of the defined sequence windows were omitted for pause analysis.

All scaffold-based transcription elongation assays used the following protocol. First, the nucleic acid scaffold was annealed in a buffer (20 mM Tris, pH 7.2, 150 mM KCl, and 5 mM DTT) with 5 μ M RNA and 10 μ M template DNA (T-DNA). Then, ECs were reconstituted by combining RNAP with the nucleic acid scaffold in transcription buffer (20 mM Tris, pH 7.2, 150 mM KCl, and 5 mM DTT) and incubated for 15 min at 37 °C. Next, the non-template DNA (NT-DNA) was added and incubation continued for 10 min at 37 °C to complete EC assembly. The ratio of RNA:T-DNA:NT-DNA:RNAP was 1:2:5:3 corresponding to concentrations of 0.5, 1, 2.5, 1.5 μ M, respectively. Next, the ECs were diluted fivefold, and heparin was added to the ECs at a final concentration of 0.1 mg/ml to sequester unbound RNAP. ECs were incubated with heparin for 3 min at 37 °C. To radiolabel the ECs, [α -³²P]ATP (Revvity) was added to the ECs for 1 min at 37 °C, which extended a fraction of RNAs by 1 nt for visualization. To extend all ECs by 1 nt, ECs were incubated with unlabeled ATP at a final concentration of 2 μ M for 3 min at 37 °C.

Transcription elongation reactions were performed by combining the ECs with an NTP mix containing 1 mM each ATP, UTP, CTP, and GTP (100 μ M final, each NTP). $MgCl_2$ was added to a final concentration of 5 mM alongside NTP introduction. Additionally, to extend pause duration and aid with pause mapping, transcription elongation reactions were performed with three of the NTPs at a final concentration of 100 μ M and the fourth NTP limiting at a final concentration of 10 μ M. Samples were removed at various time points and quenched with an equal volume of 2x stop buffer. Chase reactions were performed with each NTP at a final concentration of 500 μ M. In parallel, RNA sequencing ladders were generated to map pause locations. To make the ladder, the ECs were prepared as described above. The ECs were combined with ATP, UTP, CTP, and GTP to 100 μ M and one 3'-dNTP (Millipore Sigma) to 1 mM. The sequencing reactions proceeded for 20 min at 37 °C and were quenched with an equal volume of 2x stop buffer. Radiolabeled RNAs from the quenched time points were heat-denatured for 2 min at 95 °C and separated using denaturing PAGE (15% 19:1 acrylamide:bisacrylamide, 8 M urea). The gel was exposed to a PhosphorImager screen, and the screen was scanned using a Typhoon PhosphorImager.

Supplemental Table 2.1. Key reagents used in this study.

Proteins and associated solutions	Source	Cat no.
Q5 High-Fidelity DNA Polymerase	New England Biolabs	M0491
5X Q5 Reaction Buffer		
5X Q5 High GC Enhancer		
Vent (exo-) DNA polymerase	New England Biolabs	M0257
10X Thermo Pol Buffer	New England Biolabs	
Mth RNA Ligase	New England Biolabs	E2610
DNA Adenylation Buffer		
1 mM ATP		
T4 RNA Ligase 2 truncated KQ	New England Biolabs	M0373
10X T4 RNA Ligase Buffer		
50% PEG 8000		
ET SSB	New England Biolabs	M2401
RNase H	New England Biolabs	M0297
RNase If	New England Biolabs	M0243
TURBO DNase	Invitrogen (Thermo Fisher Scientific)	AM2239
	Invitrogen (Thermo Fisher Scientific)	AM2694
	Invitrogen (Thermo Fisher Scientific)	18064071
SuperaseIN RNase Inhibitor		
SuperScriptII	Sigma-Aldrich	SAE0094
DTT, 100 mM		
SAvPhire Monomeric Streptavidin		
T4 DNA Ligase	New England Biolabs	M0202
DpnI	New England Biolabs	R0176
<i>E. coli</i> RNAP	Purified in-house	
s ⁷⁰	Purified in-house	
NusA	Purified in-house	
TIPP	New England Biolabs	M0296
Sall	New England Biolabs	R3138
HindIII	New England Biolabs	R3104
EcoRI	New England Biolabs	R3101
PstI	New England Biolabs	R3140
KpnI	New England Biolabs	R3142
BamHI	New England Biolabs	R3136
XbaI	New England Biolabs	R0145
Nucleotides	Source	Cat no.
dNTP Solution Mix	New England Biolabs	N0447S
Biotin-11-dATP	AAT BioQuest	17014
Biotin-11-dGTP	AAT BioQuest	17015
Biotin-11-dUTP	AAT BioQuest	17016
Biotin-11-dCTP	Biotium	40036
High-Purity rNTPs set	Cytiva	27-2025-01
[a- ³² P]ATP	Revvity	BLU003H250
ddATP, ddUTP, ddCTP, ddGTP (Sequencing Grade)	Millipore Sigma	UC
ApU dinucleotide	Trilink	3732738001
ATP	Sigma-Aldrich	0-31004
UTP	Sigma-Aldrich	A26209
CTP	Sigma-Aldrich	94370
GTP	Sigma-Aldrich	C1506
	Sigma-Aldrich	G8877
Kits	Source	Cat no.
HiScribe T7 High Yield RNA Kit	New England Biolabs	E2040
Qubit ssDNA Assay Kit	Invitrogen (Thermo Fisher Scientific)	Q10212
	Invitrogen (Thermo Fisher Scientific)	
Quant-iT dsDNA Assay Kit (HS)	Invitrogen (Thermo Fisher Scientific)	Q33120

Supplemental Table 2.1. Key reagents used in this study.

Quant-iT RNA Assay Kit	Invitrogen (Thermo Fisher Scientific)	Q33140
Columns	Source	Cat no.
HiLoad 16/600 Superdex 200 pg (1x120 ml)	Cytiva	28989335
HisTrap HP	Cytiva	17524802
HiTrap Heparin HP	Cytiva	17040703
Other chemicals and reagents	Source	Cat no.
10 mg/ml Dynabeads MyOne Streptavidin C1 beads	Invitrogen (Thermo Fisher Scientific)	65001
Agencourt AMPure XP beads	Beckman Coulter	A63881
Benzoyl Cyanide (BzCN) 98%	Sigma-Aldrich	115959
Chloroform	Fisher	C298-500
Dimethyl Sulfate (DMS) 99+%	Sigma-Aldrich	D186309
Dimethyl Sulfoxide (DMSO), Anhydrous	Invitrogen (Thermo Fisher Scientific)	D12345
Ethanol, 200 proof	Fisher	BP2818-500
Formamide	Fisher	BP227-500
GlycoBlue Coprecipitant	Invitrogen (Thermo Fisher Scientific)	AM9515
Manganese (II) Chloride Tetrahydrate	Fisher	AA3652636
o-Nitrophenyl-B-D-Galactopyranoside (ONPG)	Thermo Fisher Scientific	34055
Phenol (Buffer Saturated Phenol)	Invitrogen (Thermo Fisher Scientific)	15513-039
Protease Inhibitor Cocktail	Sigma-Aldrich	P2714-1BTL
Rifampicin	Fisher	BP2679250
Trizol Reagent	Invitrogen (Thermo Fisher Scientific)	15596026
SpeedBead Magnetic Carboxylate Beads		651521050502
SPRI beads	Cytiva	50'
	Prepared as in ref 50	N/A
Nuclease Free Water	Invitrogen (Thermo Fisher Scientific)	10977023
Streptavidin Mag Sepharose	Cytiva	28985799

The modification codes correspond to those used for IDT ordering:
 /5bioSG/: “standard” 5’ biotin; /5 Phos/: 5’ phosphate; /3AmMO/: 3’ amino modifier

Supplemental Table 2.2. Oligonucleotides used in this study.				
Stock #	Name	Sequence (5' to 3')	Purification	Source
#0005	M13/pUCfor	CCCAGTCACGACGTTGTAAAAC	Desalt	IDT
#0026	M13/pUCrev	AGCGGATAACAATTTACACACAGG	Desalt	IDT
#0110	PRE T7 wt template for	GGATCCGATGACACTAATAC	Desalt	Eton
#0139	9N_VRA3_Oligo	/5Phos/rNrNrNrNrNrNNNGATCGTCGGACTGT AGAACTCTGAAC/3AmMO/	HPLC	IDT
#0155	PRA1_SC1_F_1	acctctggcggTgataatggtgcATGGCCTTCGGGCCAA TGCAAAGGGGAGTAACTTC	Desalt	Eton
#0156	PRA1_SC1_F_2	ttatcaaaaagagtattgacTCttttacctctggcggTgataatg	Desalt	Eton
#0157	PRA1_NoMod.F	TTATCAAAAAGAGTATTGACT CTTTTACCTCTGGCGGTGATA ATGGTTGCAT	HPLC	IDT
#0159	dRP1_5Bio.R	/5Biosg/AATGATACGGCGACCACCGAGATCT ACACGTTACAGTTCTACAGTCCGACGATC	HPLC	IDT
#0160	SC1_Brdg_PLUS	CCTTGGCACCCGAGAATTCCARRRYATGGC CTTCGGGCCAA	HPLC	IDT
#0161	SC1_Brdg_MINUS	CCTTGGCACCCGAGAATTCCAYYYRRATGGC CTTCGGGCCAA	HPLC	IDT
#0162	dRP1_NoMod.R	AATGATACGGCGACCACCGAGATCTACACG TTCAGAGTTCTACAGTCCGACGATC	HPLC	IDT
#0163- 0178; #0235- 0248	RPI (Index 1-30 sequences derived from Illumina Truseq Small RNA Kit)	CAAGCAGAAGACGGCATAACGAGAT[INDEX] GTGACTGGAGTTCCTTGGCACCCGAGAATTC CA	HPLC	IDT
#0187	Alx' extension	GTGCCCCACGACGCCCTGCAACAACAGGTC GATAGCCAGCATAATGGCGACAACAACAGC G	Desalt	Eton
#0188	alx extension rev	GTGCCCCACGACG	Desalt	Eton
#0189	0189_alx_extension_ rev_5bio	/5Biosg/GTGCCCCACGACGCCC	Desalt	IDT
#0202	0202_DAN1	AGC GGT ACT GCA GTA ATA CGA CTC ACT ATA G	Desalt	Eton
#0203	0203_DAN2	TAG AAG TTC CTT ACA CAT AAA AAA ACG GCC AAC G	Desalt	Eton
#0204	0204_DAN3	GTA AGT GAG ACC TTG CCG GAA GGC GAG	Desalt	Eton
#0229	0229_PCR1 for	GAGCTCGAATTAATTCCCCTGCA	Desalt	Eton
#0232	0232_PCR2 rev	CACGACGTTGTAAAACGACGG	Desalt	Eton
#0257	mntP_txn_template_ F cloning	GAGAACGGGATCCGATCGTTGAAATATCG ACGATCTGG	Desalt	IDT
#0258	mntP_txn_template_ R cloning	GCAAGATATTGAATTCCATCGACATACCAAA CGCAAG	Desalt	IDT
#0259	mntP_PRA1_SC1_F 1	acctctggcggTgataatggtgcATGGCCTTCGGGCCAA TCATTGGGGAGTAGCCG	Desalt	IDT
#0262	mntP_txn_template f	CGTTCGAAATATCGACGATCTGG	Desalt	IDT
#0263	mntP_txn_template r	CATCGACATACCAACGCAAG	Desalt	IDT
#0264	PRA1_SC1_F_2_mnt P cloning	GGAACGGGATCCGATttatcaaaaagagtattgacTCttt acctctggcggTgataatg	Desalt	IDT
#0265	cotrans_mntP_clonin g_R_2	GCAAGATATTGAATTCCATCGACATACC	Desalt	IDT
#0267	mntP_cotrans_templa te_R_5bio	/5Biosg/GCAAGATATTGAATTCCATCGACATA CCAAACGCAAGAAG	Desalt	IDT
#0273	0273_alx_aptamer_tx n rev	CCGCAAGGTCTCACTTA	Desalt	Eton

Supplemental Table 2.2. Oligonucleotides used in this study.				
#0274	0274_mntP_apramer txn rev	CATATGGTCTCGCCTGACTG	Desalt	Eton
#0277	0277_RT_primer_SH APEcassette	gaaccggaccgaagcccg	Desalt	Eton
#0278	0278_Step1_rev_SH APEcassette	CCCTACACGACGCTCTCCGATCTNNNNNgaa ccggaccgaagcccg	Desalt	IDT
#0279	0279_Step1_fwd_SH APEcassette	CCTTGGCACCCGAGAATTCCANNNNNggccttcg ggccaa	Desalt	IDT
#0287	mntP_elemental_map ping 1 RNA	rArCrGrUrArCrGrG	Desalt	IDT
#0288	mntP_elemental_map ping 1 tDNA	CGATTGATGTGCATATGGTCTCGCCTGACTG AAAGTATTCAGTCCGTACGTAGGACGC	Desalt	IDT
#0289	mntP_elemental_map ping 1 ntDNA	GCGTCCTACGTACGGACTGAATACTTTTCAGT CAGGCGAGACCATATGCACATCAATCG	Desalt	IDT
#0290	mntP_elemental_map ping 2 RNA	rCrArGrUrCrArGrGrCrG	Desalt	IDT
#0291	mntP_elemental_map ping 2 tDNA	GCAGGCATAGCGATTGATGTGCATATGGTCT CGCCTGACTGAAAGTATTCAAGGACGC	Desalt	IDT
#0292	mntP_elemental_map ping 2 ntDNA	GCGTCCTTGAATACTTTTCAGTCAGGCGAGAC CATATGCACATCAATCGCTATGCCTGC	Desalt	IDT
#0297	mntP_FL_SHAPE_tx n template F	GGTACCTCGCGAATGCATCTAG	Desalt	IDT
#0298	mntP_FL_SHAPE_tx n template R	GAACCGGACCGAAGCCC	Desalt	IDT
#0305	mntP_mapping_scaff old 3 ntDNA	CAGGCGAGACCATATGCACATCAATCGCTAT GCCTGCATGAACGCGAAAACCATCACGG	Desalt	IDT
#0306	mntP_mapping_scaff old 3 tDNA	CCGTGATGGTTTTTCGCGTTCATGCAGGCATA GCGATTGATGTGCATATGGTCTCGCCTG	Desalt	IDT
#0307	mntP_mapping_scaff old 3 RNA1	rCrCrArUrArUrGrCrArC	Desalt	IDT
#0308	mntP_mapping_scaff old 3 RNA2	rCrUrArUrGrCrCrUrGrC	Desalt	IDT
#0452	0452_PRE_SL_RBS termbio	/5Biosg/GCAACGGCGTGCCGAC	None	IDT
#0455	0455_mntP_lacZ_fw d	CAT CAA TCG CTA TGC CTG CAT GAA CG	Desalt	Eton
#0458	0458 PRE A37U F	CGGTGATCGTCTTTACGATGTGTG	Desalt	Eton
#0459	0459 PRE A37U R	CACACATCGTAAAGACGATCGACCG	Desalt	Eton
RAV23	RAV23	CATGGCATGTGGATCCGCCCCACGACGCCCC TGCAACAACA	Desalt	Eton
RAV126	RAV126	CTCGTTCGGTCGACTCCATCGACATACCAAA C	Desalt	Eton

The table below summarizes the linear double-stranded DNA templates prepared for this study, including the primers used for PCR amplification, the template for PCR, modifications (if applicable), the purification method for the DNA template, and the figure(s) in which each DNA template was used.

Supplemental Table 2.3. DNA templates used in this study.							
Template #	Description	Fwd primer	Rev primer	Template	Modifications	Clean-up	Used in Fig(s)
1	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>alx</i> riboswitch (nt 17-223), and nt 1-100 of the <i>alx</i> gene.	#0157	#0188	pMIS313	N/A	Phenol extraction/Ethanol precipitation	N/A
2	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>alx</i> riboswitch (nt 17-223), and nt 1-100 of the <i>alx</i> gene.	#0157	#0189	Template 1	Terminal biotin	Phenol extraction/Ethanol precipitation	N/A
3	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>alx</i> riboswitch (nt 17-223), and nt 1-100 of the <i>alx</i> gene.	#0157	#0189	Template 2	Internal biotin-11 dNMPs, Terminal biotin	Phenol extraction/Ethanol precipitation	Figs. 4, 5, 7, and 8; SI Figs. 7,8,9,10,11,15,16,17,19
4	Template contains wild-type <i>mntP</i> promoter, wild-type <i>mntP</i> riboswitch (nt 1-225), and nt 1-45 of the <i>mntP</i> gene.	#0262	#0263	pMIS309	N/A	Monarch PCR & DNA Cleanup Kit (New England Biosciences)	SI Fig. 4
5	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>mntP</i> riboswitch (nt 17-241), and nt 1-45 of the <i>mntP</i> gene.	#0157	#0267	pMIS315	Terminal biotin	Phenol extraction/Ethanol precipitation	N/A
6	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>mntP</i> riboswitch (nt 17-241), and nt 1-45 of the <i>mntP</i> gene.	#0157	#0267	Template 5	Internal biotin-11 dNMPs, Terminal biotin	Phenol extraction/Ethanol precipitation	Figs. 3,5, and 6; SI Figs. 3,6,9,10,11,12,13,18
7	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>alx</i> riboswitch (nt 17-223), and nt 1-25 of the <i>alx</i> gene	#0157	#0452	Template 1	Terminal biotin	Monarch PCR & DNA Cleanup Kit (New England Biosciences) or Phenol extraction/Ethanol precipitation	SI Fig. 19B

Supplemental Table 2.3. DNA templates used in this study.							
8	Template contains T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch (nt 15-238), and a 3' linker and RT primer anneal site (nt 239-281)	#0026	#0277	pMIS511	N/A	DpnI digest	SI Fig. 20
9	Template contains T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch aptamer (nt 15-144), and a 3' linker and RT primer anneal site (nt 145-187)	#0026	#0277	pMIS513	N/A	DpnI digest	Fig. 2 and SI Fig. 1
10	Template contains T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>mntP</i> riboswitch (nt 15-239), and a 3' linker for RT primer annealing (nt 240-264)	#0297	#0298	pMIS314	N/A	DpnI digest	SI Fig. 14

Supplemental Table 2.4. *E. coli* K12 strains used in this study.

Strain	Genotype
MC4100	$\Delta(argF-lac)U169 rpsL150 relA1spoT1araD139 flbB5301 deoC1 ptsF25$
RAS31	MC4100 $\Delta alx::Kan$

[#]All strains employed in this study are *E. coli* K12 derivatives. The $\Delta alx::Kan$ deletion mutation was sourced from the library of Keio collection (SI ref 8).

Supplemental Table 2.5. Plasmids used in this study.		
Plasmid	Source	Description
pMIS301	This work (purchased from Bio Basic)	pUC57-Kan plasmid with synthetic construct encoding wild-type <i>alx</i> riboswitch (nt 1-207) and the first 59 nt of <i>alx</i> , transcribed from the <i>alx</i> promoter
pMIS306	Landick Lab, University of Wisconsin Madison	Overexpression plasmid for wild-type <i>E. coli</i> s^{70} with an N-terminal His ₆ tag
pMIS309	This work	pUC19-Amp plasmid with insert encoding wild-type <i>mntP</i> riboswitch (nt 1-225) and the first 45 nt of <i>mntP</i> , transcribed from the <i>mntP</i> promoter
pMIS313	This work	pUC19-Amp plasmid with insert encoding PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>alx</i> riboswitch (nt 17-223) and the first 100 nt of <i>alx</i>

Supplemental Table 2.5. Plasmids used in this study.		
pMIS314	This work (purchased from Bio Basic)	pUC57-Kan plasmid with insert encoding T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>mntP</i> riboswitch (nt 15-239), and a 3' linker for RT primer annealing (nt 240-264)
pMIS315	This work	pUC19-Amp plasmid with insert encoding PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>mntP</i> riboswitch (nt 17-241) and the first 45 nt of <i>mntP</i> .
pMIS349	This work	pMU2386 containing translational reporter of <i>alx</i> clone in the <i>PstI</i> and <i>BamHI</i> sites. Contains A37T mutation in the <i>alx</i> riboswitch sequence
pMIS511	This work	pUC57-Kan plasmid with insert encoding T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch (nt 15-238), and a 3' linker and RT primer anneal site (nt 239-281)
pMIS513	This work	pUC57-Kan plasmid with insert encoding T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch aptamer (nt 15-144), and a 3' linker and RT primer anneal site (nt 145-187)
pMIS447	This work (purchased from BioBasic)	pUC57-Kan plasmid with insert encoding a 50 nt spacer, -35 element, -10 element, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch (nt 15-298), 3' linker and RT primer anneal site (nt 299-341), and <i>rrnB</i> T1 and T2 terminators
pMIS518	This work	pUC19-Amp plasmid with insert encoding a 50 nt spacer, -35 element, -10 element, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch (nt 15-298), 3' linker and RT primer anneal site (nt 299-341), and <i>rrnB</i> T1 and T2 terminators
pMIS501	This work	pMU2386 containing translational reporter of T7 promoter- <i>alx</i> in <i>PstI</i> and <i>BamHI</i> sites
pMIS517	This work	pMU2386 containing translational reporter of T7 promoter- <i>mntP</i> in <i>PstI</i> and <i>Sall</i> sites
pRA46	This work	Overexpression plasmid for wild-type <i>E. coli</i> NusA with an N-terminal His ₆ tag
pRA53	Sharma et. al., Journal of Bacteriology, 2024 (ref 19)	promoter-less pMU2386 (empty vector control for lacZ reporter fusion assays)
pRA54	Sharma et. al., Journal of Bacteriology, 2024 (ref 19)	pMU2386 containing translational reporter of <i>alx</i> clone in the <i>PstI</i> and <i>BamHI</i> sites
pRA57	Sharma et. al., Journal of Bacteriology, 2024 (ref 19)	pMU2386 containing translational reporter of <i>mntP</i> clone in the <i>PstI</i> and <i>Sall</i> sites
pRM756	Landick Lab, University of Wisconsin Madison	Wild-type <i>E. coli</i> RNAP ($\alpha 2\beta\beta'$) overexpression plasmid. His ₁₀ -ppx tag at <i>rpoC</i> -C-terminus

Supplemental Table 2.6. Sequences of DNA templates used in this study.		
Template #	Template description	Sequence
#1-3	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>alx</i> riboswitch (nt	ttatcaaaaagagtattgacTCttttacctctggcggTgataatggttgcATGGCCTTCGGGC CAATGCAAAGGGGAGTAACCTTCATTGCCGGTCGATCGTCATTA CGATGTGTGAAAAAACACATCCGGTCACCGGGCAACCCGAAA GGAATACGCAGACGTATTCCTTTTGTGTGAAGTGAGACCTTG

Supplemental Table 2.6. Sequences of DNA templates used in this study.		
	17-223), and nt 1-100 of the <i>alx</i> gene.	CCGGAAGGCGAGGTCTATGCATAAAAAAGCAGCGGCTGACGTCT TCCGACGTTGGCCGTTTTTTTATGTGTAAGGAACCTTCTATGAAT ACTGTCGGCACGCCGTTGCTATGGGGCGGATTGCTGTTGTTGT CGCCATTATGCTGGCTATCGACCTGTTGTTGCAGGGGCGTCGTG GGGCAC
#4	Template contains wild-type <i>mntP</i> promoter, wild-type <i>mntP</i> riboswitch (nt 1-225), and nt 1-45 of the <i>mntP</i> gene.	CGTTCGAAATATCGACGATCTGGAAAAAATTTATAAGCTTCTC GTTTCAACTCAATAAGTTATGAATTTAGCCAAAGCTATGTTTAG TGTATTTTTTAATAATCAGACATAGCTTAGGCTATATTACCTCTT CCCTTATTTGTTATTTATTTTAACGTTTCATTGATATATAAAATCC AAATGAAAATCGTTATCAATAAAGCAATGAAATAATATATTCC AACAGTTGTTTTATATTCTCAAAATATGTTAAGGTTGCGCCCTC ATTTGGGGAGTAGCCGATTTCAGATTCCGAAATGTACGTGT CAACATACTCGTTGCAAAACGTGGCACGTACGGACTGAATACT TTCAGTCAGGCGAGACCATATGCACATCAATCGCTATGCCTGC ATGAACGCGAAAACCATCACGGCTTGTTGTTTCATGTATGCATG TTTGCTGGGGGCGATGATGTGTTTTATGGATAACCCCGGTCAGG ACATTGTCatgAATATCACTGCTACTGTTCTTCTTGCCTTGGTAT GTCGATG
#5-6	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>mntP</i> riboswitch (nt 17-241), and nt 1-45 of the <i>mntP</i> gene.	tatcaaaaagagtattgacTCttttacctctggcggTgataatggttgcATGGCCTTCGGGC CAATCATTTGGGGAGTAGCCGATTTCAGATTCCGAAATGTA CGTGTCAACATACTCGTTGCAAAACGTGGCACGTACGGACTGA ATACTTTTCAGTCAGGCGAGACCATATGCACATCAATCGCTATG CCTGCATGAACGCGAAAACCATCACGGCTTGTTGTTTCATGTAT GCATGTTTGCTGGGGGCGATGATGTGTTTTATGGATAACCCCGGT CAGGACATTGTCatgAATATCACTGCTACTGTTCTTCTTGCCTT GGTATGTCGATG
#7	Template contains PRA1 promoter, 5' SC1 hairpin (nt 1-16), wild-type <i>alx</i> riboswitch (nt 17-223), and nt 1-25 of the <i>alx</i> gene	tatcaaaaagagtattgacTCttttacctctggcggTgataatggttgcATGGCCTTCGGGC CAATGCAAAGGGGAGTAACCTTCATTGCCGGTCGATCGTCATTA CGATGTGTGAAAAAACACATCCGGTCACCGGGCAACCCGAAA GGAATACGCAGACGTATTCCTTTTTTGTGTAAGTGAGACCTTG CCGGAAGGCGAGGTCTATGCATAAAAAAGCAGCGGCTGACGTCT TCCGACGTTGGCCGTTTTTTTATGTGTAAGGAACCTTCTATGAAT ACTGTCGGCACGCCGTTGC
#8	Template contains T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch (nt 15-238), and a 3' linker and RT primer anneal site (nt 239-281)	TAATACGACTCACTATAGGGggccttcgggccaTGCAAAGGGGAGTA ACTTCATTGCCGGTCGATCGTCATTACGATGTGTGAAAAAACA CATCCGGTCACCGGGCAACCCGAAAGGAATACGCAGACGTATT CCTTTTTTGTGTAAGTGAGACCTTGCCGGAAGGCGAGGTCTAT GCATAAAAAAGCAGCGGCTGACGTCTTCCGACGTTGGCCGTTTT TTTATGTGTAAGGAACCTTCTATGAATACTGTCTCGATCCTCGATCC GGTTCGCCGGATCCAAATcgggcttcggtcgggttc
#9	Template contains T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>alx</i> riboswitch aptamer (nt 15-144), and a 3' linker and RT primer anneal site (nt 145-187)	TAATACGACTCACTATAGGGggccttcgggccaTGCAAAGGGGAGTA ACTTCATTGCCGGTCGATCGTCATTACGATGTGTGAAAAAACA CATCCGGTCACCGGGCAACCCGAAAGGAATACGCAGACGTATT CCTTTTTTGTGTAAGTGAGACCTTGCCGGTCGATCCGGTTCGC CGGATCCAAATcgggcttcggtcgggttc
#10	Template contains T7 promoter, 5' SC1 hairpin (nt 1-14), wild-type <i>mntP</i> riboswitch (nt 15-239), and a 3' linker for RT primer annealing (nt 240-264)	TTAATACGACTCACTATAGGGGGCCTTCGGGCCAATCATTTGG GGAGTAGCCGATTTCAGATTCCGAAATGTACGTGTCAACAT ACTCGTTGCAAAACGTGGCACGTACGGACTGAATACTTTTCAGT CAGGCGAGACCATATGCACATCAATCGCTATGCCTGCATGAAC GCGAAAACCATCACGGCTTGTTGTTTCATGTATGCATGTTTGCTG GGGGCGATGATGTGTTTTATGGATAACCCCGGTCAGGACATTGT CTCGATCCGGTTCGCCGGATCCAAATCGGGCTTCGGTCCGGTTC

Supplemental Table 2.8. BioSample information for all single-length, equilibrium refolded chemical probing experiments performed in this work.

[illegible]

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Chapter 2, in full, is a reprint of the material as it appears in Structurally distinct manganese-sensing riboswitch aptamers regulate different expression platform architectures. Stephen, Christine; Palmer, Danae; Bautista, Clarisa; and Mishanina, Tatiana. Nucleic Acids Research, 2025. The dissertation author was a primary researcher and co-author of this paper.

Chapter 3 pH-dependent allosteric remodeling of a bacterial riboswitch couples alkaline activation to metal sensing

Title

pH-dependent allosteric remodeling of a bacterial riboswitch couples alkaline activation to metal sensing

Danea Palmer¹, Adrien Chauvier², Tomás F. D. Silva³, Avery Ontiveros¹, Giovanni Bussi³, Nils G. Walter^{2,*}, and Tatiana V. Mishanina^{1,*}

¹Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Dr, La Jolla, CA, USA.

²Single Molecule Analysis Group and Center for RNA Biomedicine, Department of Chemistry, University of Michigan, Ann Arbor, MI, USA.

³Scuola Internazionale Superiore di Studi Avanzati, via Bonomea 265, Trieste, 34136, Italy.

*Address correspondence to: nwalter@umich.edu; tmishanina@ucsd.edu

Abstract

The widespread *yybP-ykoY* riboswitches control bacterial manganese (Mn) homeostasis by activating exporter expression in response to intracellular Mn²⁺ levels. The *E. coli alx* riboswitch distinctively couples Mn²⁺ sensing to cytoplasmic alkalinity, but the mechanism is unknown. We show that pH tunes the *alx* aptamer's conformational sampling to modulate Mn²⁺ sensitivity. Single-molecule FRET reveals that Mn²⁺ stabilizes a docked three-way-junction conformation, and alkaline pH shifts this equilibrium to sensitize metal-dependent folding. Molecular dynamics

simulations identify a loop whose low-pH-induced base pairing perturbs the adjacent helix, predicted to allosterically disrupt the Mn^{2+} -binding state. *In vivo* reporters indicate that both this loop and the Mn^{2+} -binding core are required for optimal pH-dependent translational activation: replacing the core with the non-pH-responsive *mntP* sequence abolishes activation. These results define how RNA allosterically integrates orthogonal metal and proton cues to enable combinatorial environmental sensing during alkaline stress.

Introduction

Bacteria inhabit diverse, fluctuating environments and have evolved rapid responses to numerous, often overlapping stresses, such as alkaline pH and changes in micronutrient metal availability^{1,2}. Transition metals are essential cofactors for nearly half of all cellular enzymes, but an excess of these metal ions can compromise cell survival^{3,4}. Riboswitches are one gene regulatory mechanism by which bacteria achieve metal ion homeostasis^{5,6}. These structured non-coding RNA elements are embedded within the 5' untranslated regions (5'-UTR) of messenger RNAs (mRNAs). Upon binding their cognate ligands with exquisite selectivity, riboswitches undergo conformational changes that promote or inhibit expression of a downstream gene⁷, thereby acting as allosteric biomolecular sensors of the cellular state. Each riboswitch comprises a ligand-binding aptamer domain and an expression platform that either controls transcription termination or affects translation initiation by dictating mRNA ribosome binding site (RBS) accessibility⁷. Metal ion-binding riboswitches typically modulate the transcription or translation of genes encoding metal ion transport proteins⁶. A variety of metal ions serve as ligands, including magnesium (Mg)^{8,9}, manganese (Mn)^{10,11}, cobalt (Co)¹², nickel (Ni)¹², lithium (Li)¹³, and sodium (Na)¹⁴. Among these, Mn²⁺-sensing motifs constitute the largest class of metal-ion sensing riboswitches and belong to the *yybP-ykoY* family that is broadly distributed among bacteria^{5,15}.

One unique member of the *yybP-ykoY* family found in *Escherichia coli* (*E. coli*), *alx*, was originally identified as a “pH-responsive element” that activated translation of the downstream gene at alkaline pH¹⁶. Later, *alx* was shown to be a Mn²⁺-sensing riboswitch¹⁰, and the protein product of the downstream gene was recently characterized as an Mn²⁺ exporter¹⁷. *In vivo* gene reporters showed that, while Mn²⁺ supplementation alone boosts *alx* translation ~20-fold, translation increases ~70-fold when *E. coli* are cultured at alkaline pH¹⁷, demonstrating the remarkable ability of the *alx* riboswitch to incorporate two distinct inputs (metal ion and proton

concentration) into a single gene expression output. In contrast, a second member of the *yybP-ykoY* family also found in *E. coli* (*mntP*) likewise upregulates translation of an Mn^{2+} exporter as a function of Mn^{2+} concentration, but shows no translational response to alkaline pH¹⁷.

The structures of several representative *yybP-ykoY* aptamers have been characterized, with riboswitches from *L. lactis*, *X. oryzae*, and *E. coli* (*mntP*) assuming a four-way junction (4WJ) architecture^{11,18}. The 4WJ is comprised of four helices (P1-P4), which organize into two helical legs that dock to stabilize the Mn^{2+} -binding site^{11,18}. In contrast, the *alx* aptamer assumes a three-way junction (3WJ) architecture with a “capping loop”, L2, replacing the 4WJ P2 helix¹⁹ (Fig. 1a). Despite this difference, both the 3WJ and 4WJ aptamers share a structurally conserved metal-ion binding core. This core is composed of a conserved loop L1 in the “right leg” of the aptamer and a variable loop L3 in the “left leg” that juxtapose to coordinate two metal ions (Fig. 1a)¹¹. One metal-ion binding site is occupied by a Mg^{2+} ion (M_A) – the dominant, millimolar cellular divalent metal ion – whereas the other has micromolar affinity for Mn^{2+} (M_B), specified by a direct contact with N7 of an absolutely conserved adenosine in L3 (A37 in *alx*; Fig. 1a)^{11,19,20}.

Previous single-molecule Förster Resonance Energy Transfer (smFRET) studies of 4WJ *yybP-ykoY* aptamers from *X. oryzae* and *L. lactis* demonstrated that, at physiological Mg^{2+} concentration, the RNA transiently samples the docked state, but becomes stably docked only in the presence of Mn^{2+} ^{18,21,22}. Consistent with this behavior, molecular dynamics (MD) simulations indicated that Mn^{2+} binding is required to stabilize base stacking in the L3 loop^{18,23}. A recent co-transcriptional *in vitro* folding analysis of the *E. coli* *mntP* and *alx* riboswitches found that alkaline pH alone does not appreciably alter overall folding of the *alx* riboswitch aptamer or its expression platform, but instead appears to modulate Mn^{2+} binding to the 3WJ²⁴. *In vivo* translation reporter assays further showed that an A37U mutation of the Mn^{2+} -coordinating adenosine – a mutation

that abolishes Mn^{2+} -dependent docking in the *X. oryzae* 4WJ aptamer¹⁸ – abrogates pH-dependent translation activation of the 3WJ *alx* aptamer²⁴, suggesting that coupled pH and Mn^{2+} sensing may trigger exporter gene expression before Mn^{2+} toxicity rises under alkaline stress²⁴. Notably, both *alx* and *mntP* aptamers dynamically sample ambient Mn^{2+} during co-transcriptional folding²⁴. However, the molecular mechanism underlying pH-adaptive tuning of ligand binding remains unresolved; defining it should reveal principles by which RNAs can reweight their folding ensembles to allosterically integrate orthogonal chemical inputs, with implications for natural regulatory logic and the engineering of multi-input RNA sensors.

To address this gap, we used smFRET to resolve the real-time tertiary structure dynamics of the *alx* 3WJ riboswitch aptamer and found that pH modulates both aptamer docking and Mn^{2+} binding. In the absence of Mn^{2+} , the *alx* aptamer exhibits low occupancy of a stably docked conformation at neutral pH, which is lost under alkaline conditions as the RNA remains predominantly undocked. By contrast, at sub-saturating Mn^{2+} , alkaline pH more effectively promotes the stably docked state than neutral pH, thereby priming the aptamer for increased Mn^{2+} sensitivity in alkaline environments. MD simulations identify a pH-dependent nucleobase protonation event in the flexible L2 capping loop – unique to the 3WJ aptamer – that reorients the loop and provides a structural basis for pH-dependent docking and Mn^{2+} sensitization. Consistent with this model, translation reporter assays show that the resulting alternative base pairing in L2 is required for alkaline activation *in vivo*. Moreover, *in vivo* reporter analyses establish that the native *alx* L3 docking loop within the Mn^{2+} -binding core is also required for pH-dependent Mn^{2+} responsiveness, supporting long-range allosteric coupling. Together, these results define a multipartite mechanism by which pH tunes riboswitch conformational dynamics to integrate metal and proton cues within a single aptamer and mitigate cellular Mn^{2+} toxicity under alkaline stress.

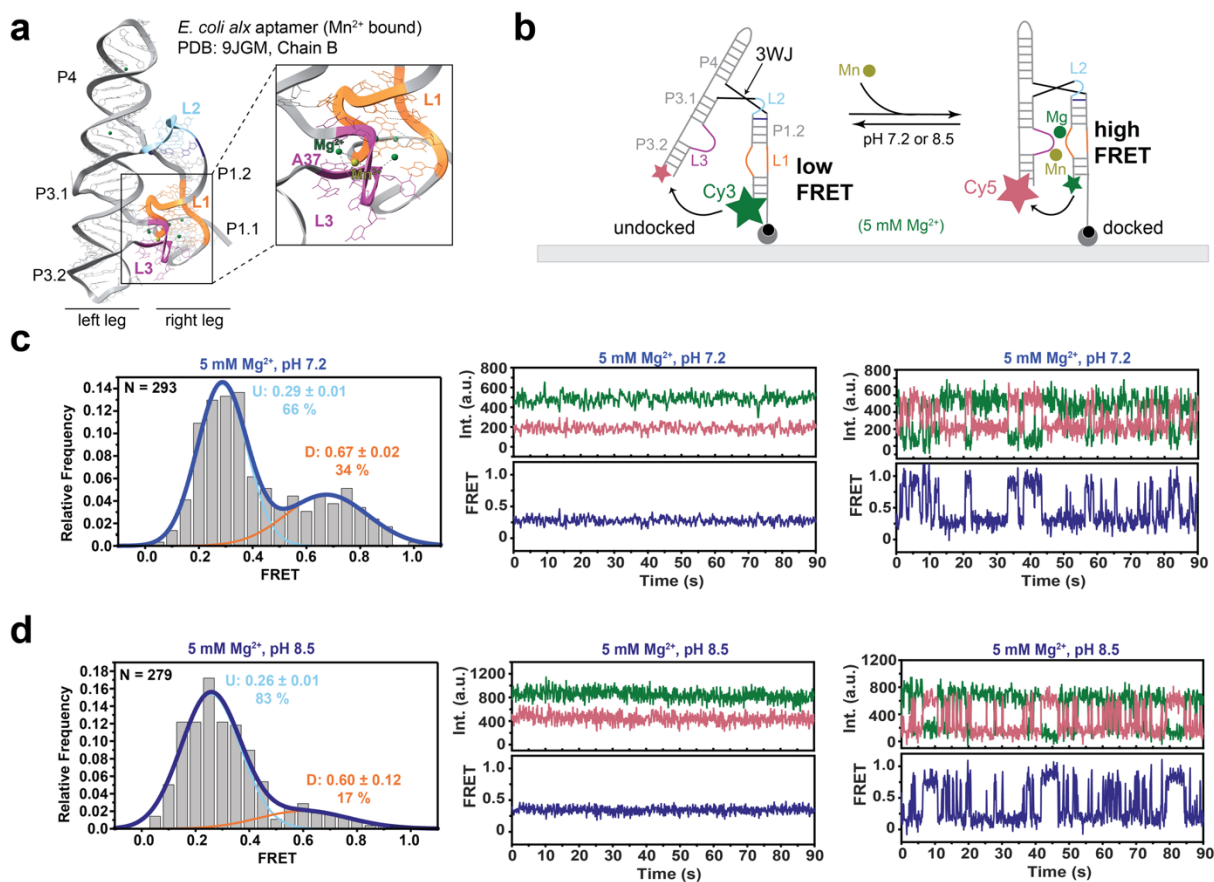


Figure 3.1. *alx* aptamer architecture and dynamics captured by smFRET at neutral and alkaline pH.

a, *alx* aptamer crystal structure with a zoomed-in panel for the Mn^{2+} binding site (PDB: 9JGM, Chain D). **b**, Experimental setup for smFRET experiments. **c,d**, Histograms and representative traces showing the fraction of low and high FRET molecules at (c) pH 7.2 and (d) pH 8.5.

Results

In the absence of Mn^{2+} , alkaline pH reduces aptamer docking

To assess the 3WJ *alx* aptamer global dynamics, we used smFRET to monitor aptamer docking in real time under alkaline pH and elevated Mn^{2+} conditions. A FRET fluorophore pair (Cy3 and Cy5) was positioned on each leg of the riboswitch aptamer, and the relative fluorophore intensities were used to identify the low-FRET (undocked) and high-FRET (docked) states (Fig. 1b and Extended Data Fig. 1). smFRET traces at near-physiological Mg^{2+} concentration (5 mM)

in the absence of Mn^{2+} revealed a highly dynamic ensemble of RNA molecules at both pH 7.2 (neutral) and pH 8.5 (alkaline). At pH 7.2, 66% of imaged aptamer molecules assumed the undocked conformation (Fig. 1c) compared to 83% at pH 8.5 (Fig. 1d). The mean low-FRET values are similar at both pHs (0.29 at pH 7.2 versus 0.26 at pH 8.5), indicating that the RNA samples very similar low-FRET states regardless of pH (Fig. 1c,d). Under all conditions, many traces captured transitions between low and high FRET states (Fig. 1c,d), consistent with smFRET data for the 4WJ Mn^{2+} -sensing aptamers at physiological Mg^{2+} conditions^{18,21,22}.

To further evaluate the docking dynamics, we employed transition occupancy density plots (TODP)²⁵, ensemble heatmaps representing each molecule's behavior: molecules undergoing at least one transition between FRET states appear off-diagonal, whereas molecules that never transition (i.e., stably remain in one state) are found on the diagonal (see Methods). At neutral pH (pH 7.2) in the absence of Mn^{2+} (with 5 mM Mg^{2+}), the TODP shows that ~17% of aptamer molecules are stably docked (SD), without noticeable undocking (Fig. 2a and Supplementary Table 5). In contrast, the 4WJ aptamers from *L. lactis* and *X. oryzae* show no significant SD complex unless Mn^{2+} is present^{18,22}. In addition, a significant fraction of the *alx* traces is dynamic and exhibits double-exponential FRET state interconversion kinetics with $k_{\text{dock,fast}} \sim 2.0 \text{ s}^{-1}$ (73%) and $k_{\text{dock,slow}} \sim 0.26 \text{ s}^{-1}$ (27%), as well as $k_{\text{undock,fast}} \sim 3.89 \text{ s}^{-1}$ (63%) and $k_{\text{undock,slow}} \sim 0.71 \text{ s}^{-1}$ (37%; Fig. 2e). Accordingly, ~30% of aptamer molecules populate a dynamically undocked (DU) state, whereas ~11% occupy a dynamically docked (DD) state. The remaining 42% are stably undocked (SU) (Fig. 2a and Supplementary Table 5). Overall, the weighted-mean docking rate constant ($k_{\text{dock,overall}} \sim 1.53 \text{ s}^{-1}$) is slower than the corresponding undocking rate constant ($k_{\text{undock,overall}} \sim 2.71 \text{ s}^{-1}$; Supplementary Table 6), favoring the undocked state.

Under alkaline conditions (pH 8.5) in the absence of Mn^{2+} , only ~9% and ~6% of traces reside in the SD and DD conformations, respectively (Fig. 2b and Supplementary Table 5). This pronounced loss of docking ability is consistent with prior smFRET analyses of the *L. lactis* and *X. oryzae* 4WJ Mn^{2+} -sensing aptamers^{18,22}. The docking kinetics of dynamic traces is again well described by a double-exponential model, with $k_{\text{dock,fast}} \sim 1.5 \text{ s}^{-1}$ (77%) and $k_{\text{dock,slow}} \sim 0.24 \text{ s}^{-1}$ (22%; Fig. 2f), whereas undocking occurs with $k_{\text{undock,fast}} \sim 3.13 \text{ s}^{-1}$ (86%) and $k_{\text{undock,slow}} \sim 0.41 \text{ s}^{-1}$ (14%; Fig. 2f). As at neutral pH, the weighted-mean docking rate constant $k_{\text{dock,overall}} \sim 1.21 \text{ s}^{-1}$ is slower than undocking at $k_{\text{undock,overall}} \sim 2.75 \text{ s}^{-1}$ (Supplementary Table 6). Relative to docking at neutral pH, $k_{\text{dock,overall}}$ decreases, whereas $k_{\text{undock,overall}}$ remains largely unchanged. Consistent with this shift, a slightly higher ~35% of traces populate the DU state, and only ~6% remain in the DD state—nearly a two-fold reduction compared to pH 7.2 (Fig. 2b and Supplementary Table 5). Notably, 50% of *alx* aptamer molecules reside in the SU state at alkaline pH, exceeding both the neutral pH fraction (42%) and the SU populations previously reported for the 4WJ *L. lactis* and *X. oryzae* aptamers^{18,22}. Together, these data indicate that, in the absence of Mn^{2+} , alkaline pH destabilizes docked conformations and slows docking transitions, biasing the 3WJ aptamer toward a more undocked ensemble that may prime it for increased Mn^{2+} sensitivity upon ligand availability.

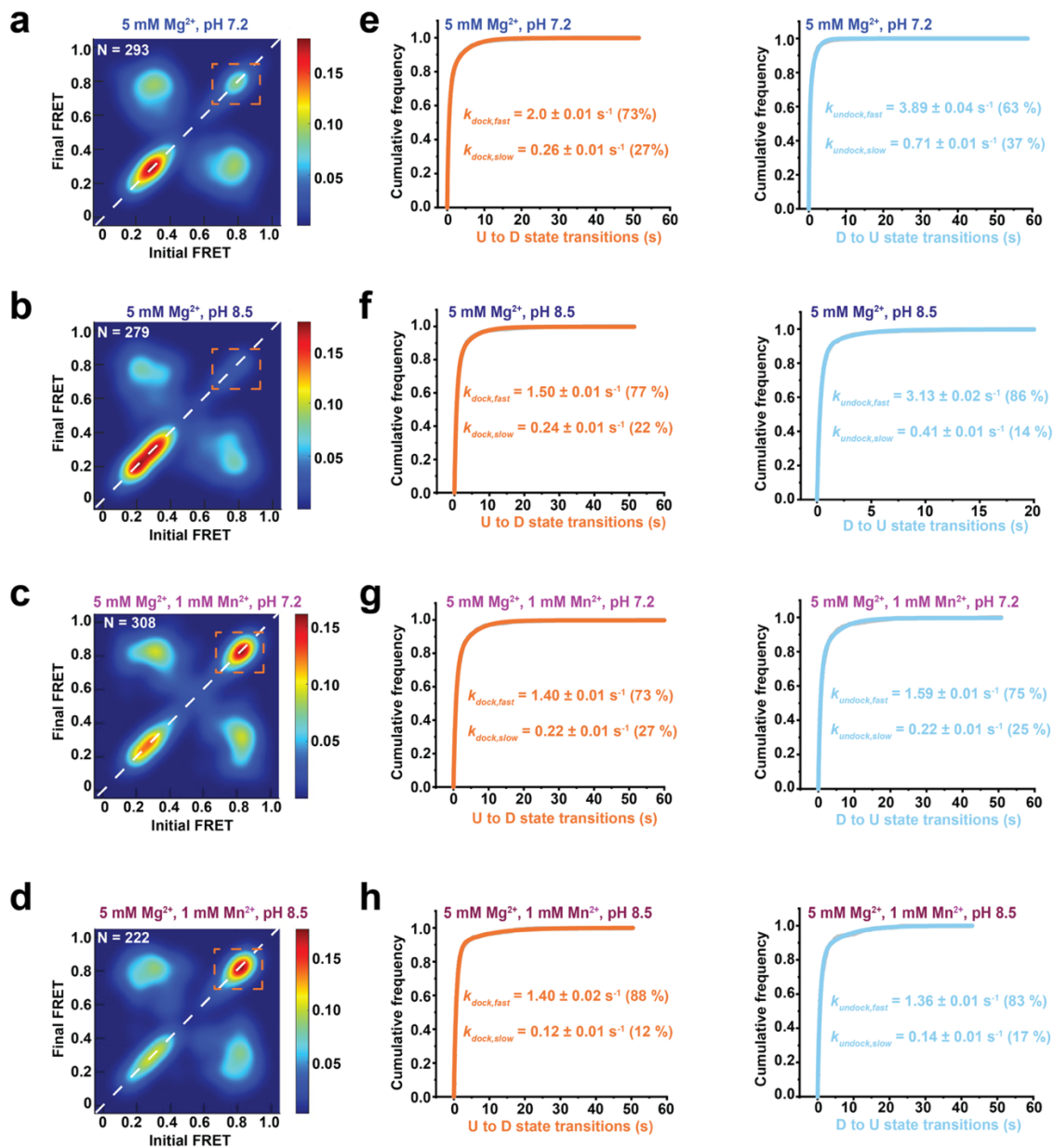


Figure 3.2. Saturating Mn²⁺ ligand stabilizes docked *alx* aptamer and impacts docking kinetics.

a-d, Transition occupancy density plot (TODP) showing initial FRET vs final FRET. Traces on the diagonal represent static molecules and traces off the diagonal represent dynamic molecules, with the stable docked (SD) conformation highlighted with the dashed box. TODPs for **(a)** pH 7.2 with 5 mM Mg²⁺, **(b)** pH 8.5 with 5 mM Mg²⁺, **(c)** pH 7.2 with 5 mM Mg²⁺ and 1 mM Mn²⁺, **(d)** pH 8.5 with 5 mM Mg²⁺ and 1 mM Mn²⁺. **e-h**, Kinetics for docking (U to D state transitions) and undocking (D to U state transitions) based on the cumulative dwell times in each state fit with double-exponential functions for **(e)** pH 7.2 with 5 mM Mg²⁺, **(f)** pH 8.5 with 5 mM Mg²⁺, **(g)** pH 7.2 with 5 mM Mg²⁺ and 1 mM Mn²⁺, **(h)** pH 8.5 with 5 mM Mg²⁺ and 1 mM Mn²⁺.

Saturating Mn^{2+} promotes docking independent of pH by slowing undocking

Despite the pH-dependent differences in the docking equilibrium of the apo *alx* aptamer, addition of 1 mM Mn^{2+} (orders of magnitude higher than physiological $\sim 20 \mu\text{M}^{17}$) to the aptamer produced remarkably similar smFRET traces at both neutral and alkaline pH, but consistently favored docking. In accordance with 4WJ Mn^{2+} -sensing aptamers, Mn^{2+} binding to the 3WJ *alx* aptamer stabilizes the SD conformation, with $\sim 29\%$ and $\sim 31\%$ SD molecules at pH 7.2 and pH 8.5, respectively (Fig. 2c,d and Supplementary Table 5). Additionally, the mean high FRET value upon Mn^{2+} binding at both pHs is 0.76, indicating that the RNA is sampling the same docked conformation (Extended Data Fig. 2a,b). Despite the presence of saturating Mn^{2+} , $\sim 30\%$ of molecules remain SU at both pHs. This is drastically different from the 4WJ *L. lactis* and *X. oryzae* aptamers, which dock completely with saturating $\text{Mn}^{2+18,22}$. At both pHs, around 40% of *alx* traces are dynamic in the absence and presence of Mn^{2+} , but significantly more of them are DD in the presence of 1 mM Mn^{2+} ($\sim 20\%$ versus $\sim 6\%$ in the absence of ligand). The increase in DD traces upon Mn^{2+} binding correlates with a decrease in DU traces (Supplementary Table 5), serving as additional evidence that Mn^{2+} promotes the docked conformation. These dynamic molecules again exhibit double-exponential kinetics, with $k_{\text{dock,overall}} \sim 1.08 \text{ s}^{-1}$ and $k_{\text{undock,overall}} \sim 1.25 \text{ s}^{-1}$ at pH 7.2 and $k_{\text{dock,overall}} \sim 1.25 \text{ s}^{-1}$ and $k_{\text{undock,overall}} \sim 1.15 \text{ s}^{-1}$ at pH 8.5 (Fig. 2g,h and Supplementary Table 6). Rather than speeding *alx* aptamer docking, ligand binding slows undocking by >2 -fold regardless of pH. Similarly, Mn^{2+} binding slows aptamer undocking for the 4WJ *L. lactis* and *X. oryzae* aptamers, indicative of a conserved mechanism for the effect of Mn^{2+} binding in the *yybP-ykoY* riboswitch family^{18,21,22}. Overall, regardless of pH, ligand binding shifts the *alx* aptamer conformational equilibrium toward the docked state, primarily by slowing undocking.

Alkaline pH sensitizes the 3WJ *alx* aptamer to Mn^{2+} *in vitro*

Because pH changes did not alter *alx* aptamer dynamics at saturating ligand, we hypothesized that alkaline pH instead biases the *alx* aptamer toward an open conformation that increases sensitivity to Mn^{2+} . To test this idea, we recorded smFRET traces at pH 7.2 and pH 8.5 in the presence of a low Mn^{2+} concentration (1 μM ; Fig. 3a,b). At pH 7.2, a 72% population resides in the undocked state with a low FRET value of 0.28 (Fig. 3a), comparable with the no- Mn^{2+} condition. Accordingly, the TODPs showed largely unchanged dynamics upon addition of 1 μM Mn^{2+} at pH 7.2 (Fig. 2a and Fig. 3c), and undocking kinetics were essentially identical to no Mn^{2+} (Extended Data Fig. 3a), indicating that 1 μM Mn^{2+} is insufficient to perturb *alx* aptamer dynamics at neutral pH. In contrast, at pH 8.5, 1 μM Mn^{2+} significantly shifted the population toward the docked conformation (37% docked; Fig. 3b) compared to 17% in the absence of Mn^{2+} (Fig. 1d). Moreover, the conformational dynamics at pH 8.5 with 1 μM Mn^{2+} closely resembled those at saturating 1 mM Mn^{2+} (Fig. 2b and Fig. 3d), with ~26% SD and ~18% DD traces (Fig. 3d and Supplementary Table 5). Fits yielded $k_{\text{dock,overall}} \sim 1.20 \text{ s}^{-1}$ and $k_{\text{undock,overall}} \sim 1.89 \text{ s}^{-1}$ at pH 8.5 with 1 μM Mn^{2+} (Extended Data Fig. 3b and Supplementary Table 6). Although 1 μM Mn^{2+} slows undocking less than 1 mM Mn^{2+} , the reduction remains substantial. Together, these data indicate that alkaline pH uniquely enables the *alx* aptamer to bind and respond to low ligand concentrations to trigger an effective gene regulatory response.

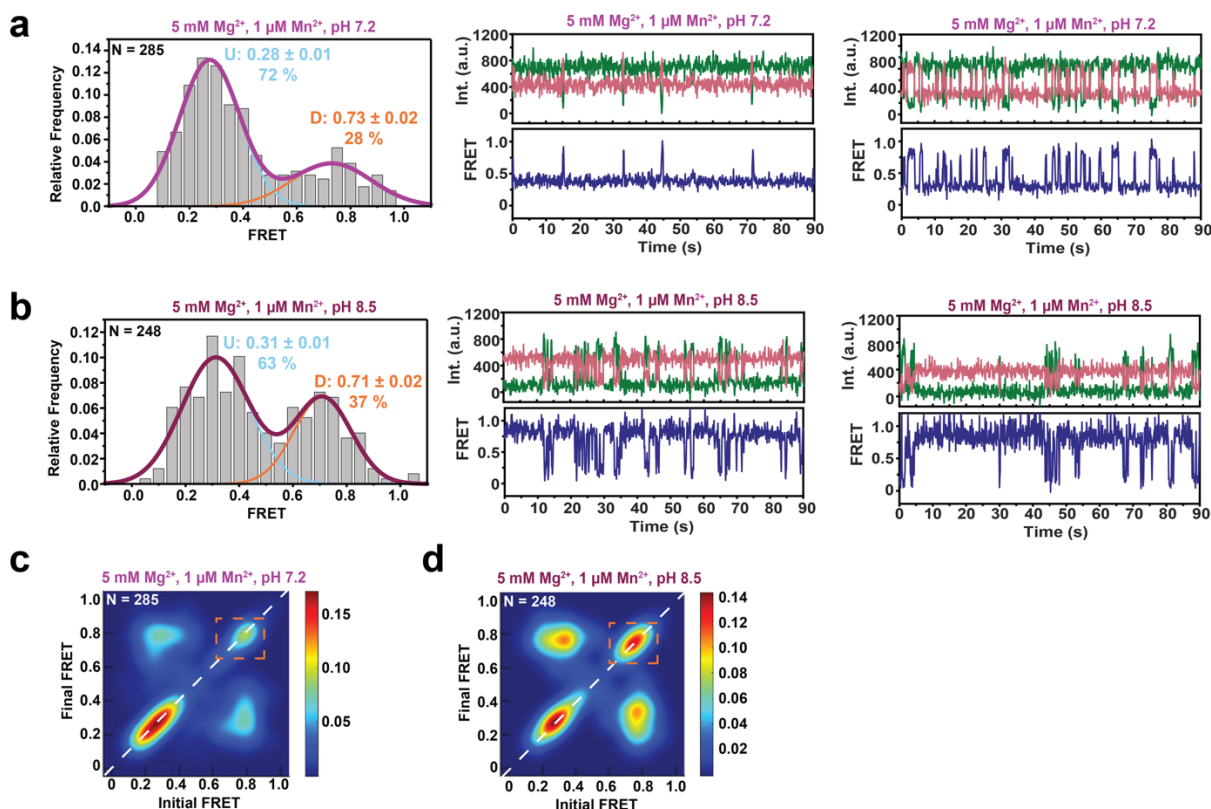


Figure 3.3. Low Mn^{2+} concentration uniquely promotes docked *alx* aptamer at alkaline pH.

a,b, Histograms and representative traces showing the fraction of low and high FRET molecules with $1 \mu M Mn^{2+}$ at **(a)** pH 7.2 and **(b)** pH 8.5. Histograms display the Gaussian peaks for low FRET, high FRET, and cumulative fit with the percentage of each FRET state and the number of molecules (N) analyzed. **c,d,** TODPs showing the initial FRET vs final FRET with $1 \mu M Mn^{2+}$ at **(c)** pH 7.2 and **(d)** pH 8.5. Traces on the diagonal represent static molecules and traces off the diagonal represent dynamic molecules, with the stable docked (SD) conformation emphasized in the dashed box.

The L2 capping loop couples pH sensing to Mn^{2+} -dependent gene activation

In contrast to the canonical 4WJ *yypP-ykoY* aptamer architecture, *alx* adopts a 3WJ fold in which the P2 helix is replaced by a single-stranded loop (Fig. 4a)¹⁹. In the *alx* aptamer crystal structure, L2 is stabilized by two P1 base pairs, U18·A114 and C19·G23, forming a 3-nucleotide (nt) “capping loop”¹⁹. Because L2 is unique to *alx* and located at the junction between the two docking legs, we hypothesized that it contributes to pH-dependent Mn^{2+} sensing.

To evaluate this idea, we reanalyzed previously reported co-transcriptional RNA structure probing data for the *alx* riboswitch collected at pH 7.2 or pH 8.5 with or without 1 mM Mn^{2+24} , matching the conditions used here. Probing was performed with DMS, which under these conditions primarily reports on A(N1) and C(N3) accessibility²⁶; thus our analysis focuses on A and C nucleobases. A114 and A115 shows reduced DMS reactivity at pH 8.5 relative to pH 7.2, independent of Mn^{2+} (Extended Data Fig. 4a,b). C19 also exhibits a marked decrease in reactivity at pH 8.5, but only beginning at transcript length of 127 nt, after the transcription of the A114 and A115 base-pairing partners (Extended Data Fig. 4c). These findings indicate that the structure of L2 is more stabilized at alkaline pH, whereas its higher DMS reactivity at pH 7.2 is consistent with greater flexibility. As expected for a loop nucleotide, A20—predicted to be highly flexible—remains highly reactive at both pH values throughout aptamer synthesis (Extended Data Fig. 4d).

These pH-dependent changes led us to hypothesize that pH may be altering the length of the L2 capping loop to modulate docking in the 3WJ *alx* aptamer: protonation of A114 could enable a 4-nt RNA via formation of a $\text{C19}\cdot\text{A114}^+$ wobble pair, whereas A114 deprotonation at alkaline pH would favor a strained 3-nt capping loop stabilized by canonical base pairing (Fig. 4b). Formation of the non-canonical $\text{C19}\cdot\text{A114}^+$ interaction, together with the disruption of the adjacent $\text{U17}\cdot\text{A115}$ base pair in the adjacent helix P1.2, could account for the higher DMS reactivity of these nucleotides at pH 7.2 (Fig. 4b and Extended Data Fig. 4a-c). Conversely, strain associated with a 3-nt loop at alkaline pH may contribute to the static undocked conformation observed by smFRET at pH 8.5 (Fig. 2b). Although adenosines have a solution pK_a of ~ 3.5 , local RNA environments can substantially shift pK_a values²⁷, and $\text{C}\cdot\text{A}^+$ wobble pairs can be functionally essential in cells^{28,29}.

To test whether C19·A114⁺ pairing is possible under our experimental conditions, we performed molecular dynamics (MD) simulations based on the published *alx* aptamer crystal structure (PDB ID: 9JGM)¹⁹, with either neutral or protonated A114. The neutral A114 maintained stable base pairing over the 200 ns (Fig. 4c). Constant-pH MD (CpHMD) simulations of the native structure yielded a neutral A114 across the tested pH range (3.0-7.0), consistent with stabilization of the U18·A114 Watson-Crick base pair^{30,31}. In contrast, during simulations initiated with a protonated adenosine, the U18·A114 Watson-Crick pair ruptured; however, A114⁺ and C19 remained separated (>3 Å), preventing spontaneous formation of an C19·A114⁺ wobble (Extended Data Fig. 5a,b). Forcing an C19·A114⁺ wobble enabled hydrogen bonding, but the interaction was unstable (Extended Data Fig. 5b).

Because flanking base pairs can stabilize wobble interactions²⁷, we next considered formation of an additional Watson-Crick pair (U18·A115) involving A115 from the adjacent helix P1.2. Enforcing simultaneous C19·A114⁺ and U18·A115 pairing, guided by a model duplex containing a wobble base pair flanked by a A·U pair³², produced a metastable structure (Fig. 4d and Extended Data Fig. 5b and 6b), supporting the potential of C19·A114⁺ formation at neutral pH with an accompanying rearrangement of helix P1.2 in the aptamer's "right" leg. Notably, the titrating proton (N1H of A114) could also be stabilized by acceptors beyond the N3 of C19 (Fig. 4e and Extended Data Fig. 7a). Across simulations, structural analysis of A114 protonation supports a 4-nt L2 capping loop, with C19·A114⁺ stabilized by at least one hydrogen bond involving the titrating proton (Fig. 5a and Extended Data Fig. 7b), whereas the absence of A114 protonation at alkaline pH favors the formation of a 3-nt L2 loop (Extended Data Fig. 8). To estimate the pK_a for the A114 N1H, we restrained the fully formed C19·A114⁺ wobble pair and performed constant pH simulations³³, yielding a microscopic pK_a of ~12 for this conformation

(Fig. 5b). Because the macroscopic pK_a reflects a weighted average across conformations, and because the $C19 \cdot A114^+$ wobble was only partially stable in our simulations, the effective pK_a could be closer to 8.5, consistent with the strong *alx* induction *in vivo*¹⁷ and pronounced smFRET changes *in vitro* (Fig. 3d).

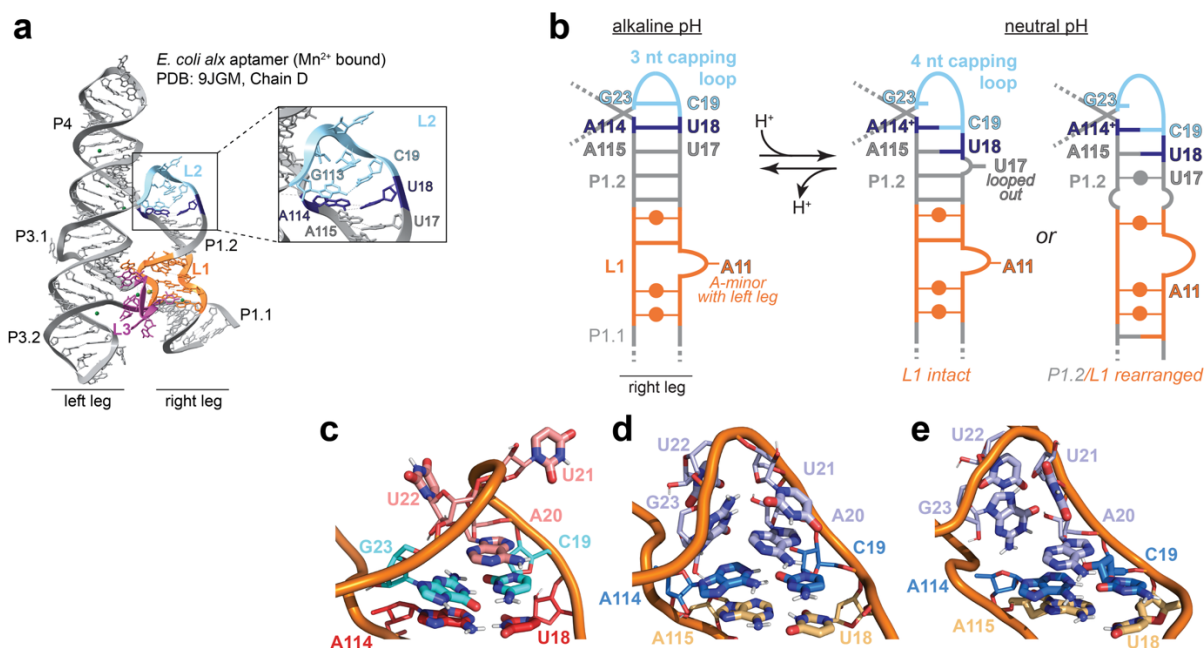


Figure 3.4. L2 capping loop length depends on A114 protonation and stability of key base pairs.

a, *alx* aptamer crystal structure with a zoomed-in panel for L2, with key nucleotides labeled (PDB: 9JGM, Chain D). **b**, Cartoon representation of proposed alternative base pairing at alkaline and neutral pH, with key nucleotides labeled. **c-e**, Cartoon representations of L2 capping loop with the **(c)** native, **(d)** wobble, and **(e)** an alternative meta-stable state, generated with PyMOL³⁴. In panel (c), the 3-nt L2 loop formed by U22, U21, and A20 (pink) requires stable U18·A114 (red) and C19·G23 (cyan) base pairs. In panels (d) and (e), the 4-nt L2 loop (light purple) requires the U18·A115 (beige) base pair and, at least, the $O2 \cdot N1H^+$ hydrogen bond formed within the $C19 \cdot A114^+$ (blue) base pair.

Guided by these simulations, we next tested the contribution of L2 to alkaline activation *in vivo*. We constructed *alx*-controlled translational *lacZ* gene reporters in single-copy plasmids bearing point mutations (A114G, C19U, and A114C) and quantified Mn^{2+} - and pH-dependent

expression using β -galactosidase assays, as previously described¹⁷. Two mutations, A114G and C19U, were designed to stabilize a 4-nt L2 capping loop, whereas A114C was expected to disrupt L2 base pairing. A114G and C19U did not significantly affect Mn^{2+} responsiveness compared to wild-type *alx* (Fig. 5c). At pH 8.5, A114G retained wild-type activation, while C19U reached 50-fold translation activation compared to 61-fold for wild-type *alx* (Fig. 5d). In contrast, A114C both abolished the Mn^{2+} response and strongly reduced pH-dependent translation activation (Fig. 5c,d). At a slightly less alkaline pH (pH 8.3), wild-type *alx* displayed 26-fold activation, while A114G and C19U were significantly reduced (12-fold and 9-fold, respectively; Extended Data Fig. 9), supporting an apparent pK_a near ~ 8.5 for the pH-sensitive nucleoside(s) within the *alx* riboswitch. Together, our MD simulations and *in vivo* mutational analyses indicate that base pairing within the flexible L2 capping loop—likely involving pH-induced alternative pairing—is critical for pH-dependent translation activation, but becomes less relevant under increasingly alkaline conditions.

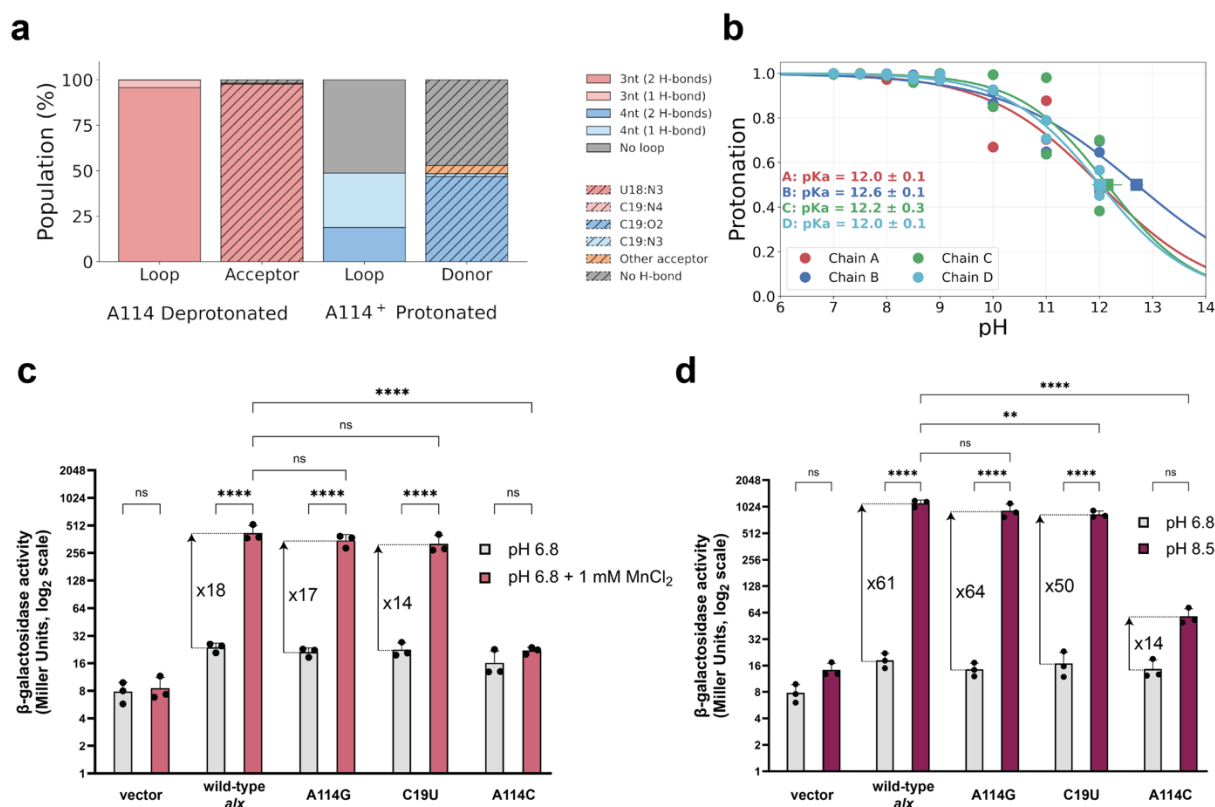


Figure 3.5. L2 capping loop mutations reduce pH-dependent *alx* translation activation.

a, Predicted relative population (%) of loop-length states and specific hydrogen-bond contacts shown by the hydrogen-bonding role (acceptor or donor) of A114 dependent on the protonation state (deprotonated/protonated) and grouped by the number of hydrogen-bonds. **b**, Titration curves and microscopic pK_a values of A114 obtained from MD simulations of the wobble conformation state at constant pH, for each crystallographic chain (A-D). Mean protonation values from independent replicates were fit to the Henderson–Hasselbalch equation. Upshifted pK_a values highlight the proton chemical stability even in highly basic environments, as a result of the specific hydrogen-bond network. **c,d**, Translational response for L2 mutants compared to wild-type *alx* for growth media **(c)** supplemented with Mn^{2+} and **(d)** buffered at alkaline pH. Statistical analysis done using a two-way ANOVA with Tukey post-hoc test for multiple comparisons to assess significance (^{ns} $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

The metal-binding L3 loop sequence is also required for the pH-dependent Mn^{2+} response

Because L2 capping loop mutations reduced—but did not abolish—pH-enhanced translation activation, we reasoned that additional riboswitch elements contribute to the full pH-dependent Mn^{2+} response of *alx*. Prior work showed that alkaline pH affects Mn^{2+} binding by *alx*

and the pH-dependent gene expression response requires Mn^{2+} binding to the aptamer²⁴. In *yybP-ykoY* riboswitches, the Mn^{2+} -binding site forms between the conserved L1 loop and the variable L3 loop, which positions the conserved adenosine (A37 in *alx*) for direct coordination of Mn^{2+} (Fig. 6a)^{11,20}. Across the family, L3 is ubiquitous: 28% of aptamers contain the *mntP* sequence (ACAUACU), whereas only 3% contain the *alx* sequence (UCAUUAC) (Fig. 6b); the remaining ~70% differ from both by at least one nucleotide. Although the overall Mn^{2+} -binding-site architecture appears similar across L3 variants (Extended Data Fig. 10a), we hypothesized that nucleotide identity within L3 modulates the stacking interactions that underlies pH-dependent Mn^{2+} binding. Specifically, we reasoned that after the riboswitch legs dock (tuned in part by the L2 capping loop), the L3 sequence further contributes to pH-dependent Mn^{2+} binding.

To test this notion, we swapped the L3 sequences between the pH-responsive *alx* aptamer and the non-pH responsive *mntP* aptamer¹⁷ in their respective translational reporter constructs and quantified output using *in vivo* β -galactosidase assays. The L3^{alx} *mntP* chimera completely lost the Mn^{2+} responsiveness and did not yield any gain in pH-dependent translation activation (Fig. 6c,d). Conversely, L3^{mntP} *alx* retained Mn^{2+} responsiveness, exhibiting a 13-fold induction at saturating Mn^{2+} compared with an 18-fold increase for wild-type *alx*, (Fig. 6c), but strikingly lost pH-dependent activation at pH 8.5 (Fig. 6d). These data demonstrate that the wild-type *alx* L3 sequence is essential for alkaline activation in cells.

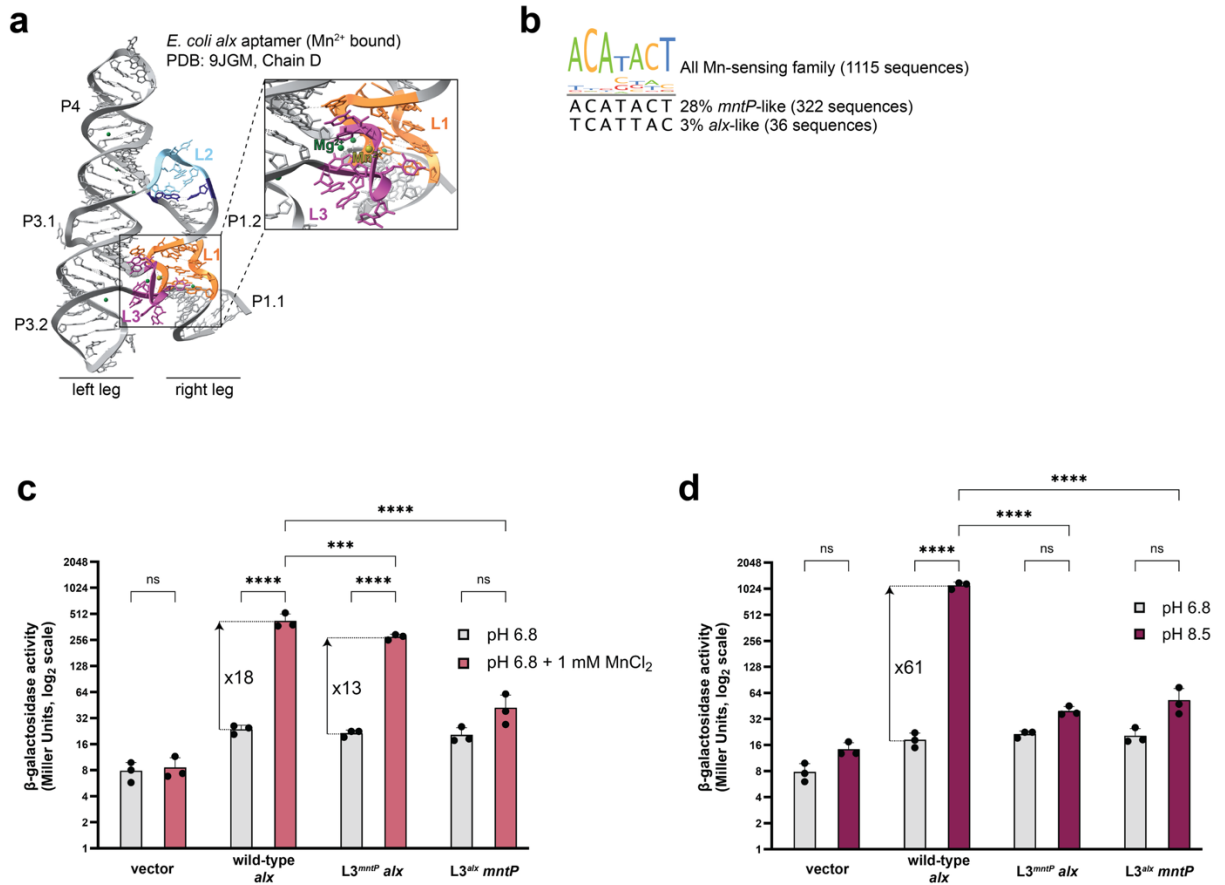


Figure 3.6. Native *alx* L3 loop sequence is essential for pH-dependent translation activation.

a, *alx* aptamer crystal structure with a zoomed-in panel for Mn^{2+} binding site, with key loops L1 and L3 labeled (PDB: 9JGM, Chain D). **b**, L3 consensus sequence from *yybP-ykoY* family members in Rfam database, with the percent of *mntP*-like and *alx*-like sequences displayed below the consensus sequence. **c,d**, Translational response for L3 loop swap (between *alx* and *mntP*) mutants compared to wild-type *alx* for growth media **(c)** supplemented with Mn^{2+} and **(d)** buffered at alkaline pH. Statistical analysis done using a two-way ANOVA with Tukey post-hoc test for multiple comparisons to assess significance (^{ns} $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

To assess whether this alkaline stress response mechanism might be conserved across Mn^{2+} -sensing riboswitches, we aligned all *yybP-ykoY* family members (1,115 sequences in Rfam)³⁵, filtered for sequences containing the *alx* L3, and used RNAfold³⁶ to identify aptamers predicted to adopt an *alx*-like 3WJ architecture, given the apparent importance of the L2 capping loop (Supplementary Table 7). Phylogenetic analysis showed that *yybP-ykoY* members with the

alx L3 sequence are restricted to the *Bacillales* order, with 67% belonging in the *Bacillaceae* family (Extended Data Fig. 10b). Predicted *alx*-like 3WJ architectures are distributed more broadly across multiple families (Extended Data Fig. 10b). Notably, organisms containing both the *alx* L3 sequence and 3WJ architecture are found in soil or ocean environments and are capable of growth at alkaline pH^{37–42}, with most organisms optimally growing at alkaline pH^{37–41}. Together, our observations suggest that both the 3WJ global structure and the specific nucleotide context within the Mn²⁺-binding site are required for bacteria optimally adapted to alkaline stress^{37–42}.

Discussion

Integrating smFRET, MD simulations, and *in vivo* gene reporters, we uncover how two distinct cellular cues—pH and Mn²⁺—are coupled to empower a rapid bacterial response to alkaline stress through riboswitch-mediated induction of a Mn²⁺ exporter. The 3WJ *alx* aptamer is strongly pH sensitive: alkaline pH favors an undocked (open) global architecture, whereas neutral pH supports more docked sampling, thereby priming the aptamer to specifically respond to low- μ M Mn²⁺ under alkaline conditions. Our data further identify two key regions that together enable this dual-input behavior: the L2 capping loop at the junction between the docking legs and the L3 loop within the metal-binding core. In this way, *alx* provides an example of combinatorial gene regulation in bacteria in which riboswitch signal integration is key for cell survival in an alkaline habitat. Whereas eukaryotes often deploy large repertoires of transcription factors to integrate inputs⁴³, bacteria frequently rely on such RNA-based integrative regulation to match metal-ion and metabolite availability to cellular demands^{44,45}.

The pH-dependent control of Mn²⁺ export may be particularly important during alkaline stress. At elevated pH, Mn(II) is oxidized to Mn(IV)^{46–48} and Mn²⁺ hydroxide can promote RNA and DNA cleavage. Alkaline pH also exasperates oxidative stress by increasing free radical oxygen

species (ROS)⁴⁹. Conversely, Mn^{2+} can mitigate oxidative stress by serving as a cofactor for enzymes such as superoxide dismutase that break down ROS^{50,51}. In fact, intracellular Mn^{2+} increases ~1.5-fold in *E. coli* at alkaline pH¹⁷. Additionally, during oxidative stress, Mn^{2+} can substitute for iron in certain enzymes to partially restore enzymatic activity while limiting damage to the enzymatic active site from oxidized iron^{52,53}. However, excess free Mn^{2+} is toxic⁵⁴, necessitating tight homeostatic control. A dual Mn^{2+} /pH sensing riboswitch that tunes Mn^{2+} exporter expression may therefore provide a robust mechanism to balance protection and toxicity in ecological niches that impose alkaline stress.

The *yybP-ykoY* aptamer core harbors binding sites for both Mg^{2+} and Mn^{2+} , underscoring a likely role for Mg^{2+} in pre-organizing docking. Indeed, prior smFRET studies of *yybP-ykoY* aptamers observed no docked traces in monovalent cation-only buffers ($K^{+18,22}$ or Na^{+21}): without divalents, these aptamers were completely undocked (SU), whereas near-physiological Mg^{2+} promoted both DU and DD conformations in which the two helical legs transiently dock^{18,21,22}. Consistent with these observations, Mg^{2+} alone is sufficient for *alx* to sample an SD conformation at pH 7.2, which may reduce the dynamic range for a Mn^{2+} -dependent response at neutral compared to alkaline pH. Similar Mg^{2+} -induced “folded-like” intermediates have been observed for other riboswitches, including preQ₁ (where ligand binding subsequently locks the fully folded state)^{55,56}, a nickel/cobalt (NiCo) riboswitch (where Mg^{2+} and Na^{+} promote folding and slow unfolding, even without ligand)⁵⁷, and a fluoride riboswitch (where Mg^{2+} pre-folds the aptamer and coordinates with the ligand to lock the folded state)⁵⁸. Together with our findings, these studies support a general model in which Mg^{2+} facilitates aptamer pre-organization, while ligand binding provides the final stabilization of the regulatory fold.

A central mechanistic theme is that the protonation state of RNA nucleotides can affect structure and catalytic activity^{27,59}. Although the solution pK_a values for guanosine and uridine are near 9.2, and those for adenosine and cytidine are ~3.5 and ~4.2, respectively²⁷, local RNA structural context can shift pK_a values toward physiological pH⁶⁰. Our MD simulations suggest that protonation of an adenosine within the strained L2 capping loop can enable alternative base pairing of C19·A114⁺ (Fig. 4d) accompanied by formation of U18·A115, reorganizing the top helix of the aptamer's "right" docking leg. We propose that this alternative pairing stabilizes a 4-nt L2 loop (Fig. 4b), thereby altering leg docking and Mn²⁺ binding in a pH-dependent manner. Because the docking timescales observed by smFRET are not readily accessible with direct simulation, it will be informative in future work to apply enhanced-sampling approaches for testing how adenosine protonation reshapes the docking landscape. More broadly, protonation-dependent non-canonical base pairing and conformational switching are established mechanisms in RNA structure and function^{61,62}, including pH-tuned wobble formation that modulates microRNA processing through a G·A⁺ pair⁶³, VS ribozyme activity via a C·A⁺ pair²⁸, bacterial group II intron splicing based on a G·A⁺ pair⁶⁴, eukaryotic spliceosomal catalysis enabled by a C·A⁺ pair^{29,65}, as well as protonation-linked conformational switches that reprogram translation readthrough⁶⁶ and frameshifting^{66,67}. Our study expands this repertoire with a regulatory example in which pH reshapes RNA dynamics to allosterically modulate ligand binding and, consequently, gene expression.

Functionally, our *in vivo* data support a key role for L2 in pH-dependent translation activation in response to Mn²⁺ binding. Mutations in L2 perturb alkaline activation, with a strongly disrupting transversion (A114C) abolishing Mn²⁺ responsiveness and markedly attenuating pH-dependent activation (Fig. 4c,d). More modest L2 mutations reduce pH-dependent activation more

prominently at slightly less alkaline pH (Extended Data Fig. 4e), indicating that additional RNA features contribute to the overall pH dependence. Consistent with this notion, prior co-transcriptional DMS probing data for *alx* showed that the absolutely conserved L3 adenosine that directly coordinates Mn^{2+} (A37) becomes substantially less reactive at alkaline pH in *alx*, whereas the corresponding position in the non-pH-responsive *mntP* does not²⁴. In the current work, we swapped the L3 sequences and found that the native *alx* L3 is required for alkaline activation in cells: replacing it with the *mntP* L3 preserves Mn^{2+} responsiveness but eliminates pH-dependent activation (Fig. 5d), whereas inserting the *alx* L3 into *mntP* disrupts Mn^{2+} responsiveness. Finally, our phylogenetic analysis showed that both the *alx* L3 sequence and *alx*-like 3WJ architecture are conserved in *Bacillales* from alkaline-associated environments (Extended Data Fig. 10b), suggesting that allosteric coupling of binding-site sequence and global fold may be conserved to support growth under alkaline stress.

Beyond its physiological role, the *alx* aptamer also offers a design principle for synthetic RNA sensors. RNA aptamers inspired by natural riboswitches have long been pursued as ligand biosensors and fluorogenic imaging tools^{68–70}, and recent efforts have sought single-molecule RNA sensors that integrate multiple inputs⁷¹. Such compact RNA sensors could complement established protein circuit strategies that require multiple interacting components^{72,73}. Natural multi-input control is often achieved by tandem riboswitches, in which multiple aptamers act as sequential checkpoints—either amplifying output by sensing the same ligand or implementing combinatorial control with distinct ligands^{74–76}. While effective *in vivo*, tandem riboswitch architectures require separate, compatible ligand-binding domains. In contrast, we show that *alx* represents a minimal, two-input allosteric sensor that readily integrates two distinct signals—pH

and Mn^{2+} —using only a single aptamer, providing a potential blueprint for engineering simpler multi-input RNA regulators.

Methods

Protein

Q5 High-Fidelity DNA Polymerase, T4 DNA Ligase, PstI-HF, and BamHI-HF were purchased from New England Biolabs. Glucose oxidase and catalase were purchased from Sigma Aldrich and resuspended according to manufacturer instructions. Details and catalog numbers for all commercially available proteins are available in Supplementary Table 1.

Oligonucleotide design

Custom oligos for smFRET were ordered from Revvity Inc (formerly Horizon Discovery). Custom oligo modifications of 5'-Cy3, 5'-Cy5, and 3'-biotin were added where indicated (Supplementary Table 2). All custom oligos were desalted, deprotected, and purified via HPLC. Other oligos used in this study were ordered from Eton Bioscience Inc and purified via desalting. All oligos used in this study are tabulated in Supplementary Table 2.

Bacterial strains

The strain used in this study is a derivative of *E. coli* K-12 (MC4100). The $\Delta alx::Kan$ mutation was sourced from the Keio collection⁷⁷ and introduced into the *E. coli* MC4100 strain via P1 transduction. All strains used in this study are tabulated in Supplementary Table 3.

Plasmids

The plasmid carrying the *alx* translation *lacZ* reporter gene fusion under the control of a native *E. coli* promoter is pRA54 and the empty vector plasmid is pMU2386. These were constructed in prior work and used as controls in this work (Supplementary Table 4)¹⁷. Translation *lacZ* reporter gene fusions of *alx* mutants were constructed via sequential PCR amplification from pRA54. The first amplification of pRA54 used forward primer 0504, which contains a PstI restriction site, and a reverse primer appropriate for introducing the desired L2 or L3 mutation(s) (Supplementary Table 2). The second amplification of pRA54 used a corresponding forward primer to introduce the desired L2 or L3 mutation(s) and reverse primer 0505, which contains a BamHI restriction site (Supplementary Table 2). The final PCR used primers 0504 and 0505 to combine these two fragments into the full mutant *alx* translational fusion insert. Translation *lacZ* reporter gene fusions of *mntP* mutants were constructed via sequential PCR from pRA57. The first amplification of pRA57 used forward primer 0506, which contains a PstI restriction site, and reverse primer 0511 appropriate for introducing the desired L3 mutations (Supplementary Table 2). The second amplification of pRA57 used corresponding forward primer 0510 to introduce the desired L3 mutations and reverse primer 0507, which contains a SalI restriction site (Supplementary Table 2). The final PCR used primers 0506 and 0507 to combine these two fragments into the full mutant *mntP* translation fusion insert. Then, the *alx* translational fusions were cloned into the PstI and BamHI sites of the single copy plasmid pMU2386. The *mntP* translation fusions were cloned into the PstI and SalI sites of the single copy plasmid pMU2386. The plasmids were assembled via restriction-ligation using T4 DNA Ligase (New England Biolabs) and sequence-verified by Sanger sequencing. All plasmids used in this study are tabulated in Supplementary Table 4.

smFRET data acquisition

smFRET experiments were performed using the Oxford Nanoimager (ONI) microscope in total internal reflection fluorescence (TIRF) mode. Movies were collected at 22 °C for 1,200 frames with 100 ms exposure time. PEG-passivated quartz slides were prepared as described in previous work⁷⁸. The surface of the slide was incubated with 0.2 mg/mL streptavidin for 10 minutes, then washed three times with the appropriate 1X smFRET buffer (50 mM HEPES pH 7.2 or pH 8.5, 100 mM KCl). RNA oligos were hybridized in a 1:20 ratio (10 nM alx_apt_oligo_2_Cy5_Bio:200 nM alx_apt_oligo_1_Cy3) (Supplementary Table 2), heat denatured at 90 °C for 2 min, snap cooled on ice for 3 min, and refolded in 1X folding buffer (50 mM HEPES pH 7.5, 100 mM KCl, 1 mM MgCl₂) at room temperature for 15 min. Diluted hybridized RNA samples (10-30 pM relative to alx_apt_oligo_2_Cy5_Bio) were immediately applied to the slide, incubated for 5 min at room temperature, then washed three times with appropriate 1X smFRET buffer (50 mM HEPES pH 7.2 or pH 8.5, 100 mM KCl). A freshly prepared enzymatic oxygen scavenging system (1X smFRET buffer, 5 mM MgCl₂, 44 mM glucose, 165 U/mL glucose oxidase from *Aspergillus niger*, 5 mM Trolox, 2,170 U/mL catalase from *Corynebacterium glutamicum*) was added along with MnCl₂, where indicated. Raw movies were collected for 120 sec with direct green laser (532 nm, 18% power) excitation for 100 s to detect smFRET, followed by addition of direct red laser (640 nm, 8% power) excitation for the final 20 s to identify the presence of Cy5.

smFRET data analysis

Location of molecules and their corresponding fluorophore intensity traces were extracted from the raw movie files (see above) using custom MATLAB codes²². Traces were visualized and

manually selected using MATLAB based on the following criteria: minimum intensity for Cy3 (200 A.U.), single step photobleaching of the dyes, and either longer than 20 s or having ≥ 1 clear transition between low and high FRET states. Selected traces were then background-subtracted to correct and minimal bleed through. The FRET ratio was calculated as I_A/I_A+I_D where I_A and I_D are the background-corrected intensities of Cy5 (acceptor) and Cy3 (donor), respectively. FRET histograms were made using the first 10 frames of all traces in each condition and were fit with a sum of Gaussians using OriginPro 10.2. For kinetic analysis, traces were idealized using a two-state model corresponding to low FRET (undocked) and high FRET (docked) states using the segmental k-means algorithm in QuB software as previously described^{25,79}. Cumulative dwell time histograms were plotted from all extracted dwell times and fit with double exponential functions using OriginPro 10.2 to obtain lifetimes in undocked (t_{undock}) and docked (t_{dock}) states. The rate constants for docking and undocking were calculated as follows: $k_{\text{dock}} = 1/t_{\text{undock}}$ and $k_{\text{undock}} = 1/t_{\text{dock}}$. Kinetics were calculated using both the short and long dwell times to calculate the fast and slow rate constants, respectively. Idealized smFRET traces were used for creating transition occupancy density plots (TODPs), which show the fraction of traces molecules undergo ≥ 1 transition between FRET states. In TODPs, dynamic traces showing a FRET transition (regardless of the number of transitions in the trace) and static traces (with no transitions in the trace) are weighted equally to avoid overrepresenting traces with many fast transitions. Graphs were visualized in GraphPad Prism.

β -galactosidase assays

Overnight culture of each strain was diluted 1:100 into LBK (LB media where KCl replaces NaCl) with appropriate antibiotic at either pH 6.8 or pH 8.5, with or without 1 mM supplemented

MnCl₂, where indicated. The cultures were grown to mid-log phase and β -galactosidase assays were performed using the Miller method⁸⁰. Briefly, bacterial growth was measured using OD₆₀₀ before cultures were placed on ice. 100 μ L cells were lysed via vortexing in 900 μ L sodium phosphate buffer (100 mM sodium phosphate pH 7.0, 10 mM KCl, 2 mM MgSO₄, 38 mM 2-mercaptoethanol), 20 μ L 0.1% SDS, and 20 μ L chloroform. Substrate *O*-nitrophenyl- β -D-galactopyranoside (ONPG) was added and the reaction was stopped with 1 M Na₂CO₃ when a yellow color appeared, or after 35 min. After centrifuging 5 min at 16,000g, absorbance was measured at 420 nm (A₄₂₀) to calculate activity using Miller units. Each culture was tested in duplicate and A₄₂₀ values were averaged. Each final reported value is an average of three replicates with standard deviation. Statistical analysis was done in GraphPad Prism using a two-way ANOVA with Tukey post-hoc test for multiple comparisons.

Sequence alignment and phylogenetic analysis

Sequences for *yybP-ykoY* riboswitches were downloaded from Rfam (Rfam number: RF00080)³⁵ and aligned using Kalign multisequence alignment (<https://www.ebi.ac.uk/jdispatcher/msa/kalign>). The sequence alignment was visualized using Jalview⁸¹. The L3 consensus sequence was visualized and exported from Jalview. The sequences were filtered by the *alx* L3 sequence and *alx* L3-containing sequences were exported (Supplementary Table 7). The phylogenetic tree was generated using phyloT (<https://phylot.biobyte.de/>) and the original Rfam organism names were updated according to NCBI taxonomy⁸², where indicated in bold (Supplementary Table 7). The phylogenetic tree was visualized using Interactive Tree Of Life (iTOL, <https://itol.embl.de/>).

Structure prediction and modeling

For *yybP-ykoY* riboswitch sequences containing *alx* L3, structure predictions were generated using RNAfold with default settings³⁶ and were manually noted as having *alx* or *mntP*-like architecture (Supplementary Table 7). Two-dimensional RNA structure models were visualized in VARNA⁸³. Crystal structures of the Mn-bound *alx* aptamer (PDB: 9JGM)¹⁹ and the Mn-bound *X. oryzae* aptamer (PDB: 6N2V)¹⁸ were visualized and colored in ChimeraX⁸⁴. Crystal structure alignments were also done in ChimeraX using the default matchmaker settings.

Co-transcriptional SHAPE-MaP single-nucleotide trajectories

Previous published raw sequencing data from co-transcriptional DMS-MaP of the *alx* riboswitch is available in the SRA under project number PRJNA1197522 (<https://www.ncbi.nlm.nih.gov/sra>)²⁴. This co-transcriptional structure probing was performed *in vitro* using the Variable Length Transcription Elongation Complex RNA Structure Probing (TECprobe-VL) method⁸⁵. This data from prior work contained the reactivities with DMS for nucleotides of interest at pH 7.2, pH 8.5, and $\pm$ Mn at every RNA transcript length. In the current work, we plotted the single nucleotide trajectories for nucleotides of interest in GraphPad Prism.

Molecular dynamics simulations

System setup

Molecular dynamics (MD) simulations were performed to characterize the conformational dynamics of the *alx* riboswitch aptamer under different structural and protonation conditions. Four simulation setups were prepared for standard MD conditions, combining two starting conformations (native and C19·A114 wobble base-pair states, see below for the procedure used to

model the wobble pair) with two protonation schemes for residue A114: fixed protonated and fixed deprotonated.

Stochastic titration constant-pH MD (CpHMD) simulations were performed using a discrete protonation framework as described in other works^{33,86,87}. CpHMD simulations were prepared starting from either the native and wobble states, with a single titrable residue (A114). In all simulations, all other nucleotides were kept in their standard neutral protonation states.

All systems were derived from each chain (A-D) of the Mn-bound *alx* aptamer crystal structure (PDB: 9JGM). Structural divalent ions present in the binding pocket were retained, though the Mn^{2+} ions were replaced by Mg^{2+} due to the lack of reliable force-field parameters for Mn^{2+} . No additional divalent ions were included.

Each system was solvated in a dodecahedral box to physiological conditions using 38,642 water molecules, 130 K^+ ions, and 31 Cl^- ions, to match a salt concentration of 100 mM and ensuring charge neutrality. System topologies were generated using a modified XOL3 force field for RNA^{33,88}, Joung–Cheatham parameters for monovalent ions⁸⁹, and the microMg model for Mg^{2+} ions⁹⁰. Water molecules were described using the OPC model⁹¹.

All simulations were performed using GROMACS 2022.3⁹² coupled to PLUMED 2.8.2^{93,94} at constant temperature (310 K) using a v-rescale thermostat⁹⁵, while the pressure (1 bar) was maintained using a c-rescale barostat⁹⁶. Covalent bonds were constrained using the LINCS algorithm⁹⁷, and long-range electrostatic interactions (at distance larger than 1.0 nm) were treated using the particle-mesh Ewald (PME) method⁹⁸. Each system was minimized before equilibration and other protocols.

Generation of wobble base-pair conformations

Wobble base-pair starting structures were generated from the native conformation for each crystallographic chain (A–D). The native U18·A114 base pair was converted to an C19·A114⁺ wobble base pair using eRMSD-based steered MD simulations^{32,33}. Steering enforced the formation of C19·A114 and U18·A115 base pairs. The latter was found pivotal to the wobble base pair stability, as found in hairpin structures studied by Wilcox and Bevilacqua et al³⁰. One of these sequences was used to generate a target geometry.

Steering protocols were performed using the eRMSD metric as implemented in PLUMED 2.8.2., which accounts for nucleobase distances and relative orientation to measure structural similarity. The eRMSD was computed using nucleobases 18, 19, 114, and 115. A harmonic moving restraint with force constant $\kappa = 10\,000\text{ kJ mol}^{-1}$ was applied to drive the eRMSD from its native value toward 0, corresponding to the target wobble base pair geometry of the reference structure. Each steered MD simulation was run for 200 ns.

For each chain (A–D), two replicates were performed corresponding to fixed protonated and fixed deprotonated states of A114 (see Supplemental Information). Following the steering phase, a second equilibration run of 200 ns was performed while preserving the same eRMSD restraint parameters to allow relaxation of the remaining RNA structure. An additional unrestrained equilibration run of 1 μs was then performed prior to production simulations, which are described in the following section. Wobble base-pair formation was verified through eRMSD convergence and analysis of hydrogen-bond distances over simulation time.

Production simulations

Production simulations were carried out at 310 K and 0.1 M KCl using either standard MD or CpHMD, depending on the protonation scheme. For systems with fixed protonation states, two

independent 1 μ s MD replicates were performed for each crystallographic chain (A-D), starting from either the native or wobble base-pair conformations, except for the native protonated and wobble deprotonated schemes that were structurally unstable (Extended Data Fig. 5a & 6a).

For CpHMD simulations, eRMSD restraints were applied to preserve the structural stability of native and wobble states throughout the simulations. Two independent replicates were performed for each conformational state and each chain (A-D) across distinct pH ranges: pH 7.0–12.0 for the wobble state and pH 3.0–7.0 for the native state. The pH titration range for the wobble state was selected based on changes in activity observed at pH 8.5 in the experimental assays. The pH titration range for the native state was chosen considering that the pKa of a Watson-Crick base pair is lower than the aqueous pKa of a free adenine (3.5)^{30,31}.

Trajectory analysis

Trajectory analyses were performed to characterize the conformational stability, protonation behaviour of A114, L2 loop length, and key hydrogen-bond interactions of the *alx* riboswitch across all production simulations. Structural convergence and stability over time of native and wobble conformational states was quantified using the eRMSD metric. For CpHMD simulations, residue-specific protonation time-series were verified, while averages were computed by averaging the protonation state of A114 of each simulation at each pH values. Titration curves were generated from these averages and fit to the Henderson-Hasselbalch equation to estimate the microscopic pKa values. Hydrogen-bond interactions were identified using geometric criteria on donor-acceptor distances (<0.35 nm) and angles (>150°) analyzed with MDanalysis. L2 loop length was determined indirectly by the presence of the C19·G23 base pair, or its absence coupled with the C19·A114⁺ wobble base pair. Statistical uncertainties for all reported observables were

estimated using a block bootstrap approach applied to the full production trajectories, where time series were partitioned into 10 blocks each trajectory and resampled to obtain mean values and standard errors.

Data Availability

The modified nucleic acid parameters used in the st-CpHMD are available at <https://github.com/Tomfersil/CpH-MetaD>. All the input scripts are available at <https://github.com/Tomfersil/Alx-Riboswitch-MD/>. PLUMED input files are also available on PLUMED-NEST⁹³ with plumID:26.001. Processed trajectories are available on Zenodo at <https://doi.org/10.5281/zenodo.18462847>. Source data for single molecule FRET assays are available through the University of Michigan DeepBlue deposit ([https://doi.XXXXXX](https://doi.org/10.5281/zenodo.18462847), TBD) and will be accessible upon manuscript acceptance.

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Authors and Affiliations

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Dr, La Jolla, CA, USA

Danea Palmer, Avery Ontiveros, & Tatiana V. Mishanina

Single Molecule Analysis Group and Center for RNA Biomedicine, Department of Chemistry, University of Michigan, Ann Arbor, MI, USA

Adrien Chauvier & Nils G. Walter

Scuola Internazionale Superiore di Studi Avanzati, via Bonomea 265, Trieste, 34136, Italy

Tomás F. D. Silva & Giovanni Bussi

Contributions

Conceptualization: D.P., T.V.M., N.G.W., and G.B. Experimental design: D.P., N.G.W., A.C., T.F.D.S., and G.B. Investigation: A.C. and D.P. (smFRET experiments and data analysis); D.P. and A.O. (*in vivo* experiments and data analysis); T.F.D.S. (molecular dynamics simulations and data analysis). Writing (original draft): D.P. and T.V.M. Writing (review and editing): N.G.W., A.C., T.F.D.S., G.B., D.P., and T.V.M. Supervision: T.V.M., N.G.W., and G.B. Funding acquisition: T.V.M., N.G.W., and G.B.

Corresponding Author

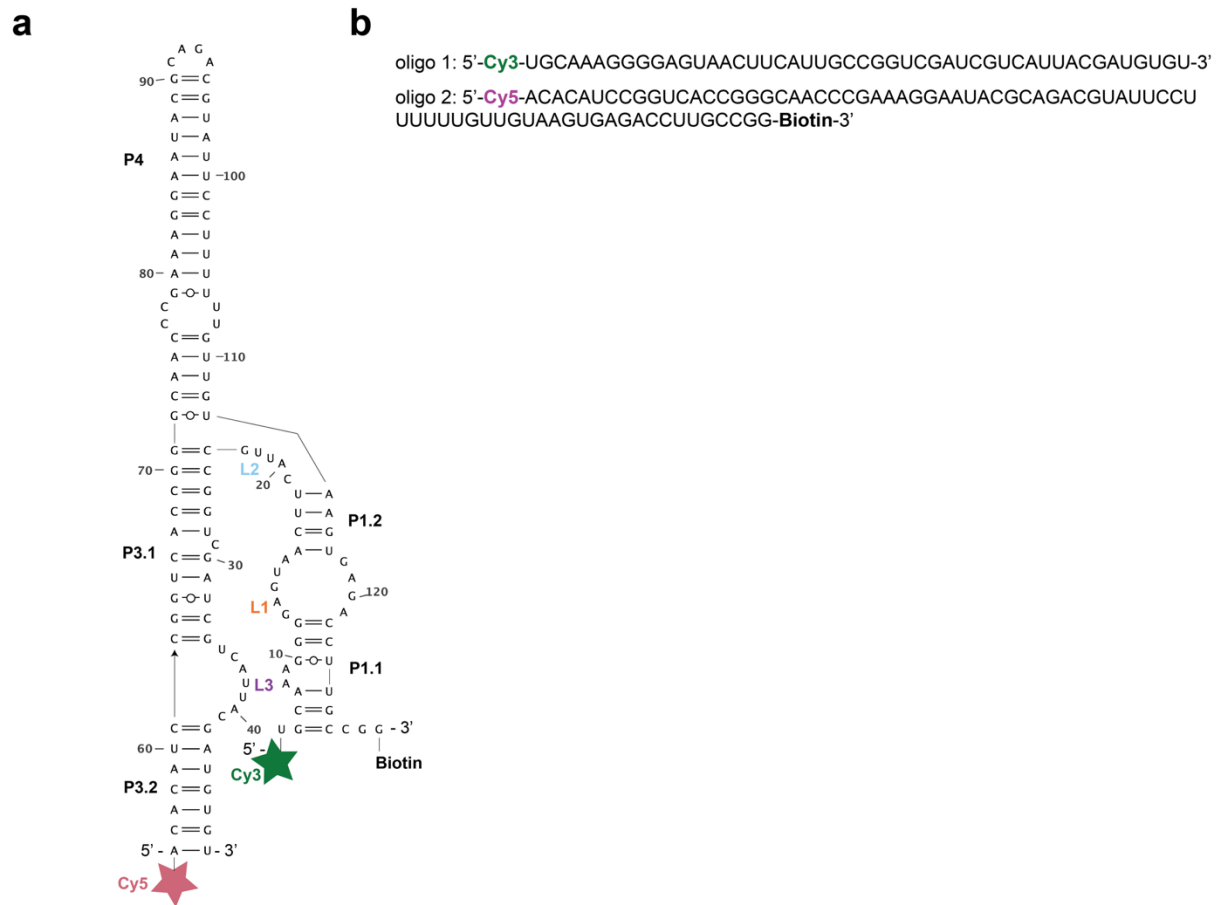
Correspondence to Nils G. Walter and Tatiana V. Mishanina

Ethics Declarations

Competing Interests

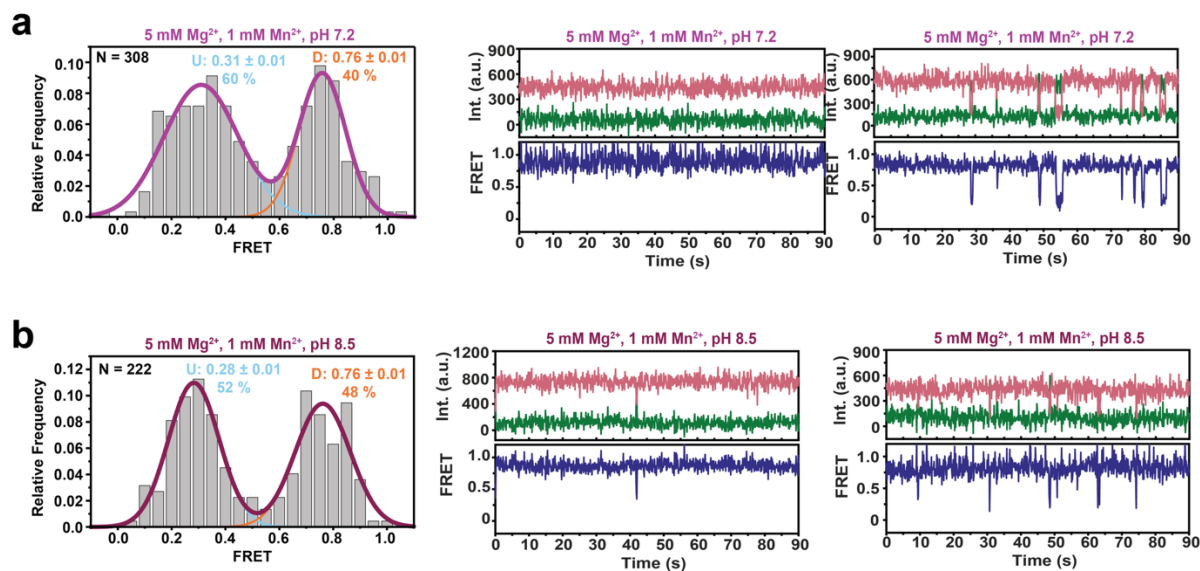
The authors declare no competing interests.

Supplementary Information for: pH-dependent allosteric remodeling of a bacterial riboswitch couples alkaline activation to metal sensing



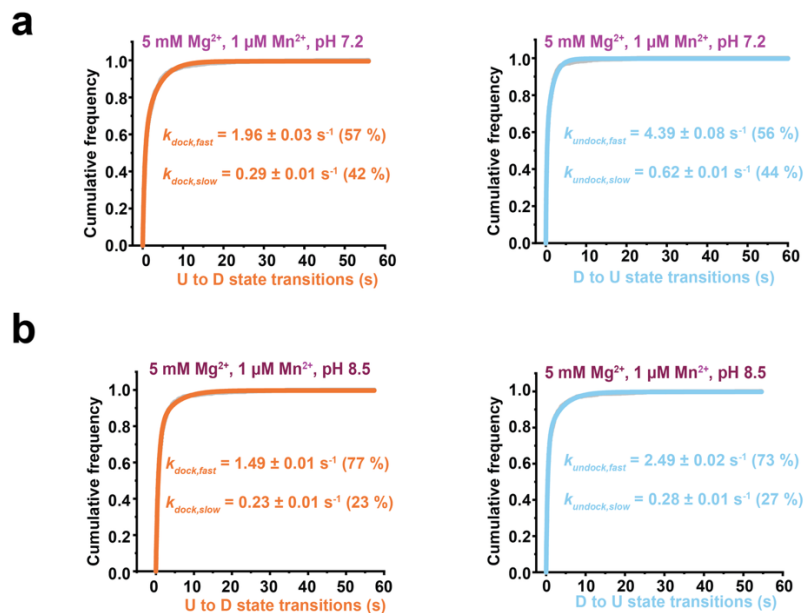
Supplemental Figure 3.1. smFRET construct design.

a, Sequence and secondary structure for the *alx* aptamer construct used for smFRET. Positions of Cy5, Cy3, and biotin labels are displayed on the secondary structure. **b**, The oligo sequence and modifications ordered for smFRET experiments.



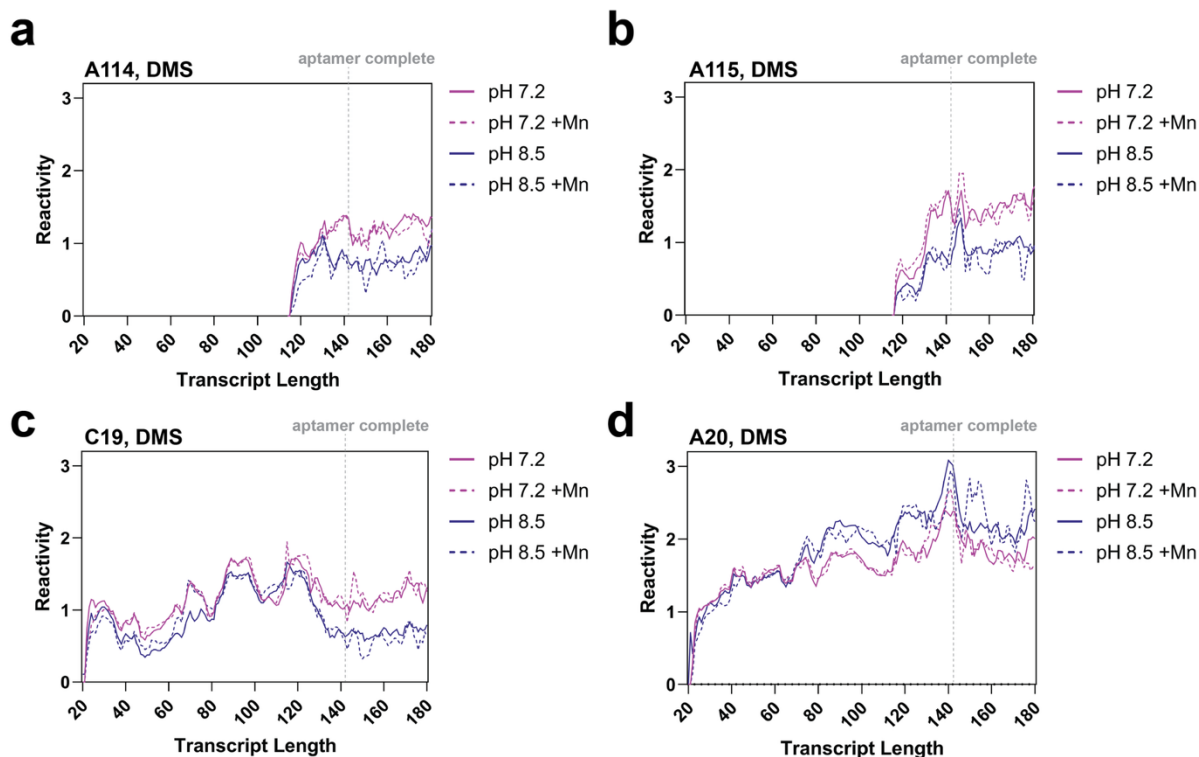
Supplemental Figure 3.2. *alx* aptamer docking at saturating [Mn²⁺] and neutral vs alkaline pH.

a,b, Histograms and representative traces showing the fraction of low and high FRET molecules with 1 mM Mn²⁺ at **(a)** pH 7.2 and **(b)** pH 8.5. Histograms display the Gaussian peaks for low FRET, high FRET, and cumulative fit with the percentage of each FRET state and the number of molecules (N) analyzed.



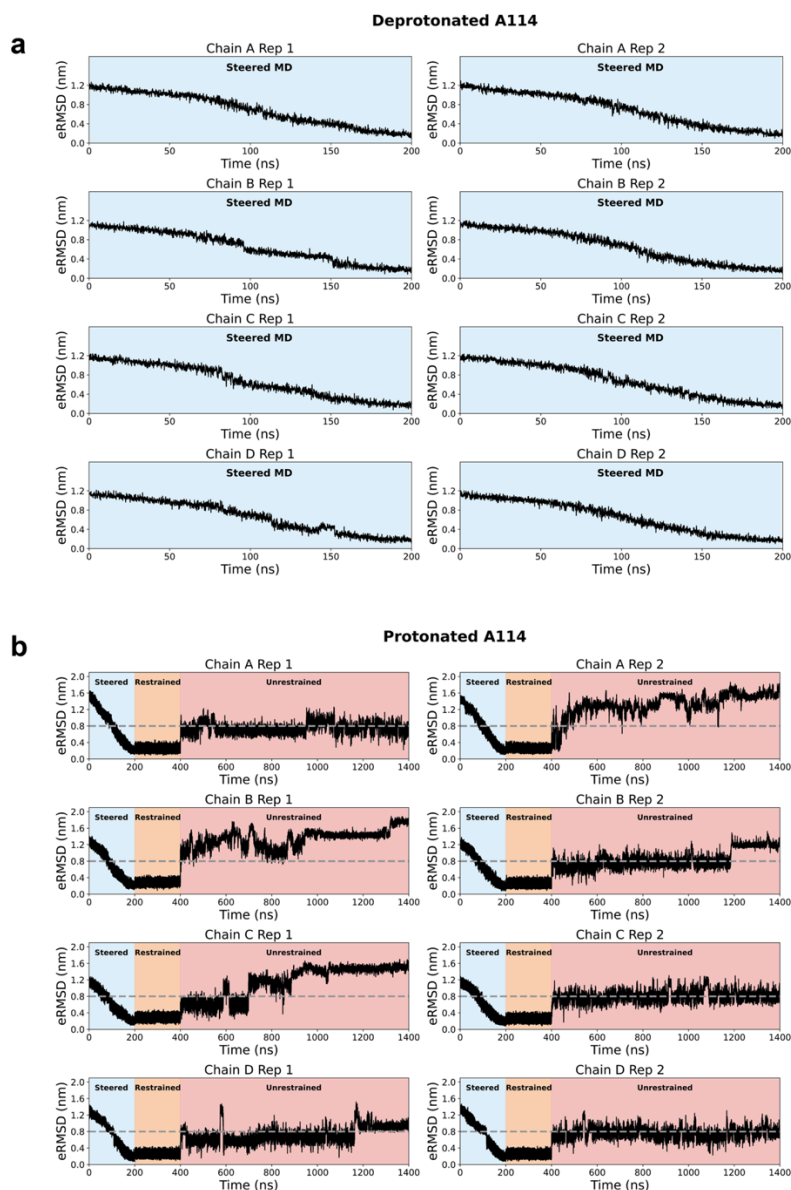
Supplemental Figure 3.3. *alx* aptamer docking kinetics at 1 μM Mn^{2+} and neutral vs alkaline pH.

a,b, Kinetics for docking (U to D state transitions) and undocking (D to U state transitions) based on the cumulative dwell times in each state fit with double-exponential functions for **(a)** pH 7.2 with 5 mM Mg^{2+} and 1 μM Mn^{2+} and **(b)** pH 8.5 with 5 mM Mg^{2+} and 1 μM Mn^{2+} .



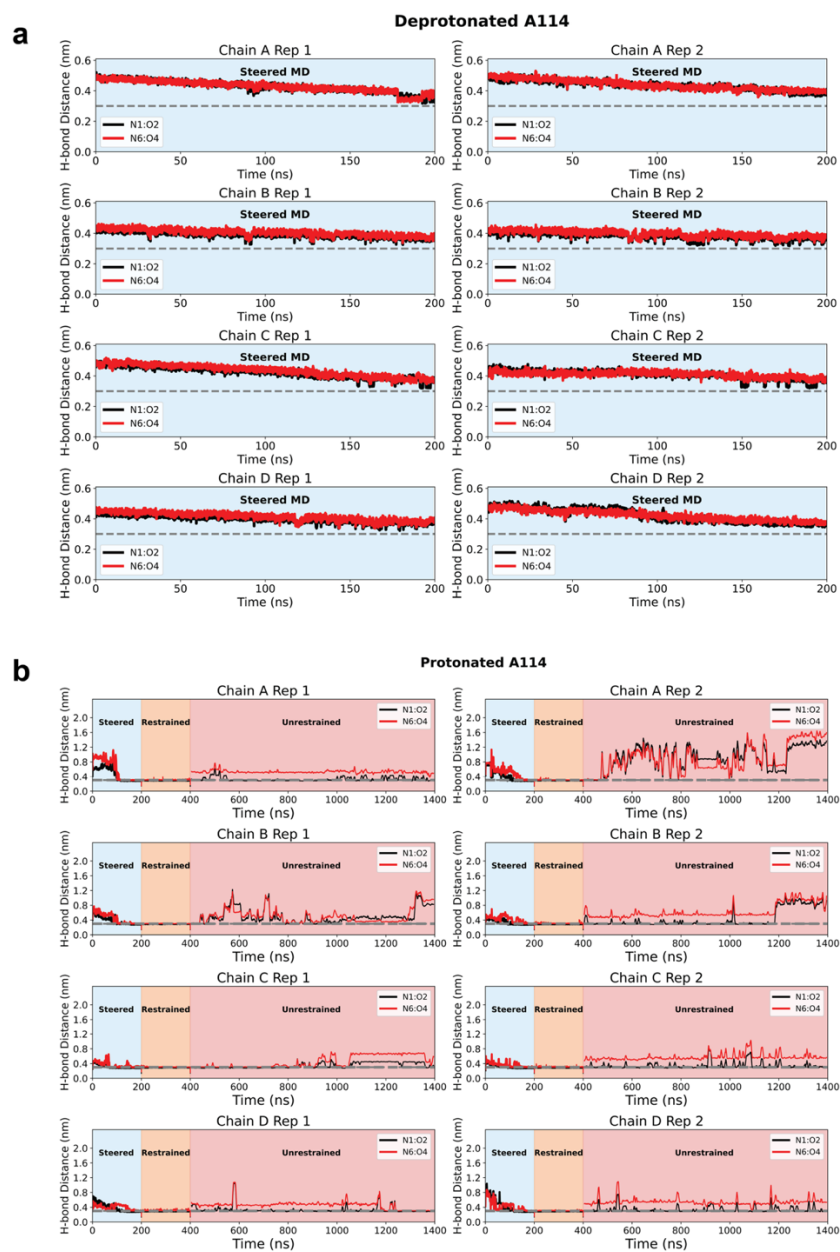
Supplemental Figure 3.4. Single-nucleotide DMS reactivity trajectories for L2 nucleotides.

a-d, Single-nucleotide trajectories displaying transcript length-dependent reactivity changes for L2 nucleotides. DMS reactivity data from Stephen et al²⁴ plotted for pH 7.2 and pH 8.5 without Mn^{2+} (solid lines) and with Mn^{2+} (dashed lines) for the following nucleotides within the *alx* aptamer: **(a)** A114, **(b)** A115, **(c)** C19, and **(d)** A20.



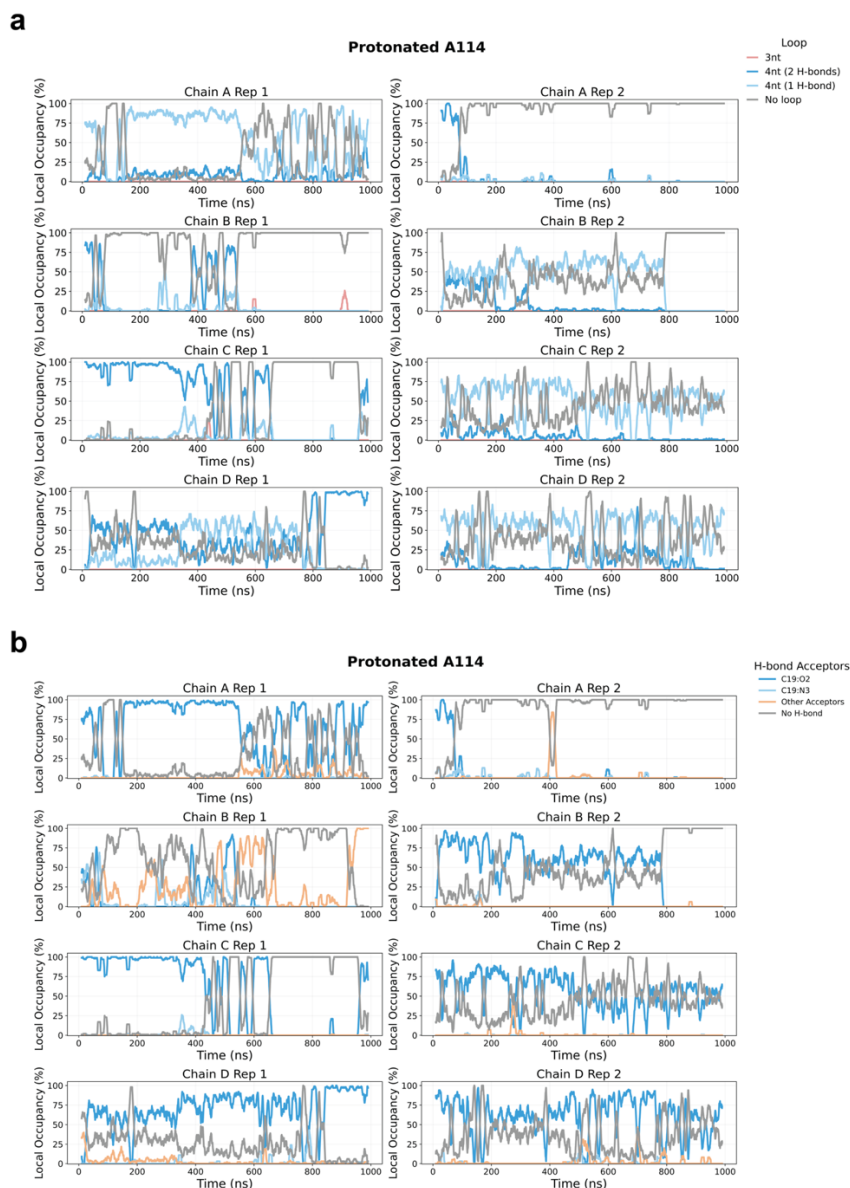
Supplemental Figure 3.5. Molecular dynamics protocol to generate wobble structure with a deprotonated and protonated A114.

a,b, Time series of the eRMSD for each crystallographic chain (A–D) and replicate obtained from the steered, restrained, and unrestrained MD simulations of *alx* aptamer with a **(a)** deprotonated A114 or **(b)** protonated A114. During the steered step, the system is driven from the native crystal structure toward the target wobble reference geometry, forming the C19·A114 and U18·A115 base pairs. The restrained step allows relaxation of the remaining structure, while the unrestrained phase assesses the stability of the generated structure. Dashed lines indicate the eRMSD cutoff (0.8), below which conformations are considered similar to the reference structure. Restrained and unrestrained simulations for the deprotonated system are not performed since hydrogen bonds necessary to maintain the wobble state could not be formed.



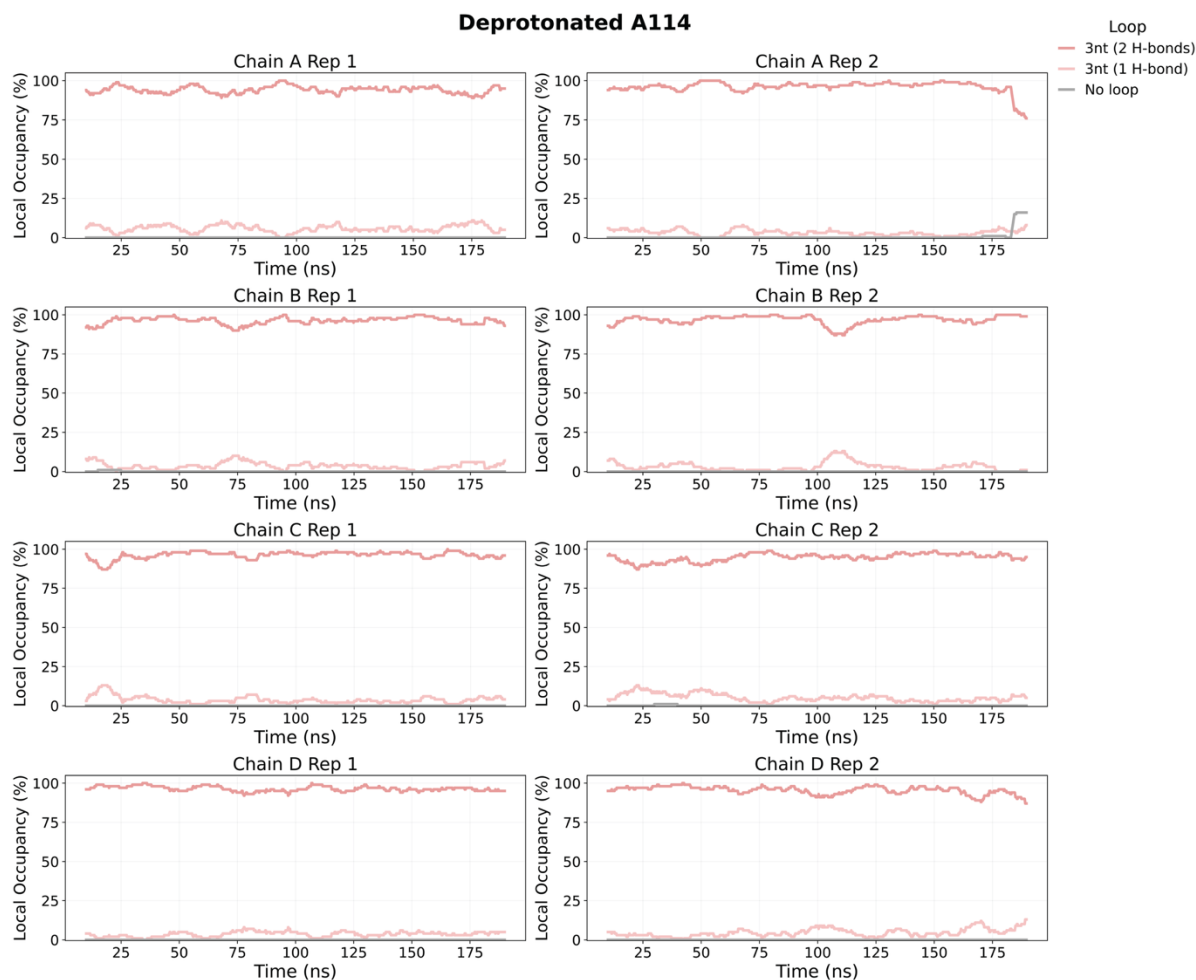
Supplemental Figure 3.6. Hydrogen-bond criterion for the formation of wobble C19·A114⁺.

a,b, Time series of hydrogen-bond distances within the C19·A114 wobble base pair. Distances between the N1-O2 and N6-O4 atom pairs are shown for each crystallographic chain (A–D) and replicate during the steered MD simulations of the *alx* aptamer with **(a)** deprotonated A114 or **(b)** protonated A114. In **(a)**, the measured hydrogen-bond distances fail to meet the distance criteria of a wobble base pair, supporting the data in Fig. 4 and 5. While in **(b)**, conformational rearrangements, as shown by the large fluctuations, occur to facilitate convergence toward the hydrogen-bond distances found in the reference wobble structure. Dashed lines indicate the heavy-atom hydrogen-bond cutoff (0.3 nm), below which a stable hydrogen bond is considered formed.



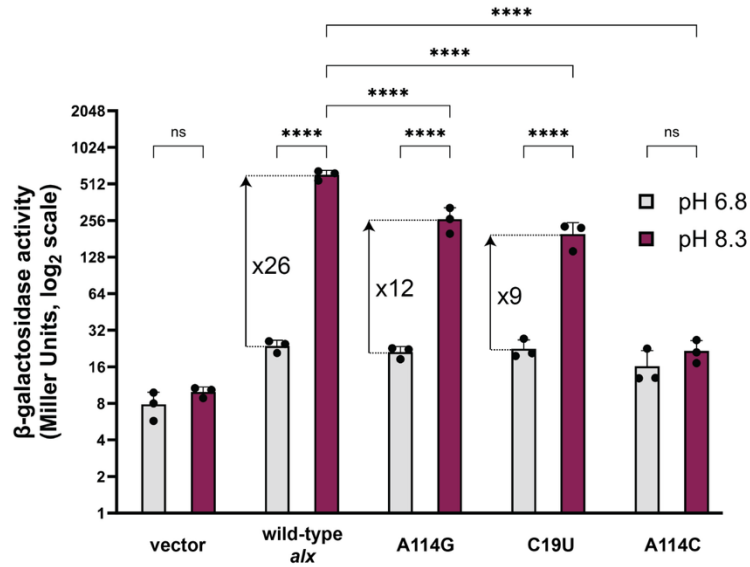
Supplemental Figure 3.7. L2 loop length depends on the number of hydrogen bonds (H-bonds) and H-bond acceptor competition in the C19·A114⁺ wobble structure.

a, Relative occupancy of L2 loop-length states is defined by the number of H-bonds formed between C19·A114⁺. Several transitions between multiple loop states are identified, whereas the 3-nt loop state is largely absent due to the lack of the stabilizing C19·G23 base pair found in the native structure. **b**, Time series of the relative occupancy of distinct H-bond acceptors interacting with the A114⁺ N1H⁺ donor. The A114⁺ N1H⁺ donor forms transient H-bonds with multiple acceptors, particularly the O2 and N3 of C19, thus preserving at least a partial wobble base pair. This partially stabilized state impacts the occupancy of L2 loop states and facilitates the observed transitions. Time series plots were generated for each crystallographic chain (A–D) and replicate of the unrestrained MD simulations. Each time series was computed using a moving average window ($n = 100$ data points).



Supplemental Figure 3.8. L2 loop length is based on the number of hydrogen bonds (H-bonds) present in C19·A114 in the native structure.

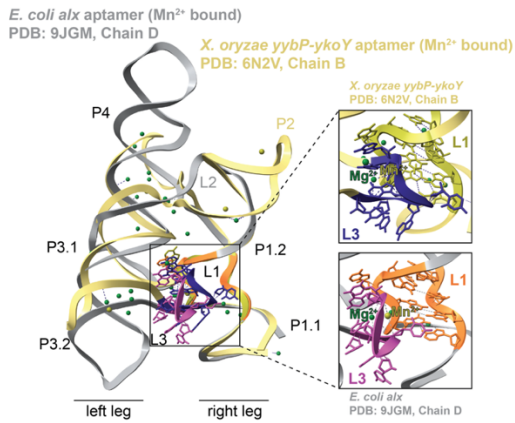
Time series of relative occupancy of L2 loop-length states defined by the number of H-bonds formed between C19·A114, for each crystallographic chain (A–D) and replicate of the unrestrained native MD simulations. Each time series was computed using a moving average window ($n = 100$ data points). No transitions are observed between loop states as the native structure is highly stable, particularly the C19·G23 and U18·A114 base pairs, in the absence of A114 protonation events to trigger conformational rearrangements.



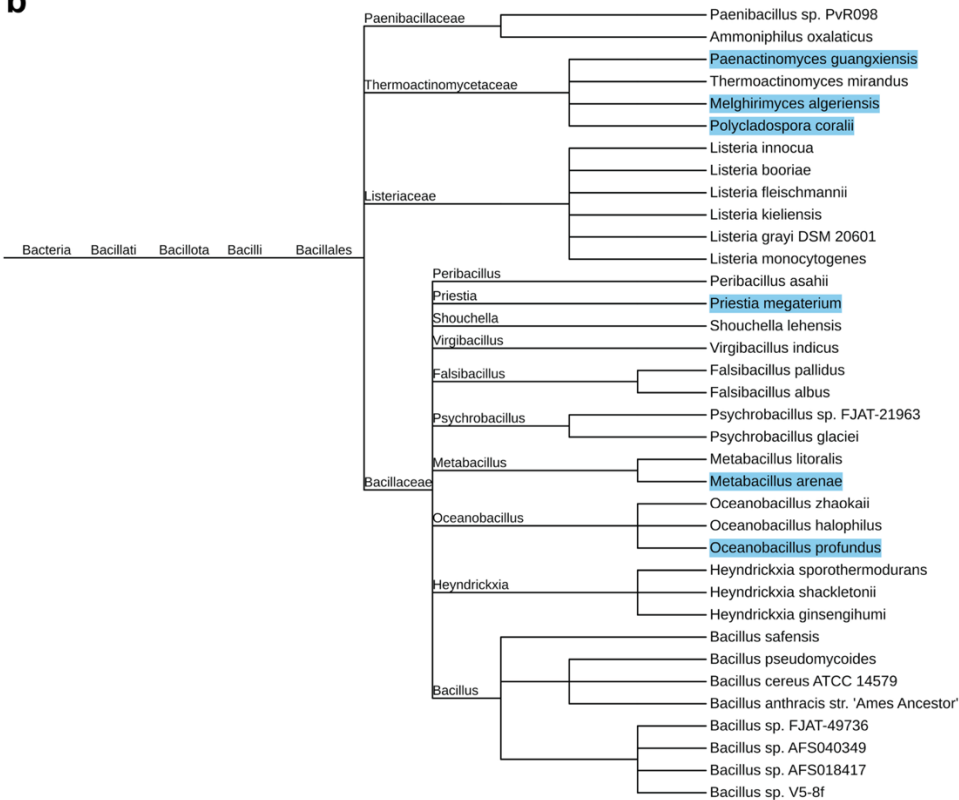
Supplemental Figure 3.9. L2 capping loop mutations reduce pH-dependent *alx* translation activation at pH 8.3

Translational response of the L2 mutants compared to wild-type *alx* for growth media buffered at pH 8.3. Statistical analysis done using a two-way ANOVA with Tukey post-hoc test for multiple comparisons to assess significance (^{ns} $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

a



b



Supplemental Figure 3.10. L3 structure, sequence conservation, and phylogenetic analysis.

a, Alignment of the *yybP-ykoY* family aptamer crystal structures for the *E. coli alx* (PDB: 9JGM, Chain D) and *X. oryzae* (PDB: 6N2V, Chain B). Individual aptamer Mn²⁺ binding sites are displayed in the zoomed-in panels for both *alx* and *X. oryzae*. **b**, Phylogenetic tree of *yybP-ykoY* family aptamers (from Rfam) with the *alx* L3 sequence. The highlighted organisms have *alx*-like aptamer architecture.

Supplemental Table 3.1. Key reagents and chemicals used in this study.

Proteins and associated solutions	Source	Cat no.
Q5 High-Fidelity DNA Polymerase	New England Biolabs	M0491
5X Q5 Reaction Buffer	New England Biolabs	M0491
T4 DNA Ligase	New England Biolabs	M0202
10X T4 DNA Ligase Buffer	New England Biolabs	M0202
PstI-HF	New England Biolabs	R3140
BamHI-HF	New England Biolabs	R3136
10X rCutSmart Buffer	New England Biolabs	B6004
dNTP Solution Mix	New England Biolabs	N0447S
Glucose Oxidase	Sigma Aldrich	G7141
Catalase	Sigma Aldrich	2071
Kits	Source	Cat no.
Monarch DNA Gel Extraction Kit	New England Biolabs	T1020
Monarch PCR & DNA Cleanup Kit	New England Biolabs	T1030
Other chemicals and reagents	Source	Cat no.
Chloroform	Fisher	C298-500
Ethanol, 200 proof	Fisher	BP2818-500
Manganese (II) Chloride Tetrahydrate	Fisher	AA3652636
o-Nitrophenyl-B-D-Galactopyranoside (ONPG)	Thermo Fisher Scientific	34055
Phenol (Buffer Saturated Phenol)	Invitrogen (Thermo Fisher Scientific)	15513-039
Nuclease Free Water	Invitrogen (Thermo Fisher Scientific)	10977023
Sodium Dodecyl Sulfate	Fisher	O267425
Magnesium Chloride Hexahydrate	Fisher	M35500
TAPS 99+%	Thermo Scientific	A17754.14
HEPES	Fisher	BP310100
Potassium Chloride	Fisher	P217500
Potassium Hydroxide	Fisher	P251500
Sodium Carbonate Anhydrous	Fisher	S263500
Glucose	Fisher	A1682836
Trolox	Sigma Aldrich	648471

Supplemental Table 3.2. Oligonucleotides used in this study.

Modifications: 5' Cy3 (labeling 5' end of RNA with Cy3); 5' Cy5 (labeling 5' end of RNA with Cy5); Bi 3' (labeling 3' end of RNA with biotin)

Stock #	Name	Sequence (5' to 3')	Purification	Source
504	0504_RAV5 copy	AAATGCAGGACTGCAGTCTCAGCCAGCCGAGCA TATTAAT	Desalt	Eton
505	0505_RAV2 3 copy	CATGGCATGTGGATCCGCCCCACGACGCCCTGC AACAACA	Desalt	Eton
506	0506_RAV1 19 copy	TAGCAGGACTGCAGAATCACGGACCTGGTACTG	Desalt	Eton
507	0507_RAV1 26 copy	CTCGTTCGGTCGACTCCATCGACATACCAAAC	Desalt	Eton
508	0508_alx_sw ap_L3_mntP longer F	GGTCGATCGACATACTGATGTGTGAAAAACACA TCCGG	Desalt	Eton
509	0509_alx_sw ap_L3_mntP longer R	CACACATCAGTATGTGATCGACCGGCAATGAAG TTAC	Desalt	Eton
510	0510_mntP swap_L3_alx longer F	GTACGTGTCATCATTACCGTTGCAAAACGTGGCA CGTACGG	Desalt	Eton
511	0511_mntP swap_L3_alx longer R	CGTTTGTCAACGGTAATGATGACACGTACATTTC GGAATC	Desalt	Eton
512	0512_alx_L 2_A114G_m ut fwd	GTTGTGAGTGAGACCTTGCCGGAAG	Desalt	Eton
513	0513_alx_L2 _A114G_mu t rev	GGTCTCACTCACAACAAAAAAGGAATACGTC	Desalt	Eton
514	0514_alx_L2 _A114C_mut fwd	GTTGTCAGTGAGACCTTGCCGGAAG	Desalt	Eton
515	0515_alx_L2 _A114C_mut rev	GGTCTCACTGACAACAAAAAAGGAATACGTC	Desalt	Eton
516	0516_alx_L2 _C19U_mut fwd	GTAACCTTATTGCCGGTCGATC	Desalt	Eton
517	0517_alx_L2 _C19U_mut rev	CGGCAATAAAGTTACTCCCTTTG	Desalt	Eton
536	alx_apt_oligo_1_Cy3	5' Cy3 UGCAAAGGGGAGUAACUUAUUGCCGGUCGAUC GUCAUUACGAUGUGU	HPLC	Revvity Inc (formerly Horizon Discovery)
537	alx_apt_oligo_2_Cy5_Bio	5' Cy5 ACACAUCCGGUCACCGGGCAACCCGAAAGGAAU ACGCAGACGUUUUUUUUGUUGUAAGUGAG ACCUGCCGG Bi 3'	HPLC	Revvity Inc (formerly Horizon Discovery)
538	mntP_apt_oligo_1_Cy3	5' Cy3 UCAUUUUUGGGGAGUAGCCGAUUUCCAGAUUCCG GAAUGUACGUGUCAACAUACUCGUU	HPLC	Revvity Inc (formerly Horizon Discovery)
539	mntP_apt_oligo_2_Cy5_Bio	5' Cy5 AACGUGGCACGUACGGACUGAAUACUUUCAGUC AGGCGAGACCAUAUG Bi 3'	HPLC	Revvity Inc (formerly Horizon Discovery)

Supplemental Table 3.3. *E. coli* K12 strains used in this study.

Strain	Genotype
MC4100	$\Delta(\text{argF-lac})\text{U169 rpsL150 relA1spoT1araD139 flbB5301 deoC1 ptsF25}$
RAS31	MC4100 $\Delta\text{alx::Kan}$

All strains employed in this study are *E. coli* K12 derivatives. The $\Delta\text{alx::Kan}$ deletion mutation was sourced from the library of Keio collection.⁷⁶

Supplemental Table 3.4. Plasmids used in this study.

Plasmid	Source	Description
pRA53	Sharma et. al., <i>Journal of Bacteriology</i> , 2024 ¹⁷	promoter-less pMU2386 (empty vector control for lacZ reporter fusion assays)
pRA54	Sharma et. al., <i>Journal of Bacteriology</i> , 2024 ¹⁷	pMU2386 containing translational reporter of <i>alx</i> cloned in the PstI and BamHI sites
pRA57	Sharma et. al., <i>Journal of Bacteriology</i> , 2024 ¹⁷	pMU2386 containing translational reporter of <i>mntP</i> cloned in the PstI and SalI sites
pMIS520	This work	pMU2386 containing translation fusion of <i>PmntP-mntP</i> with <i>mntP</i> L3 swapped to <i>alx</i> L3 in PstI and Sal sites
pMIS521	This work	pMU2386 containing translation fusion of <i>Palx-alx</i> with <i>alx</i> L3 swapped to <i>mntP</i> L3 in PstI and BamHI sites
pMIS525	This work	pMU2386 containing translation fusion of <i>Palx-alx</i> with L2 A114G point mutation in PstI and BamHI sites
pMIS526	This work	pMU2386 containing translation fusion of <i>Palx-alx</i> with L2 A114C point mutation in PstI and BamHI sites
pMIS527	This work	pMU2386 containing translation fusion of <i>Palx-alx</i> with L2 C19U point mutation in PstI and BamHI sites

Supplemental Table 3.5. smFRET dynamics.

	pH 7.2, 5mM Mg	pH 8.5, 5mM Mg	pH 7.2, 5mM Mg, 1uM Mn	pH 8.5, 5mM Mg, 1uM Mn	pH 7.2, 5mM Mg, 1mM Mn	pH 8.5, 5mM Mg, 1mM Mn
% static undocked (SU)	41.1	50.9	46.5	34.7	32.6	29.6
% static docked (SD)	17.1	8.7	19.2	25.8	28.8	30.9
% dynamic undocked (DU)	30.4	34.5	24.8	21.8	18.5	20.4
% dynamic docked (DD)	11.4	5.9	9.4	17.7	20.1	19.1

Supplemental Table 3.6. smFRET rate constants.

Mn (μ M)	pH	k_{dock} (s^{-1})	k_{undock} (s^{-1})
0	7.2	Fast: 2.0 ± 0.01 (73%) Slow: 0.26 ± 0.01 (27%) Overall: 1.53 ± 0.01	Fast: 3.89 ± 0.04 (63 %) Slow: 0.71 ± 0.01 (37 %) Overall: 2.71 ± 0.03
0	8.5	Fast: 1.50 ± 0.01 (77 %) Slow: 0.24 ± 0.01 (22 %) Overall: 1.21 ± 0.01	Fast: 3.13 ± 0.02 (86 %) Slow: 0.41 ± 0.01 (14 %) Overall: 2.75 ± 0.02
1	7.2	Fast: 1.96 ± 0.03 (57 %) Slow: 0.29 ± 0.01 (42 %) Overall: 1.24 ± 0.02	Fast: 4.39 ± 0.08 (56 %) Slow: 0.62 ± 0.01 (44 %) Overall: 2.73 ± 0.05
1	8.5	Fast: 1.49 ± 0.01 (77 %) Slow: 0.23 ± 0.01 (23 %) Overall: 1.20 ± 0.01	Fast: 2.49 ± 0.02 (73 %) Slow: 0.28 ± 0.01 (27 %) Overall: 1.89 ± 0.01
1000	7.2	Fast: 1.40 ± 0.01 (73 %) Slow: 0.22 ± 0.01 (27 %) Overall: 1.08 ± 0.01	Fast: 1.59 ± 0.01 (75 %) Slow: 0.22 ± 0.01 (25 %) Overall: 1.25 ± 0.01
1000	8.5	Fast: 1.40 ± 0.02 (88 %) Slow: 0.12 ± 0.01 (12 %) Overall: 1.25 ± 0.02	Fast: 1.36 ± 0.01 (83 %) Slow: 0.14 ± 0.01 (17 %) Overall: 1.15 ± 0.01

Supplemental Table 3.7. L3 alignment for phylogenetic tree and aptamer architecture analysis.

Sequence ID	Sequence	Organism Name (Rfam Database)	Updated Organism Name (NCBI Taxonomy)	Architecture	Organism of Origin
QWEH0100 0001.1/2698 70-270008	ACATCTGTCAAAGGGGAGTAGCGGTGGAATCA AGTGATTCCAAAATAAAGTCGTCATTACATGA TGGATTTTCATCATCGGCTTTATTGGCAGAACAT GCTAAGCGAGACCTTTGCCCAATCGTGGGTAA GGTCTCGCTT	Oceanobacillus profundus	Oceanobacillus profundus	alx-like	Oceanobacillus profundus
QQAY0100 0001.1/3526 35- 352481/1- 155	ACGAAAGTCGAAGGGGAGTAGCGTTGGGTAA ATCACCCAAAATAAAGTCGTCATTACATGGAGT ATTCCATCGGCTTTATTGACCTTGAACGGTTTA GCGAGACCTTTGCCTTAATGGCAGAGGTCTCT TTTTATTTGGATCCTGTCAATTAAGGA	Bacillus [pallidus]	Falsibacillus pallidus	mntP-like	Falsibacillus pallidus
RBZP01000 006.1/14005 9-139922/1- 138	ACTAATGTCAAAGGGGAGTAGCGTTGGAGTGA ATTAATTGCTCCAAAATAAAGTCGTCATTACA TGATGAAAATCATCGGCTTTATTGACAAGAAA TTGTAAAGCGAGACCTTTGCCCATGCGGTAGA GGTCTCGCTT	Ornithinibacillus sp. J8B(2010)	Oceanobacillus halophilus	mntP-like	Oceanobacillus halophilus
CP001983.1/ 1210555- 1210650	TTACGGAGTAAAGGGGAGTAGCGTCAGGGAA ACCTGAAACAAAGTCGTCATTACATGGAGTAT TCCATCGGCTTTGTTGGCATAATGATTATGCTT	Bacillus megaterium QM B1551	Priestia megaterium	alx-like	Priestia megaterium
JNFA01000 002.1/57454 -57621	ACATTCGTCAAAGGGGAGTAGCGTTAGCCTTT TTGGAGGCTAAGATAAAAGTCGTCATTACATGA ATTCGTTTCATCGGCTTTATTAACATTTTATACA ATGTCAGCGAGACCTTTGCCAATTTGGGCAGG GGTCTCGTTTTTTGTTGTTTGAAGCAGACGCT TGTGAC	Listeria booriae	Listeria booriae	mntP-like	Listeria booriae
UAWT0100 0033.1/6880 1-68955	ACAATAGTCAAAGGGGAGTAGCGCTGGTTTTTC GGACCAGGATAAAATCGTCATTACATGAATGG ATCATTTCATCGGTTTTATTAACATGTAACATGT CAGCGAGACCTTTGCCCGTAGTGGGTGAGGTC TCGTTTTTTATTACTCGGCTTTGTC	Listeria fleischmannii subsp. fleischmannii	Listeria fleischmannii subsp. fleischmannii	mntP-like	Listeria fleischmannii subsp. fleischmannii
GL538352.1 /366619- 366788	ACATGTGTCAAAGGGGAGTAGCATCAAATCCA GGACTGGATTTGAGATAAAGTCGTCATTACGT GGCATCCGTGCCATCGGTTTTATCAACATTTAA AATGTCAGCGAGACCTTTGCCCTATTTATAGG GTGGGTCTCGTTTTTTTATTGTCAGACCTAGCG CAAGGAA	Listeria grayi DSM 20601	Listeria grayi DSM 20601	mntP-like	Listeria grayi DSM 20601
CP024848.1/ 3816039- 3815897/1- 143	TGAATTGTCAAAGGGGAGTAGCGGTGGGATTT AAATGCTAATTCCAAGATAAAGTCGTCATTAC ATGATTTTTTAAATCATCGGCTTTATTGGCAAGA AATTGCTCAGCGAGACCTTTGTCCTAATAAGG CAGAGGTCTCGCTT	Oceanobacillus zhaokaii	Oceanobacillus zhaokaii	mntP-like	Oceanobacillus zhaokaii
JACEOL010 000043.1/17 601-17730	TTATCTGTCAAAGGGGAGTAGCGTTGAAATCA TCCGTGATTTCAAATAAAGTCGTCATTACAT GGGTTACCCATCGGCTTTATTGGCATTGTTTGC TTAGCGAGACTTTGCCTGAAGGCAATGTC T	Thermoactinomyces mirandus	Thermoactinomyces mirandus	mntP-like	Thermoactinomyces mirandus
JRUN01000 039.1/5450- 5623	ACAATTGTCAAAGGGGAGTAGCGTTAAGGATG TTGGCATCCTTAAACAAAGTCGTCATTACAT GATTTCTGTTTTTCAGAAGTCATCGGCTTTGTTG GCATTCTGAAAAAATGTCAGCAAGACCTTTG CCTATAAGGGCAGAGGTCTTGCTTTTGTCTTT ATAGGAAGATT	Bacillus ginsengihumi	Heyndrickxia ginsengihumi	mntP-like	Heyndrickxia ginsengihumi
CP043404.1/ 231032- 230862/1- 171	AATCCCGCCAAAGGGGAGTAGCGTCCGGTTTT TACCGGAAACAAAGTCGTCATTACATGGACTG ATGTTTCATCGGCTTTGTTGGCATGATTCTACAT ATCACATGTCTAGCAAGACCTTTGCCATTACA GGCAAAGGTCTTTTTTGCATTACATAAGCCTTTG TCTGTTGTG	Bacillus safensis	Bacillus safensis	mntP-like	Bacillus safensis

Supplemental Table 3.7. L3 alignment for phylogenetic tree and aptamer architecture analysis.

LJIY010000 01.1/167560 -167402/1- 159	ACATATGTCAAAGGGGAGTAGCTATAGGGAA ACCTATTTTCATAATCGTCATTACATGGCGTAAG CCATCGGTTATGATAGTAATTTTAAAAATTGCT TAGCAAGACCTTTGCCCTTTAATAGGCATGGG TCTTTTATTGCAAAAAAGGAGGATATTT	Psychrobac illus sp. FJAT- 21963	Psychrobacillu s sp. FJAT- 21963	mntP-like	Psychrobac illus sp. FJAT- 21963
FXTI010000 03.1/260431 -260344/1- 88	TCTCCTGTTAAAGGGGAGTAGCTATTACAACA AAGTCGTGATTACGGGGTTTGAACCCCGGCT TTGTTGGCAACGCCAACCGTTGTT	Thermoacti nomycetace ae bacterium NariEX	Melghirimyce s algeriensis	alx-like	Melghirim yces algeriensis
LJJC010000 04.1/332647 5- 3326312/1- 164	GACAAATGTCAAAGGGGAGTAGCTTTGGGTGTG AAAACATCTAAACAAAGTCGTCATTACATGAA GAAATTCATCGGCTTTGTTGGCATGATACATAT GTCTAGCAAGACCTTTGCCTATAAAGGGCAGA GGTCTTTTTTTCGCTCCTTTATAGGGTTTGTGT T	Bacillus shackletoni i	Heyndrickxia shackletonii	mntP-like	Heyndrickx ia shackletonii
LQYN01000 016.1/49359 -49166/1- 194	ACGAATGTCAAAGGGGAGTAGCGTTAAATGAT TTAAGACACGATAATTTGTGACTTAAACATTTA AAACAAAGTCGTCATTACATGGGTGTAATAATC CATCGGCTTTGTTGACATGAATAATGTCTAGC AAGACCTTTGCCTATAATAGGCAGAGGCTTTT TTGCTTTTATAGGGTTTTTCGTCACACTTCATT	Bacillus sporotherm odurans	Heyndrickxia sporothermo durans	mntP-like	Heyndrickx ia sporotherm odurans
PGUW0100 0004.1/6078 03-607901	TCCTCCGTAAAGGGGAGTAGCGTCAGGGATA TCCTGAAATAAAGTCGTCATTACATGGAATTTT CCATCGGCTTTATTGGCCTATTGCTCTAGGCT C	Bacillus sp. V5-8f	Bacillus sp. V5-8f	mntP-like	Bacillus sp. V5-8f
NUMC0100 0082.1/2283 6-22933	TCCCACGTAAAGGGGAGTAGCGTCAGGTTTA ACCTGAAATAAAGTCGTCATTACATGAACAAG GTTTCATCGGCTTTATTGGCATAATTTTTGTGCT T	Bacillus sp. AFS04034 9	Bacillus sp. AFS040349	mntP-like	Bacillus sp. AFS040349
CP031223.1/ 1186154- 1186250	ACATATGTCAAAGGGGAGTAGCTATAGGGAA ACCTATTTTCATAGTCGTCATTACATGGCTTTTG CCATCGGTTATGATAGTAATTTTACAATTACTT	Psychrobac illus glaciei	Psychrobacillu s glaciei	false hit	Psychrobac illus glaciei
CP003923.1/ 3138131- 3138304	CATTCTTCAAAGGGGAGTAGCTGCAAGTGAT TTTTACTTGAAACCAAAGTCGTCATTACGTGGAT TATTCCACCGCTTTGTTTGGCATGATACATA AATGTCTAGCAAGACCTTTGCCTCGTTCGTACA CAAGGCAGGGGTCTTTTTTGTATTCTATAAAGAT CCCTCATTTT	Bacillus lehensis G1	Shouchella lehensis	mntP-like	Shouchella lehensis
JAGYPI010 000001.1/58 4288-584447	GACAAATGTCAAAGGGGAGTAGCTTTGGATGTG AAAGCATCTAAACGAAGTCGTCATTACATGGA GCATTCCATCGGCTTTGTTGACATTACATATGT CTAGCAAGACCTTTGCCTATAAAGGGCAGAGG TCTTTTTTTCGCTCCTTTATAGGGTTATTGTT	Bacillus sp. FJAT- 49736	Bacillus sp. FJAT-49736	mntP-like	Bacillus sp. FJAT- 49736
RCVZ01000 002.1/55950 -55802/1- 149	ACGAAAGTCGAAGGGGAGTAGCGTTAGGTTTT ACCTAAAAACAAAGTCGTCATTACATGGATGAT CCATCGGCTTTGTTGGCACGATAGTGCCAGC GAGACCTTTGCCTATAGGCAAGGTCTTTTTCT ATTTCTCTGCACCTTGCCAAT	Falsibacillu s albus	Falsibacillus albus	mntP-like	Falsibacillu s albus
AE017334.2 /1269290- 1269125/1- 166	CATATCTCCAAAGGGGAGTAGCGTCCAAGAAT ATTTCTTGGAACAAAGTCGTCATTACGTAGA ACTTAGTTCTCCGGCTTTGTTGGCATTTCATT ACATATGTCTAGCAAGACCTTTGCCTATTAAC GGCAGAGGTCCTTTTTTGCATTATTGCCAAATT CTT	Bacillus anthracis str. 'Ames Ancestor'	Bacillus anthracis str. 'Ames Ancestor'	mntP-like	Bacillus anthracis str. 'Ames Ancestor'
JACXAI010 000026.1/52 566- 52472/1-95	TTACCCTGTAAAGGGGAGTAGCGTCAGGAATT TCCTGAAACAAAGTCGTCATTACGTGGATCGT ATCCACCGCTTTGTTGGCATACTTATGCTT	Metabacillu s arenae	Metabacillus arenae	alx-like	Metabacill us arenae
LARY01000 001.1/34892 3-349086	ACAGATGTCAAAGGGGAGTAGCACTGATTTTT AGGAATCAGGATAAAATCGTCATTACATGGAT ACGGCGTATTATCGGTTTTATTAACATGTCAC ATGTCAGCGAGACCTTTGCCATAAATCGGGT GAGGTCTCGCTTTTTTATCCTCACTCGGACTG TT	Listeria kielensis	Listeria kielensis	mntP-like	Listeria kielensis

Supplemental Table 3.7. L3 alignment for phylogenetic tree and aptamer architecture analysis.

MCHY0100 0001.1/1275 6-12574/1- 183	TACCCCTACAAAGGGGAGTAGCTGTCAGGGAT TTTAAAAACACCCTGAAACAAAGTCGTCATTA CGTGGTTTTCTTCCACCGGCTTTGTTGGTACAT ATGAATGACGTGCTTAGCAAGACCTTTGCCTT CTTATCTTTGATAAAGGCAGGGGCTTTTTTGT TTTTTAACAGCAACAAGCATT	Ammoniph ilus oxalaticus	Ammoniphilu s oxalaticus	mntP-like	Ammoniphili us oxalaticus
NUDY0100 0031.1/2136 06- 213441/1- 166	CATATTTCCAAAGGGGAGTAGCGTCCAAGAAT ATATTCTTGGAAACAAAGTCGTCATTACGTAG AACTCAGTTCTTCCGGCTTTGTTGGCATTTTTA TTACATATGTCTAGCAAGACCTTTGCCTATCAA CGGCAGAGGTCTTTTTTGTATTCTTTTCCAATC CCT	Bacillus pseudomyc oides	Bacillus pseudomycoid es	mntP-like	Bacillus pseudomyc oides
NPMS01000 002.1/16233 3-162181/1- 153	ACAACCGTCAAAGGGGAGTAGCCTTGGAGTTG GTTTAGTGTGCGATTCCAAAATAATAGTCGTCAT TACATGATTTACGAATCATCGGCTTTATTGGCA AAAAATTGCTTAGCGAGACCTTTGTCTTAGT GGCAGAAGTCTCGCTTTTGTATT	Virgibacill us indicus	Virgibacillus indicus	mntP-like	Virgibacillu s indicus
QWVS0100 0011.1/4720 3-47303	TCCTTAATCAAAGGGGAGTAGCGTTAGGGTTC GCCCTAAAAACAAAGTCGTCATTACATGGATGG AGACATTATCGGCTTTGTTGACATGACTTCAT GTTC	Bacillus asahii	Peribacillus asahii	mntP-like	Peribacillus asahii
JACEIQ010 000014.1/61 491- 61407/1-85	CTTTCCGGCAAAGGGGAGTAGCTGCACAACAA AGTCGTCATTACGGGTTAAAACCCCGGCTTTG TTGGCAACATTTAATGTTGTT	Paenactino myces guangxiens is	Paenactinomy ces guangxiensis	alx-like	Paenactino myces guangxiens is
JACXAH01 0000002.1/8 2967-83055	GAACATGTCAAAGGGGAGTAGCTCATAAACA AAGTCGTCATTACGGGATGAATGTCCCGGCT TTGTTGGCAACGGATAATCAGTTGTT	Polyclados pora corali	Polycladospor a corali	alx-like	Polyclados pora coralii
JAFIBU010 000001.1/13 83099- 1383182	ACACCTGTAAAGGGGAGTAGCTGATAAACA AGTCGTCATTACGAGATCAAAAGTCTCCGGCT TTGTTGGCAACGTTGTTGTT	Paenibacill us sp. PvR098	Paenibacillus sp. PvR098	n/a	Paenibacill us sp. PvR098
NTTM01000 061.1/11845 -12017	CATATTTCCAAAGGGGAGTAGCGTCAGAGAAA ATATTCTCTGAAACAAAGTCGTCATTACGTGG AACTATGTTCTCCGGCTTTGTTGGCATATTTT ATTACATATGTCTAGCAAGACCTTTGCCTATTA ACGGCAGAGGTCTTTTTTGTGTTCTTTTTGAAA TTTCTCTGCC	Bacillus sp. AFS01841 7	Bacillus sp. AFS018417	mntP-like	Bacillus sp. AFS018417
AE016877.1 /1289019- 1288853/1- 167	CATATCTCCAAAGGGGAGTAGCGTCCAAGAAT ATTTCTTGGAAACAAAGTCGTCATTACGTAGA ACTTAGTTCTTCCGGCTTTGTTGGCATTTTTTAT TACATATGTCTAGCAAGACCTTTGCCTATTAAC GGCAGAGGTCTTTTTTGCATTCACTTCCAGATT CTT	Bacillus cereus ATCC 14579	Bacillus cereus ATCC 14579	mntP-like	Bacillus cereus ATCC 14579
LWSG01000 045.1/14385 1-143755/1- 97	TTCCACCGTAAAGGGGAGTAGCGTCAGGATTA TCCTGAAACAAAGTCGTCATTACATGAACCTT GTTTCATCGGCTTTGTTGGCATATTATTGTGCTT	Bacillus litoralis	Metabacillus litoralis	mntP-like	Metabacillu s litoralis
AL591977.1 /131463- 131588	ACAAAUGUCAGAGGGGAGUAGCGCUGAUUAG CUUUUUAAUCAGGAUAAAGUCGUAUUACA GAUAGAGAUUAUCAGGUUUUAUCACAUUUUA ACUUUUAAUGUUAGCGAGACCUUUGCCUUUA CG	Listeria monocytog enes	Listeria monocytogene s	mntP-like	Listeria monocytog enes
AL596167.1 /48063- 48187	ACAAAUGUCAGAGGGGAGUAGCGCUGAUUAG CUUUUUAAUCAGGAUAAAGUCGUAUUACA AUAGAGAUUAUCAGGUUUUAUCACAUUUUA CUUUUUAAUGUUAGCGAGACCUUUGCCUUUA G	Listeria innocua	Listeria innocua	mntP-like	Listeria innocua

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Chapter 3, in full, has been submitted for publication of the material as it may appear in pH-dependent allosteric remodeling of a bacterial riboswitch couples alkaline activation to metal sensing. Palmer, Danae; Chauvier, Adrien, Fernandes da Silva, Tomás; Ontiveros, Avery; Bussi, Giovanni; Walter, Nils; and Mishanina, Tatiana. In peer review at Nucleic Acids Research, 2026. The dissertation author was the primary investigator and author of this paper.

Opportunities for Riboswitch Inhibition by Targeting Co-Transcriptional RNA Folding Events

Christine Stephen [†], Danea Palmer [†] and Tatiana V. Mishanina *

Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA 92093, USA

* Correspondence: tmishanina@ucsd.edu

[†] These authors contributed equally to this work.

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Abstract

Antibiotic resistance is a critical global health concern, causing millions of prolonged bacterial infections every year and straining our healthcare systems. Novel antibiotic strategies are essential to combating this health crisis and bacterial non-coding RNAs are promising targets for new antibiotics. In particular, a class of bacterial non-coding RNAs called riboswitches has attracted significant interest as antibiotic targets. Riboswitches reside in the 5'-untranslated region of an mRNA transcript and tune gene expression levels *in cis* by binding to a small-molecule ligand. Riboswitches often control expression of essential genes for bacterial survival, making riboswitch inhibitors an exciting prospect for new antibacterials. Synthetic ligand mimics have predominated the search for new riboswitch inhibitors, which are designed based on static structures of a riboswitch's ligand-sensing aptamer domain or identified by screening a small-molecule library. However, many small-molecule inhibitors that bind an isolated riboswitch aptamer domain with high affinity *in vitro* lack potency *in vivo*. Importantly, riboswitches fold and respond to the ligand during active transcription *in vivo*. This co-transcriptional folding is often not considered during inhibitor design, and may explain the discrepancy between a low K_d *in vitro* and poor inhibition *in vivo*. In this review, we cover advances in riboswitch co-transcriptional folding and illustrate how intermediate structures can be targeted by antisense oligonucleotides—an exciting new strategy for riboswitch inhibitor design.

Keywords: bacteria; antibiotics; drug target; antisense oligonucleotide; riboswitch; riboswitch inhibitor; transcription; translation; RNA folding

Introduction

Antibiotic resistance is a major threat to human health and causes millions of prolonged bacterial infections each year, risking overwhelming our healthcare systems ¹. Due to the widespread use of antibiotics to control infection, pathogenic bacteria have developed resistance to antibiotics that were previously effective, causing deaths from once-treatable infections ². Motivated by the pressing need for innovative antibiotic strategies, the World Health Organization (WHO) creates lists of priority bacterial pathogens to focus on when designing novel antibiotics ³. One promising avenue for discovering new antibiotics is to target bacterial non-coding RNAs that control the expression of essential genes in pathogenic bacteria, such as riboswitches ⁴.

Riboswitches are 5'-untranslated regions of mRNA that typically respond to small-molecule ligands to control downstream gene expression via regulating transcription, translation, or RNA decay ⁵⁻⁷. Importantly, with the exception of the thiamine pyrophosphate (TPP) riboswitch, riboswitches are only found in bacteria and archaea ⁸, making them promising targets for selective antibiotics with low side effects to the human host. Riboswitches are composed of two domains: an aptamer domain that binds a ligand, typically with high affinity, and an expression platform that undergoes structural changes to affect gene expression (Figure 1A,B) ⁹. There are at least 55 classes of characterized riboswitches that are grouped by what ligand they bind, typically with a highly conserved aptamer domain ¹⁰. Riboswitch expression platforms exhibit more sequence and structural diversity, and either promote or inhibit gene expression through ligand-dependent structural changes (Figure 1A,B) ⁵. Expression platforms are designated as “off” or “on” depending on whether ligand binding prompts structural changes that downregulate (“off”) or upregulate (“on”) gene expression levels. “Off” riboswitch expression platforms form either an intrinsic terminator hairpin that prompts transcription termination (Figure 1A) or a hairpin stem

that sequesters the ribosome binding site, leading to inhibition of translation (Figure 1B). Conversely, for “on” riboswitch expression platforms, ligand binding leads to the formation of an anti-terminator stem or release of the ribosome binding site, promoting downstream gene expression ^{5,11}.

Riboswitches are typically found upstream of essential genes for metabolite biosynthesis and transport, thereby tuning expression of proteins that are essential for survival or virulence in some bacterial pathogens ^{12,13}. Since riboswitches are not found in human cells and often bind their ligands with high affinity in vitro (low nM K_d values) and high selectivity, the design of structural analogs that inhibit bacterial growth has attracted significant research interest ¹⁴. Designing synthetic ligands to bind riboswitches with high affinity is the dominant focus of current research aiming to inhibit gene expression by targeting riboswitches (Figure 1C). These specific riboswitch inhibitors have been extensively reviewed in several recent papers ^{4,14–16}.

To be an effective antibiotic that targets a riboswitch, compounds need to have high affinity for the riboswitch RNA, lack off-target effects on other metabolic pathways, have potential to avoid the bacteria developing antibiotic resistance, and pose low toxicity to human cells ¹⁵. Importantly, many compounds that have high affinity for a riboswitch in vitro will not end up affecting gene expression in vivo, leading some researchers to turn to alternative methods, such as RNA-based gene silencing, to target riboswitches and inhibit bacterial gene expression (Figures 1D,E) ^{17,18}. Riboswitches fold co-transcriptionally and ligands often bind an early RNA folding intermediate, leading to the rearrangement of the expression platform as it is emerging from RNA polymerase (RNAP) ¹⁹. Therefore, an inhibitor compound that has high affinity for the aptamer domain in isolation may lack potency in vivo if the inhibitor never reaches thermodynamic equilibrium with the riboswitch target, leading to a discrepancy whereby a low K_d does not indicate an effective

inhibitor ^{17,20}. An exciting new strategy for synthetic RNA silencing of riboswitches, antisense oligonucleotides (ASOs), could potentially bridge this gap ¹⁸. The aim of this review is to illustrate how ASOs can be a new tool for the development of antibiotics that target riboswitches, helping to overcome challenging cases where ligand mimics are unsuccessful. In the following sections, we first review the limitations of riboswitch small-molecule inhibitors, then shift the discussion to RNA-based gene silencing, covering how this occurs in nature and how such discoveries have inspired the design of synthetic oligonucleotides as a therapeutic strategy. From there, we discuss investigations into ASOs currently being researched to inhibit riboswitch-controlled gene expression in pathogenic bacteria. Lastly, we lay the foundation for how advances in riboswitch co-transcriptional folding can be leveraged to expand the available sequence space for ASO inhibitors targeting riboswitches.

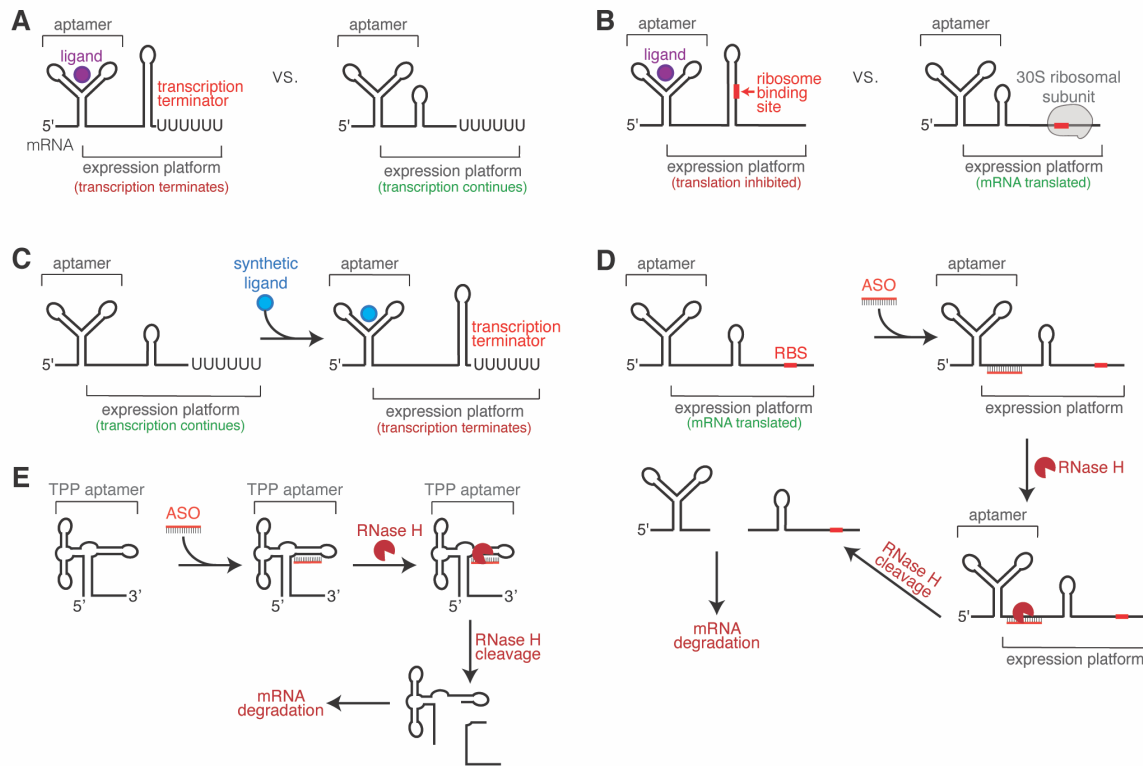


Figure 4.1. Overview of riboswitch-controlled gene expression and riboswitch inhibition strategies.

(A) Riboswitch responding to ligand by terminating transcription, turning gene expression “off”. (B) Riboswitch responding to ligand by inhibiting translation, turning gene expression “off”. (C,D) Mechanism for inhibiting gene expression by targeting a riboswitch with a synthetic ligand analog (C) or a DNA-based antisense oligonucleotide (ASO) that marks mRNA for degradation via RNase H cleavage (D). (E) Example ASO-targeting of the TPP riboswitch aptamer domain, followed by RNase H cleavage and mRNA degradation.

Limitations in Designing Synthetic Ligands That Target Riboswitches in Pathogenic Bacteria

Despite some success in designing small-molecule riboswitch inhibitors, we introduce here some key riboswitches that are present in pathogenic bacteria for which significant limitations exist in the context of designing small-molecule inhibitors. Further, these riboswitches are excellent candidates for novel inhibition strategies, since they control expression of essential

genes in bacteria. In Section 5, we continue the discussion of these riboswitches by highlighting how co-transcriptional folding studies can inspire the design of novel ASO inhibitors. This section is not intended to be a comprehensive review of small-molecule inhibitors targeting riboswitches present in pathogenic bacteria. For recent extensive reviews focused on small-molecule inhibitors, readers are directed to the following references ^{4,14–16}.

2.1. *TPP Riboswitch*

The thiamine pyrophosphate (TPP) riboswitch is one of the most widespread riboswitches, found in a variety of pathogenic bacteria ²¹. Thiamine is a critical cofactor for carbohydrate metabolism, and its biosynthetic pathway requires the expression of many unique proteins ^{22,23}. The TPP riboswitch is the only known riboswitch to occur in eukaryotic cells, with examples in plants and fungi ²⁴. The TPP aptamer, or THI-box, is highly conserved, and is made up of five stems that compact stably upon TPP binding ^{25,26}. The TPP riboswitch expression platform differs between bacterial species, and can either promote transcription termination or prevent translation initiation upon TPP binding the aptamer domain ²⁷. For example, in *E. coli*, there are multiple important TPP riboswitches that control downstream gene expression at both the transcriptional and translational level ²⁸. To highlight this diversity, the *E. coli thiC* riboswitch regulates the expression of proteins directly involved in TPP biosynthesis, while the *tpbA* riboswitch regulates the expression of an ABC transporter necessary to import thiamine and TPP into the bacterial cell ^{22,23}.

A synthetic analog of thiamine (pyrithiamine, or PT) binds the thiamine pyrophosphate (TPP) riboswitch with nearly identical affinity to the natural TPP ligand ²⁹. PT was first characterized as an inhibitor of TPP-dependent enzyme production in *E. coli* in 1967, but mutant *E. coli* strains that resist PT inhibition by derepressing TPP-dependent enzyme synthesis by an unknown

mechanism were isolated by 1969^{30,31}. In 2005, it was demonstrated that bacteria develop antibiotic resistance to PT through mutations to the TPP riboswitch, with mutations occurring in both the aptamer domain and expression platform²⁹. Another study characterized binding by thiamine, methylene diphosphoric acid (MDP, a pyrophosphate analog), and a covalently linked version of the two molecules (TPPc) to the TPP riboswitch³². The individual thiamine and MDP bound with low affinity, but the conjugate molecule exhibited 6-fold higher affinity than TPP itself. The thiamine moiety of TPPc binds the riboswitch in a different conformation than the thiamine moiety of TPP, making it an interesting candidate to test *in vivo*³². Other studies have identified limited candidate inhibitors that bind the TPP riboswitch with sufficient affinity *in vitro*, but those have not been studied extensively *in vivo*¹⁵.

2.2. FMN Riboswitch

The flavin mononucleotide (FMN) riboswitch class is widely distributed across bacteria and is found upstream of a variety of genes involved in flavin synthesis and transport²⁷. Flavin-based cofactors are essential for proteins involved in the electron transport processes³³. Since FMN is an essential cofactor for bacterial survival, inhibiting the flavin synthesis or transport pathways has attracted interest for antibiotic development. The FMN riboswitch aptamer domain has a six-stem junction that folds into a butterfly-like scaffold by identically folding the peripheral domains³⁴. Additionally, the FMN riboswitch aptamer is largely folded in the absence of a ligand, and FMN binding only leads to local conformational changes³⁵. These smaller-scale conformational changes ultimately alter the fold of the expression platform, leading to transcription termination or inhibition of translation initiation. For example, in *B. subtilis*, the *ribD* riboswitch promotes transcription termination upon FMN binding, leading to a downregulation of several FMN biosynthetic genes located in a single operon controlled by the riboswitch³⁶.

The FMN riboswitch can successfully be targeted by roseoflavin, which is phosphorylated by cellular proteins, but this requirement limits its efficiency ³⁷. Additionally, roseoflavin binds many off-target flavoproteins that are also present in humans, which gives it limited potential as an antibiotic ^{38,39}. Another ligand-mimic, ribocil, targets the FMN riboswitch aptamer with high affinity ⁴⁰. However, ribocil-resistant *E. coli* mutants have been identified, and importantly, all mutations conferring resistance mapped to the riboswitch aptamer domain or expression platform ^{40,41}.

2.3. *glmS* Ribozyme

The *glmS* ribozyme is a self-cleaving ribozyme that responds to glucosamine-6-phosphate (GlcN6P) and is located upstream of GlcN6P synthetase ⁴². GlcN6P synthetase is responsible for producing GlcN6P ⁴², which is the essential first step in the pathway to building a bacterial cell wall, so inhibiting this production prevents bacterial growth ⁴³. GlcN6P binding initiates the rapid self-cleavage of the *glmS* ribozyme, causing the release of a 25-nucleotide (nt) leader mRNA sequence that promotes subsequent degradation of the mRNA by RNase J1 ⁴³. The *glmS* ribozyme has a compact structure containing multiple pseudoknots that is largely unchanged by ligand binding ⁴⁴. Instead, GlcN6P participates directly in catalyzing the self-cleavage event ^{43,44}.

Thousands of ligand analogs for the *glmS* ribozyme have been identified and screened, but very few were found to be active in promoting ribozyme self-cleavage. Of these, none activated the ribozyme as strongly as the natural ligand, making them less promising antibiotics ^{45–47}.

2.4. Purine Riboswitch

Purine riboswitches respond to purine molecules and include guanine and adenine riboswitches ⁴⁸. These riboswitches occur upstream of genes involved in essential purine

metabolism pathways, purine salvage pathways, and purine transport ^{48,49}. Thus, designing inhibitors for these essential pathways would lead to a loss of bacterial virulence ^{50,51}. Purine riboswitches have conserved aptamer domains consisting of three helices that nearly fully surround the three-way junction that coordinates the ligand ⁵². In fact, the only difference between the adenine and guanine riboswitch aptamer binding pockets is one nucleobase that directly base-pairs with the ligand ^{53(p20)}.

Purine riboswitches can be successfully targeted by rationally designed small molecules ⁵⁴. However, these compounds often show limited affinity for the riboswitch aptamer ⁴⁹. The compounds that are identified to have high affinity often show little to no effect on gene expression ^{17,20}. One example study generated 16 rationally designed small molecules and identified 6-*N*-hydroxylaminopurine (6-*N*-HAP) as a promising guanine analog that binds the guanine riboswitch aptamer with high affinity *in vitro* ⁴⁹. Unfortunately, when added to media, 6-*N*-HAP demonstrated low potency, since a high concentration was required to inhibit bacterial growth. Additionally, other designed compounds in this study inhibited growth, but exhibited off-target effects ⁴⁹, and 6-*N*-HAP has even been demonstrated to be a general and potent mutagen in bacterial and mammalian cells ^{55,56}. These limitations to using small-molecule ligand mimics to inhibit riboswitch-controlled gene expression have led some researchers to explore RNA-based silencing methods, which will be reviewed in the next sections.

RNA-Based Silencing in Natural Contexts

RNA-based gene silencing (RNA interference, or RNAi) was described in eukaryotes in 1998, when double-stranded RNAs were identified as post-transcriptional gene silencing elements in *C. elegans* ^{57,58}. RNAi is a conserved eukaryotic mechanism that regulates gene expression at the transcriptional or post-transcriptional level ^{59(p202)}. RNA silencing relies on double-stranded

RNA (dsRNA) cleavage that results in small RNAs ^{60,61}. These small RNAs are then recruited to guide protein complexes to silence the target genes ⁶². This silencing process is a key mechanism by which eukaryotes defend against viruses and transposable elements ⁶³, but is also used to regulate endogenous genes within eukaryotic cells ⁶⁴.

In prokaryotes, small non-coding RNAs (sRNAs) are responsible for diverse gene silencing events ⁶⁵. The expression of sRNAs allows for the fine-tuning of gene expression in response to various environmental signals ⁶⁶. These sRNAs can prevent translation of genes by binding and occluding ribosome binding sites, or by affecting RNA stability and promoting degradation by RNase enzymes ^{67,68}. Some sRNAs associate with chaperone proteins, such as Hfq or ProQ, to achieve this translation inhibition and RNase-dependent degradation ^{69,70}. Other sRNAs sequester CsrA-like RNA-binding proteins that affect mRNA stability and translation ⁷¹. Additionally, sRNAs associate with Cas proteins to promote the degradation of DNA or RNA targets as part of the CRISPR/Cas defense system against viral infection ^{65,72,73}.

Diverse RNA-based gene silencing events are thus widespread among all kingdoms of life and allow for versatile and specific changes in gene expression in response to environmental signals. This versatility makes sRNAs a promising avenue for designing drugs to target and silence specific genes, especially in bacteria ⁵⁷.

Synthetic RNA-Based Silencing

Drawing inspiration from natural RNA-based gene silencing, synthetic antisense oligonucleotides (ASOs) can be designed to achieve silencing of target genes. ASOs are single-stranded synthetic oligonucleotides that are complementary to a target gene ⁷⁴. ASOs can be used to silence specific genes by Watson–Crick base-pairing with target RNA transcripts and inhibiting

gene expression as a result ¹⁸. For example, an ASO may inhibit translation by targeting the RNA transcript for cleavage (Figure 2A) or by creating a double-stranded region that serves as a steric block for translating ribosomes or for proper RNA folding (Figure 2B) ⁷⁵. The first ASO shown to inhibit gene expression *in vivo* was discovered in 1978 and targeted viral RNA translation ⁷⁶. Since then, the development of ASOs as potential drugs has been widely pursued, and over a dozen ASOs have been approved by the FDA and others are in clinical trial ^{77,78}.

Studies in the Penchovsky lab have shown that ASOs can be designed to target specific riboswitches (Figure 1D), including SAM-I, *glmS*, FMN, and TPP riboswitches (Figure 1E) from pathogenic bacteria ⁷⁷. These riboswitch-targeting ASOs have been previously reviewed ²⁷, and the main findings are summarized briefly below.

All four riboswitch-targeting synthetic DNA ASOs were designed to target a portion of the conserved riboswitch aptamer domains ²⁷. These ASOs enabled cleavage of the riboswitch RNA by RNase H to degrade the RNA and prevent downstream gene expression (Figure 1D). For each riboswitch, the ASO was designed to be fully complementary to a 13–16 nt single-stranded sequence of riboswitch RNA that does not self-hybridize. For all four ASOs, the average minimum concentration of ASO inhibitor needed to inhibit 80% of bacterial growth (MIC₈₀) was around 700 nM ²⁷. To avoid toxicity of the ASO, the sequences were chosen to be dissimilar to sequences occurring in human cells, and the ASOs were shown to be non-toxic at their MIC₈₀ to a lung cancer human cell line (A549) ²⁷. A few different methods were used to demonstrate that the ASO was specific for a particular riboswitch. For the TPP and SAM-I riboswitches, the ASO was shown to be ineffective in targeting *E. coli*, which was expected as *E. coli* lacks these target riboswitches ^{21,79}. For the FMN riboswitch, a second ASO was designed with eight mismatches with the target RNA to be used as a negative control, and this mismatched ASO was ineffective at binding the

FMN riboswitch⁸⁰. Finally, the *glmS* ribozyme ASO was designed to be fully complementary to the aptamer domain of a target *glmS* ribozyme in *S. aureus*. This ASO did not inhibit the growth of *E. coli* or *B. subtilis*, demonstrating the desired specificity for the *glmS* ribozyme in *S. aureus*⁸¹.

The advances in ASO-directed degradation of riboswitch RNAs show that riboswitches are a promising target for new antibacterials²⁷. However, the requirements that the target RNA be unable to self-hybridize, single-stranded, and absent from human cells limit the options for ASO sequences that can be designed and tested for efficacy²⁷. An exciting potential direction for antibacterial ASO design is to target single-stranded regions of RNA co-transcriptional folding intermediates to inhibit riboswitch activity and degrade the mRNA⁸².

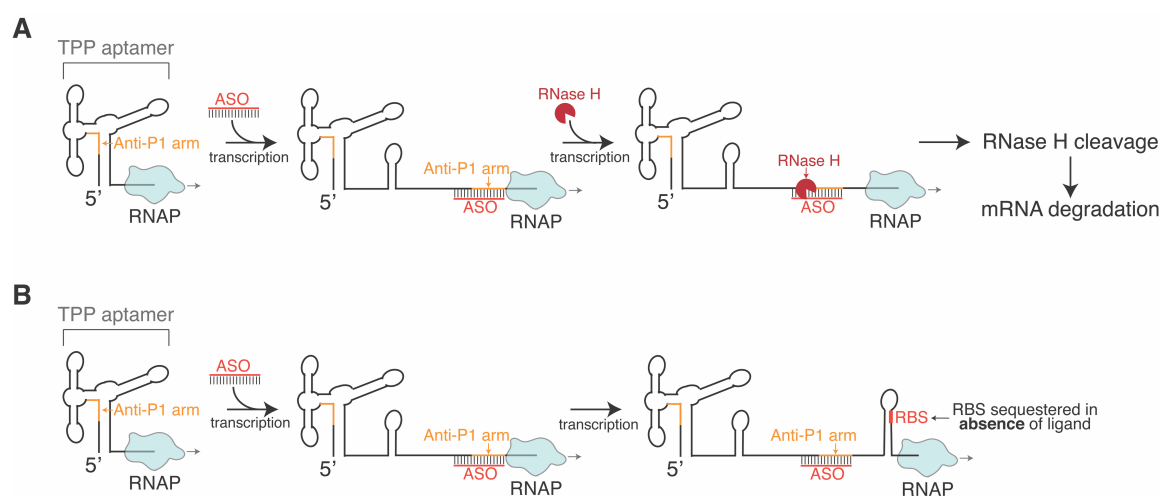


Figure 4.2. Overview of ASO-directed co-transcriptional silencing of target riboswitch-controlled genes, using the TPP riboswitch as an example.

(A) ASO binding recruits RNase H, which cleaves the DNA-RNA hybrid, prompting mRNA degradation. (B) ASO binding creates a steric block that prevents a structural rearrangement and favors the formation of the RBS sequestering stem.

Advances in Riboswitch Co-Transcriptional Folding for Riboswitch Families Found in Pathogenic Bacteria

RNA folding is by nature a co-transcriptional process, since RNA secondary structural elements can form as fast as $\sim 10^{-6}$ s⁸³—multiple orders of magnitude faster than the transcription elongation rate by RNAPs, e.g., $\sim 10^{-3}$ s/nt for *E. coli* RNAP⁸⁴. Experimental evidence pointing towards the *in vivo* relevance of co-transcriptional RNA folding emerged from early studies on the *Tetrahymena* group I intron, where it was observed that the autocatalyzed splicing of this intron occurs in a few seconds⁸⁵ and thus must take place within the timeframe of transcription. In stark contrast, when the same *Tetrahymena* group I intron RNA is synthesized *in vitro*, denatured and refolded, the majority of the RNA becomes “trapped” in intermediate folds that rearrange into the native state on a slow timescale (~ 1 – 100 min)⁸⁶. Therefore, developing a model of how an RNA acquires its functionally relevant folds requires incorporating the transcription machinery into experimental methods.

Many structural studies of riboswitch aptamer domains follow classic *in vitro* protocols: the RNA is transcribed with T7 RNAP, followed by denaturation and refolding for structural characterization^{87,88}. While such work has provided seminal insights into the architecture of riboswitch aptamer domains⁸⁹, this does not recapitulate how RNA folds *in vivo*. A key example stems from initial efforts to crystallize the *B. subtilis* *yvrC* cobalamin riboswitch, where the traditional method of denaturing gel purification, refolding, and incubation with a cobalamin ligand did not yield the ligand-bound aptamer for X-ray crystallography⁹⁰. These traditional *in vitro* methods used to study folded RNA differ from how an RNA folds in the cellular environment in a few key ways. First, RNA is transcribed 5'-3', and local secondary structures can begin forming before the full-length transcript is synthesized. Rather than adhering to a single free-

energy landscape wherein the full-length transcript folds all at once, for RNA, the folding free energy landscape changes as the transcript is lengthened by RNAP⁹¹. Second, the rate of RNAP transcription alters RNA's folded structure by dictating the time window for forming structural motifs. In particular, transcriptional pausing—a temporary halt in nucleotide addition during transcript elongation⁹²—creates a temporal window for key folding transitions and subsequent biological activity⁹³. Lastly, the experimental conditions for studying RNA folding in a test tube do not recreate the local environment experienced by an RNA in the cell, which is a topic that has been extensively reviewed elsewhere⁹⁴.

To better recapitulate how riboswitches fold and respond to a ligand in a native context, more laboratories are incorporating the transcriptional machinery into their experimental methods. Structural approaches such as Selective 2'-Hydroxyl Acylation analyzed by Primer Extension and Mutational Profiling, or SHAPE-MaP⁹⁵, have been updated to incorporate RNAP, permitting the interrogation of riboswitch RNA structures in the context of transcription complexes⁹⁶. Single-molecule approaches such as optical tweezers⁹⁷ and single-molecule FRET (smFRET)⁹⁸ have been used to study the folding events and dynamics of riboswitch constructs in the context of transcription complexes. Such studies have provided invaluable insights into structural intermediates traversed by riboswitch RNAs during active transcription that illustrate how a riboswitch aptamer domain “talks” to the expression platform^{19,99}. In light of these advances, we posit that further consideration of riboswitch folding events during active transcription will present opportunities for designing novel inhibitors. We first review methods that hold significant potential to aid rational ASO design for targeting riboswitches co-transcriptionally: Co-transcriptional SHAPE-seq¹⁰⁰ and, more recently, variable length Transcription Elongation Complex RNA structure probing (TECprobe-VL)¹⁹. Further, we present specific examples of

riboswitch co-transcriptional folding studies with the aim of highlighting the diversity of techniques that have uncovered novel insights into kinetic control of riboswitch folding. Table 1 presents all riboswitch classes found in pathogenic bacteria where specific family members have had their co-transcriptional folding characterized, with references listed for further reading. Additionally, the riboswitches we focus on in this section are poised as excellent targets for novel inhibition strategies, since they are all present in pathogenic bacteria and control the expression of essential genes for bacterial survival. Thus, upon repression of gene expression by a riboswitch inhibitor, the bacterial cell will be starved of an essential metabolite and die ¹².

Table 4.1. Riboswitch classes in pathogenic bacteria whose co-transcriptional folding has been experimentally characterized.

Riboswitch Class	Occurrence in Human Pathogenic Bacteria	Co-Transcriptional Folding Studies	Experimentally Validated Secondary/Tertiary Structures for Folding Intermediates
TPP	Present in 48 species of human pathogenic bacteria ²⁴	<i>E. coli thiC</i> riboswitch: ^{101,102} <i>E. coli tbpA</i> (a.k.a. <i>thiB</i>) riboswitch: ^{103,104}	<i>E. coli thiC</i> riboswitch: ^{101,102} <i>E. coli tbpA</i> (a.k.a. <i>thiB</i>) riboswitch: ¹⁰³
FMN	Present in 41 species of human pathogenic bacteria ²⁴	<i>B. subtilis ribD</i> riboswitch: ¹⁰⁵	N/A
<i>glmS</i> ribozyme	Present in 26 species of human pathogenic bacteria ²⁴	<i>B. subtilis glmS</i> ribozyme ^{106,107}	N/A
Purine	Present in 17 species of human pathogenic bacteria ²⁴	Guanine: <i>B. subtilis yxjA</i> and <i>xpt-pbuX</i> riboswitches: ^{108,109} Adenine: <i>B. subtilis pbuE</i> and <i>V. vulnificus add</i> riboswitches: ^{97,110,111}	Guanine: <i>B. subtilis yxjA</i> and <i>xpt-pbuX</i> riboswitches: ^{108,109} Adenine: N/A
Cobalamin	Present in 36 species of human pathogenic bacteria ²⁴	<i>E. coli btuB</i> riboswitch: ¹¹²	N/A
c-di-GMP	Present in 7 species of human pathogenic bacteria ¹¹³	<i>C. difficile</i> c-di-GMP riboswitch: ¹¹⁴	<i>C. difficile</i> c-di-GMP riboswitch: ¹¹⁴
preQ ₁	Present in 15 species of human pathogenic bacteria ¹¹³	<i>B. subtilis preQ₁</i> riboswitch: ^{115,116}	<i>B. subtilis preQ₁</i> riboswitch: ¹¹⁶

5.1. Co-Transcriptional SHAPE-Seq and TECprobe-VL

The Lucks and Strobel laboratories have been pioneers in their development of strategies to model RNA structure in the context of transcription elongation complexes. Initially, Strobel and colleagues reported on the co-transcriptional SHAPE-seq method ¹⁰⁰, which involves the following steps: (1) Preparation of a DNA template encoding the RNA sequence of interest with biotinylated dNMPs randomly distributed throughout the template. Streptavidin binds at the randomly distributed biotinylation sites, creating roadblocks to RNAP movement across every position on the DNA template. (2) Reconstitution of transcription *in vitro*, resulting in the formation of stalled transcription elongation complexes that are exposing the nascent, structured RNA transcript. (3) Chemical probing of the displayed RNA with either a SHAPE reagent (e.g.,

benzoyl cyanide) or a base-specific reagent (e.g., dimethylsulfate) followed by RNA processing steps to read out where the modification sites are and inform structure models ¹⁰⁰. More recently, SHAPE-seq has been superseded by the TECprobe-VL method, which improved upon the co-transcriptional SHAPE-seq by simplifying the library preparation steps to make the method more broadly accessible ¹⁹. The introduction of TECprobe-VL by Szyjka and Strobel studied the intermediate folds traversed early on in transcription of the *C. bacterium* ppGpp riboswitch, demonstrating regions of the RNA that are initially single-stranded and ultimately rearrange to be base-paired in the full-length folded riboswitch ¹⁹. While the ppGpp riboswitch itself is not an attractive antibiotic candidate since it does not regulate the expression of an essential gene for bacterial survival ¹¹⁷, the accessibility of TECprobe-VL permits its application to any riboswitch sequence of interest.

5.2. TPP Riboswitches

5.2.1. *E. coli thiC* Riboswitch

The Lafontaine lab applied a suite of complementary biochemical approaches to define how ligand binding to the TPP-sensing *E. coli thiC* riboswitch induces riboswitch structural rearrangements during active transcription ¹⁰¹. Oligonucleotide hybridization/RNase H digestion assays were performed to compare ligand-dependent structural changes when TPP is added co-transcriptionally vs. post-transcriptionally. Measurements of ligand binding kinetics from these data demonstrated that co-transcriptional ligand binding is approximately four-fold more efficient compared to post-transcriptional ligand binding ¹⁰¹. Further, *in vitro* transcription–translation assays elucidated that co-transcriptional TPP binding is required for the riboswitch to fold into a translationally inactive conformation ¹⁰¹. These observations strongly suggest that ligand sensing and subsequent structural rearrangements are coupled to transcription ¹⁰¹.

This study identified three transcriptional pauses encountered by RNAP during synthesis of the riboswitch expression platform ¹⁰¹. Motivated by the known importance of transcriptional pausing for RNA folding and gene regulation ⁹², the researchers explored the structure of nascent RNA transcripts at these pause sites using two complementary *in vitro* techniques that interrogate RNA structure—oligonucleotide hybridization/RNase H digestion assays and SHAPE ¹¹⁸. These data showed that while the nascent RNA adjacent to the first two pauses was competent to sense TPP (Figure 3A), the riboswitch structure at the third pause was no longer able to sense TPP; this loss of sensitivity is due to the nucleation of a hairpin stem that disrupts the folded aptamer domain in favor of an alternative fold (Figure 3B) ¹⁰¹. Taken together, this study defined a key time window for ligand-sensing and subsequent structural rearrangements that are “bookmarked” by transcriptional pauses; therefore, a rationally designed ASO that prevents the disruption of the folded aptamer domain within this window could bias the riboswitch towards the “off” state regardless of the intracellular TPP concentration (Figure 3C). Alternatively, an ASO containing DNA bases can anneal to a riboswitch folding intermediate and prompt RNase H degradation (Figure 3C).

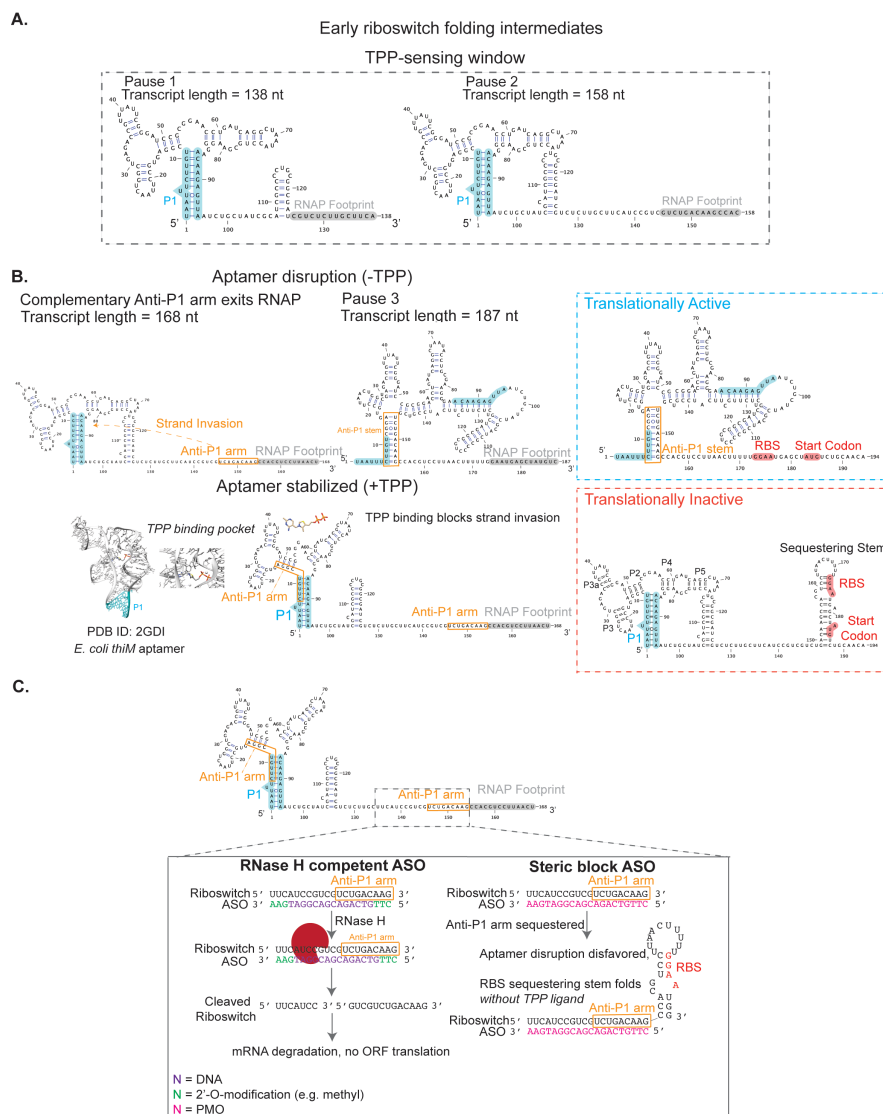


Figure 4.3. Folding pathway for *E. coli thiC* riboswitch in the presence and absence of TPP ligand and its proposed targeting with ASO.

(A) The riboswitch structural intermediates in the vicinity of RNAP pause sites are modeled based on published structure-probing experiments. (B) In the absence of TPP ligand, the Anti-P1 arm invades the already-folded TPP-sensing aptamer domain, promoting a translationally active conformation. In the presence of TPP ligand, TPP stabilizes P1 in the aptamer domain, blocking strand invasion by the Anti-P1 arm and promoting a translationally inactive conformation. (C) Opportunities for ASO inhibition by sequestering the Anti-P1 arm and preventing its invasion of the folded aptamer domain. One strategy is an RNase H-competent ASO, which contains a complementary DNA strand flanked by 2'-OMe nucleotides at its 5' and 3' ends to enhance stability. Alternatively, an ASO can act as a steric block that remains stably base-paired to the riboswitch RNA. Since the ASO remains base-paired, strand invasion by the Anti-P1 arm is blocked, favoring the folding of a hairpin stem that sequesters the RBS. A phosphorodiamidate morpholino oligonucleotide (PMO) is a charge-neutral nucleic acid that is commonly used for steric block ASOs and is described further in Section 7.

5.2.2. *E. coli* *tbpA* Riboswitch

In the case of the TPP-sensing *E. coli* *tbpA* riboswitch (a.k.a. *thiB* riboswitch), smFRET was used by the Lafontaine lab to monitor the dynamics of nascent riboswitch transcripts in the context of transcription complexes¹⁰⁴. The *tbpA* riboswitch has a critical feature in common with the *thiC* riboswitch above, in that there are three transcriptional pauses during expression platform folding. Since prior work had found pausing to set a temporal window for ligand-sensing for the *thiC* riboswitch¹⁰¹, this smFRET study investigated RNA dynamics at the *tbpA* pauses using roadblocked transcription complexes. Importantly, this study found that while nascent riboswitch transcripts were competent to bind the ligand at the first two pauses, ligand-induced structural changes were no longer observed at the third pause¹⁰⁴. The analysis of riboswitch structural dynamics in this smFRET setup was expanded to monitor the folding of nascent riboswitch RNAs in real time, providing insight into TPP-induced folding transitions during active transcription¹⁰⁴. These data reveal that TPP sensing occurs within a precise transcriptional window bound by the nucleation of a hairpin stem that competes with a paired region in the aptamer domain¹⁰⁴. This insight mirrors the findings highlighted above for the *thiC* riboswitch, demonstrating the generality of an ASO-targeting approach that focuses on nucleating RNA structures in the vicinity of transcriptional pause sites (Figure 3C).

The Lucks lab reported new insights into intermediate folds traversed by the *E. coli* *tbpA* riboswitch. The co-transcriptional SHAPE-seq approach¹⁰⁰ was applied to nascent *tbpA* transcripts to define key positions during riboswitch folding that commit the RNA structure to either a translationally active or inactive conformation. These data reveal that the linker region between the aptamer domain and expression platform is involved in nucleating a hairpin stem. The nucleation of this hairpin stem requires breaking a key pairing interaction in the aptamer domain,

and the completion of the hairpin folding results in RBS exposure¹⁰³. Unlike the smFRET study highlighted above, the co-transcriptional SHAPE-seq experiments performed in this work map intermediate RNA folds to specific riboswitch transcript lengths. Therefore, analysis of the transcript lengths where competing RNA structures begin to fold will define specific sequence targets that, upon annealing of an ASO, could bias RNA folding towards the translationally inactive conformation, starving the bacterial cell of an essential cofactor. We present secondary structure models of these experimentally validated *tbpA* riboswitch folding intermediates in Figure 4 and highlight how these intermediates could be targeted by an ASO.

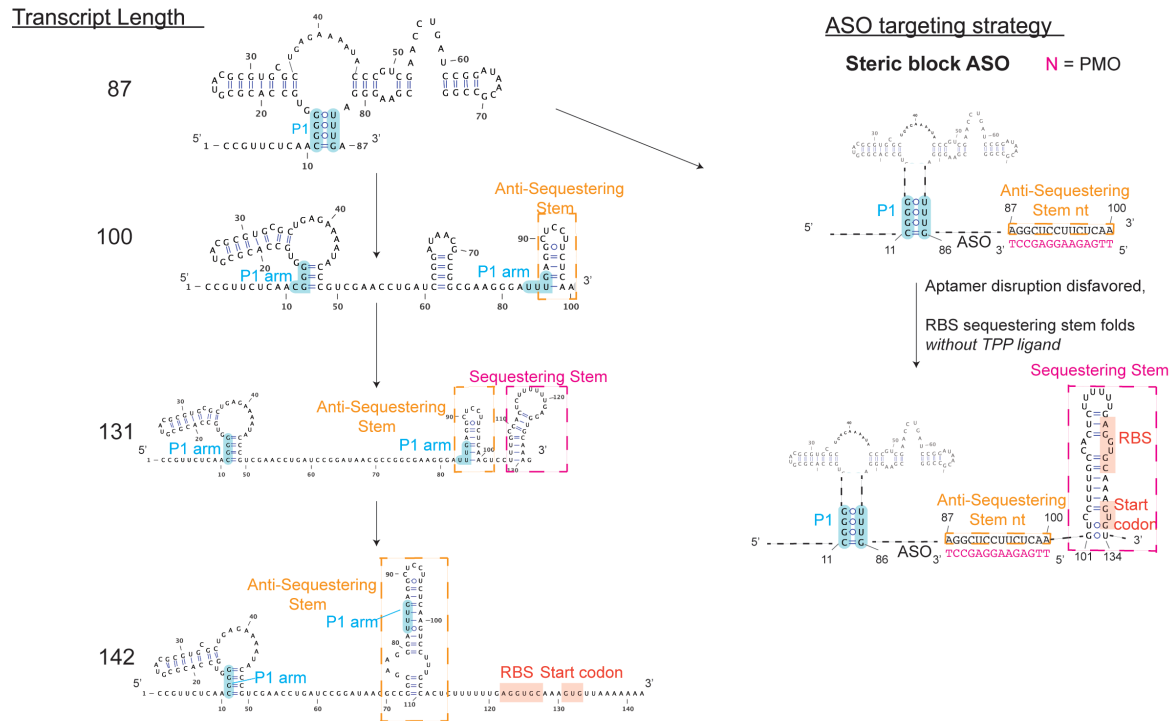


Figure 4.4. *E. coli* *tbpA* riboswitch folding intermediates experimentally validated by co-transcriptional SHAPE-seq.

A steric block ASO (e.g., PMO) can prevent formation of the anti-sequestering stem and favor instead folding of the sequestering stem, leaving RBS occluded in the absence of TPP ligand.

5.3. FMN Riboswitches

B. subtilis *ribD* Riboswitch

The Breaker laboratory clearly demonstrated that FMN-sensing by the *ribD* riboswitch in *B. subtilis* is kinetically driven, wherein ligand binding must occur within a precise time window to prompt transcription termination ¹⁰⁵. Specifically, transcriptional pauses were found to facilitate coordination between ligand binding and expression platform folding ¹⁰⁵, similar to the TPP riboswitch examples highlighted above. Further, this study ascertained that FMN never reaches thermodynamic equilibrium with the riboswitch aptamer due to the kinetic constraints on the folding regime ¹⁰⁵, highlighting the importance of transcription kinetics for effective riboswitching and downstream gene regulation.

Notably, further efforts to identify FMN riboswitch structural intermediates have not yet been reported. Approaches described earlier in this section such as co-transcriptional SHAPE-seq ¹⁰⁰, and more recently TECprobe-VL ¹⁹, will be useful strategies to precisely characterize intermediate RNA structures in the vicinity of transcriptional pause sites and provide new targets for ASO inhibitor design.

5.4. *glmS* Ribozyme

B. subtilis glmS Ribozyme

Recently, the Woodson lab explored whether the *glmS* ribozyme executes its self-cleavage co-transcriptionally ¹⁰⁷. Prior to the publication of this study, few key datapoints suggested that co-transcriptional self-cleavage was possible: (1) the ribozyme self-cleavage reaction is fast (80 min⁻¹) ¹¹⁹, so it is a reasonable assumption that cleavage occurs before the 1803-nt *glmS* open reading frame (ORF) is transcribed; (2) co-transcriptional cleavage permits RNase J1 to promptly begin mRNA degradation and prevent ribosomes from initiating translation of the *glmS* mRNA. If cleavage were to occur after ORF transcription, initiating ribosomes may impede RNase J1 degradation ^{120–122}.

Motivated by the above observations, the Woodson lab employed a combination of single-molecule fluorescence microscopy and biochemical methods to analyze co-transcriptional ribozyme cleavage¹⁰⁷. First, *in vitro* transcription reactions with radiolabeled RNAs showed that the ribozyme was able to self-cleave during transcription¹⁰⁷. However, this bulk ensemble approach could not define the sequence window where co-transcriptional cleavage began. To address this, a single-molecule TIRF (smTIRF) microscopy assay was developed to monitor transcription and ribozyme activity in real time. These data were fit to a quantitative model, which ultimately demonstrated that the nascent ribozyme can initiate cleavage before completion of its synthesis¹⁰⁷. Armed with the knowledge that the *glmS* ribozyme induces self-cleavage co-transcriptionally, the modeling of structural intermediates traversed by this ribozyme could identify sequence regions amenable to ASO targeting.

5.5. Purine Riboswitches

5.5.1. Adenine Riboswitches

The Block lab employed an optical-trapping assay to monitor co-transcriptional folding events for the *B. subtilis pbuE* adenine-sensing riboswitch⁹⁷. These assays clearly demonstrated that the adenine-induced formation of an anti-terminator stem occurs co-transcriptionally⁹⁷, highlighting a recurring theme for riboswitches that control transcription termination/anti-termination: the RNA structural switch must occur within the time-frame of transcription¹²³. Once an anti-terminator stem is formed, RNAP continues transcribing the mRNA, and any subsequent RNA structural rearrangements in the 5'-UTR can no longer exert control over transcription.

More recent work from the Batey laboratory employed a structure-guided mutagenesis approach to characterize specific regions of the *pbuE* riboswitch that confer ligand-dependent regulatory activity¹¹⁰. First, it was observed that a hairpin stem in the aptamer domain facilitates

strand invasion by the expression platform to promote the formation of an intrinsic terminator hairpin ¹¹⁰. Second, the introduction of a strong transcriptional pause towards the end of aptamer domain synthesis produced the greatest induction of reporter gene expression of all the riboswitch variants tested in this study ¹¹⁰. This finding underscores the importance of pause sites for effective riboswitching, and provides new clues for the rational design of ASO inhibitors. For example, since pauses provide a temporal window for RNA structural elements to form ⁹³, transient single-stranded regions of RNA in the vicinity of a pause site could be targeted by an ASO inhibitor, as illustrated for the TPP-sensing riboswitches described in Section 5.2 (Figures 3 and 4).

5.5.2. Guanine Riboswitches

The Lucks lab investigated the co-transcriptional folding mechanism of the *B. subtilis yxjA* guanine-sensing riboswitch, which is an example of a riboswitch where there are no overlapping sequences between the aptamer domain and expression platform ¹⁰⁸, unlike the above *pbuE* adenine-sensing riboswitch example. Interestingly, the riboswitch relies on the formation of a central helix that dictates the switching mechanism depending on whether guanine is bound to the aptamer domain (Figure 5A) ¹⁰⁸. Binding of guanine effectively prevents this central helix from folding, biasing the downstream riboswitch folding toward the “off” state ¹⁰⁸. The use of co-transcriptional SHAPE-seq defined the precise transcriptional window where this key central helix forms (Figure 5B) and led the authors to the hypothesis that this central helix forms through a strand-invasion process that is disfavored in the presence of ligand ¹⁰⁸. This hypothesis was further tested using gene reporter fusion assays and MD simulations, supporting a model wherein the formation of this central helix competes with a stable pairing interaction in the aptamer domain ¹⁰⁸. These findings underscore the importance of considering a riboswitch’s intermediate folds in efforts to bias its structural ensemble to the “off” state using novel inhibitors. Since this work on

the *yxjA* riboswitch has experimentally validated folding intermediates, we present some of the secondary structure models in Figure 5B,C to highlight how these intermediates could be targeted by an ASO. Specifically, we propose that a steric block ASO designed to mimic the central helix could promote the folding of the downstream terminator hairpin in the absence of guanine ligand.

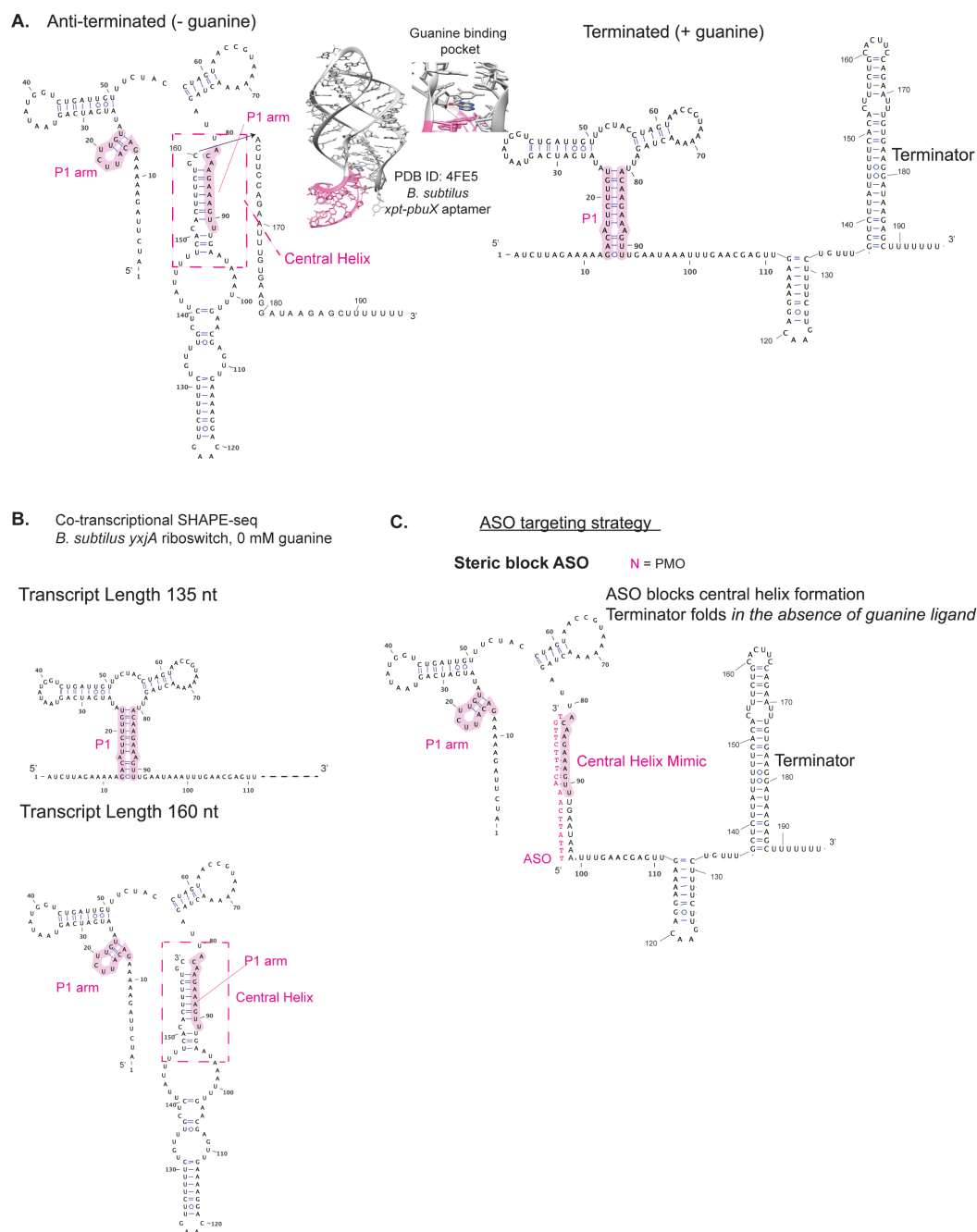


Figure 4.5. Folding outcomes for *B. subtilis* *yxjA* riboswitch in the presence and absence of guanine ligand and its proposed targeting with ASO.

(A) Alternative structures of the *B. subtilis* *yxjA* riboswitch in the presence and absence of guanine ligand. (B) Riboswitch structural intermediates based on co-transcriptional SHAPE-seq experiments. (C) ASO targeting strategy to mimic the central helix, which can promote the folding of the downstream terminator in the absence of guanine ligand.

5.6. Cobalamin, c-di-GMP, and preQ₁ Riboswitches

While less widely distributed in pathogenic bacteria, the cobalamin, c-di-GMP, and preQ₁ riboswitches present an enticing opportunity for narrow-spectrum inhibitors that target specific bacterial strains. The co-transcriptional folding of the *E. coli btub* cobalamin-sensing riboswitch was first characterized by the Pan lab in a study that used oligonucleotide hybridization/RNase H digestion assays. First off, comparison of cobalamin-dependent structural changes between denatured and refolded and co-transcriptionally folded RNA elucidated that ligand-dependent structural rearrangements occur during active transcription ¹¹². Further, transcriptional pausing was found to facilitate cobalamin-dependent structural rearrangements in the expression platform ¹¹². While the oligonucleotide hybridization/RNase H digestion assays performed in this work were not intended to be applied to riboswitch inhibitor design, a revisitation of these biochemical studies could inspire initial efforts towards ASO design. For example, since this study employed 57 different oligo probes, a fresh look at these data could provide useful starting points for ASOs that can anneal to the nascent riboswitch RNA and prompt degradation during active transcription.

The Schwalbe lab has employed NMR to study folding intermediates of the *C. difficile* c-di-GMP riboswitch. Using a method to rapidly produce and purify a library of intermediate RNA transcripts ¹²⁴, this study measured K_d values for riboswitch folding intermediates with both NMR and ITC ¹¹⁴. These data defined a 61-nt window for c-di-GMP sensing by the riboswitch, and the K_d values obtained for the riboswitch transcripts within this window spanned two orders of magnitude ¹¹⁴. The wide range of K_d values was explained by the presence of competing riboswitch folds present at specific transcript lengths, altering the riboswitch structural ensemble and in turn ligand binding ¹¹⁴.

The Walter lab has employed both smFRET and cryo-EM to investigate the folding and dynamics of the *B. subtilis* preQ₁ riboswitch. Investigation of the preQ₁ riboswitch with smFRET uncovered that a transcriptional pause situated between the aptamer domain and expression platform is stabilized by a pseudoknot that directly contacts RNAP ¹¹⁵. Further, it was observed that binding of the preQ₁ ligand signals RNAP release from the pause, underscoring the coupling between riboswitch folding and transcriptional pausing ¹¹⁵. Elaborating on this work, cryo-EM structures of paused elongation complexes containing the nascent preQ₁ riboswitch RNA were solved in both the ligand-free and ligand-bound states ¹¹⁶. This work revealed that in the absence of preQ₁, the local riboswitch RNA structure “retracts” the RNA 3' end within the RNAP active site, leading to a halt in nucleotide incorporation ¹¹⁶. However, preQ₁ binding to the riboswitch induces large-scale conformational changes in RNAP that reorient the RNA 3' end at the active site and release the pause ¹¹⁶.

5.7. Other Advances in Riboswitch Co-Transcriptional Folding

In this section, we summarize additional riboswitch co-transcriptional folding studies that point to recurring themes in riboswitch-mediated gene regulation, which will likely prove useful for the design of ASO inhibitors.

There has been significant research effort directed towards the co-transcriptional folding of the ZTP riboswitch class, which is a signaling molecule produced in response to 10-formyltetrahydrofolate deficiency in the bacterial cell ¹²⁵. The Hua lab designed a single-molecule vectorial folding assay using an engineered superhelicase that sequentially releases fluorescently labeled riboswitch RNA at a speed comparable to that of bacterial RNAPs (~60 nt/s) ¹²⁶. This technique was applied to the *F. ulcerans* ZTP riboswitch, which elucidated that the riboswitch is kinetically controlled, since the rate of RNA release by the helicase altered ligand-dependent

structural changes ¹²⁶. The folding of the *C. beijerinckii pfl* ZTP riboswitch has been extensively studied by the Lucks and Strobel laboratories using co-transcriptional SHAPE-seq, and more recently TECprobe-VL ^{19,99}. These studies uncovered the specific sequence and structural elements that modulate an internal RNA strand displacement, which is disfavored in the presence of a ligand ^{19,99}. Building upon this work on the *C. beijerinckii pfl* ZTP riboswitch, the Lucks lab discovered that altering the rate of strand displacement via mutagenesis tunes its dynamic range ¹²⁷, and that altering the expression platform sequence and structure to slow RNA folding enhances riboswitch sensitivity ¹²⁸. Additionally, the Schwalbe lab has investigated the *T. carboxydivorans* ZTP riboswitch co-transcriptional folding with NMR to define the precise window of transcript lengths where the ligand can bind ¹²⁹.

Another well-studied class is the fluoride-sensing riboswitch, which controls expression of a fluoride exporter to prevent fluoride from accumulating to toxic levels in the cytosol ¹³⁰. The co-transcriptional folding of the *B. cereus crcB* fluoride-sensing riboswitch has been characterized by the Walter lab using smFRET. These studies highlighted a dynamic interplay between the transcription factor NusA and the fluoride binding the co-transcriptionally folded RNA ¹³¹. A further smFRET study on this riboswitch by the Walter lab characterized the dynamics of intermediate transcript lengths and established the role of Mg^{2+} in fluoride-mediated conformational changes ¹³². Another study on this riboswitch from the Lucks lab employed a combination of *in vitro* transcription and cellular gene expression assays to demonstrate the role of pseudoknot base pairs on co-transcriptional structure switching ¹³³. There are additional recent works on riboswitch co-transcriptional folding for the manganese sensing riboswitch class ¹³⁴, 2'-dG sensing riboswitch class ^{135,136}, and the SAM-VI riboswitch class ¹³⁷, which we direct the reader to for further reading on this topic.

Antisense Oligonucleotides That Act on RNA Co-Transcriptionally: A Case Study on a Group I Intron

Building off of prior work from the Turner laboratory ¹³⁸, the Zhang laboratory reported the use of an ASO to target co-transcriptional self-splicing of the group I intron located within the 26S rRNA of *C. albicans*, a fungus ⁸². First, *in vitro* transcription assays with radiolabeled RNA were used to monitor the efficacy of different ASO sequences to inhibit co-transcriptional splicing ⁸². It was found that eight different ASOs targeting the P3-P7 core of the intron (Figure 6A) achieved half-maximal inhibition in the ~0.2–0.3 μM concentration range ⁸². The addition of a 2'-O-Me moiety to an ASO targeting the P3-P7 core (2'-O-Me 240-261R) enhanced splicing inhibition *in vitro* by an order of magnitude, exhibiting an IC_{50} of ~37 nM ⁸². Prior work established that modifications at the ribose 2' position enhance ASO annealing to its target by promoting a 3'-*endo* pucker conformation of the ribose, thus making it more RNA-like and increasing its propensity to base-pair with the RNA target ^{139,140}. Since the 2'-O-Me 240-261R ASO delivered promising results *in vitro*, it was used for the subsequent *in vivo* experiments described here. Confocal fluorescence microscopy confirmed the presence of the core-targeted ASO in the nucleus of *C. albicans*, and reverse-transcription PCR (RT-PCR) targeting the pre-rRNA confirmed ASO inhibition *in vivo*, with an IC_{50} of 2.30 μM ⁸². The authors proposed that splicing inhibition would be lethal to *C. albicans* because if the intron-containing RNA is assembled into the ribosome, association with the small ribosomal subunit is blocked (Figure 6B) ¹⁴¹. The authors demonstrated that the core-targeted ASO specifically inhibits the growth of an intron-containing *C. albicans* and was unable to inhibit growth for a *C. albicans* strain lacking the intron ⁸². Further, the ASO was found to inhibit *C. albicans* growth in a dose-dependent manner, with an IC_{50} of 4.7 μM ⁸².

Taken together, these data illustrate that ASOs can be rationally designed to target RNA co-transcriptional folding. For the group I intron example highlighted above, the best-performing ASO for splicing inhibition targets a region of the RNA that is base-paired in its final folded structure, and therefore would not have been an obvious target resulting from the inspection of the fully folded intron (Figure 6A). Thus, assessing the final-folded RNA structure alone limits opportunities for the design of efficacious ASO inhibitors. In the remaining sections of this review, we cover advances made in the delivery of oligonucleotide therapeutics and progress in the use of ASOs in the clinic as antibacterials.

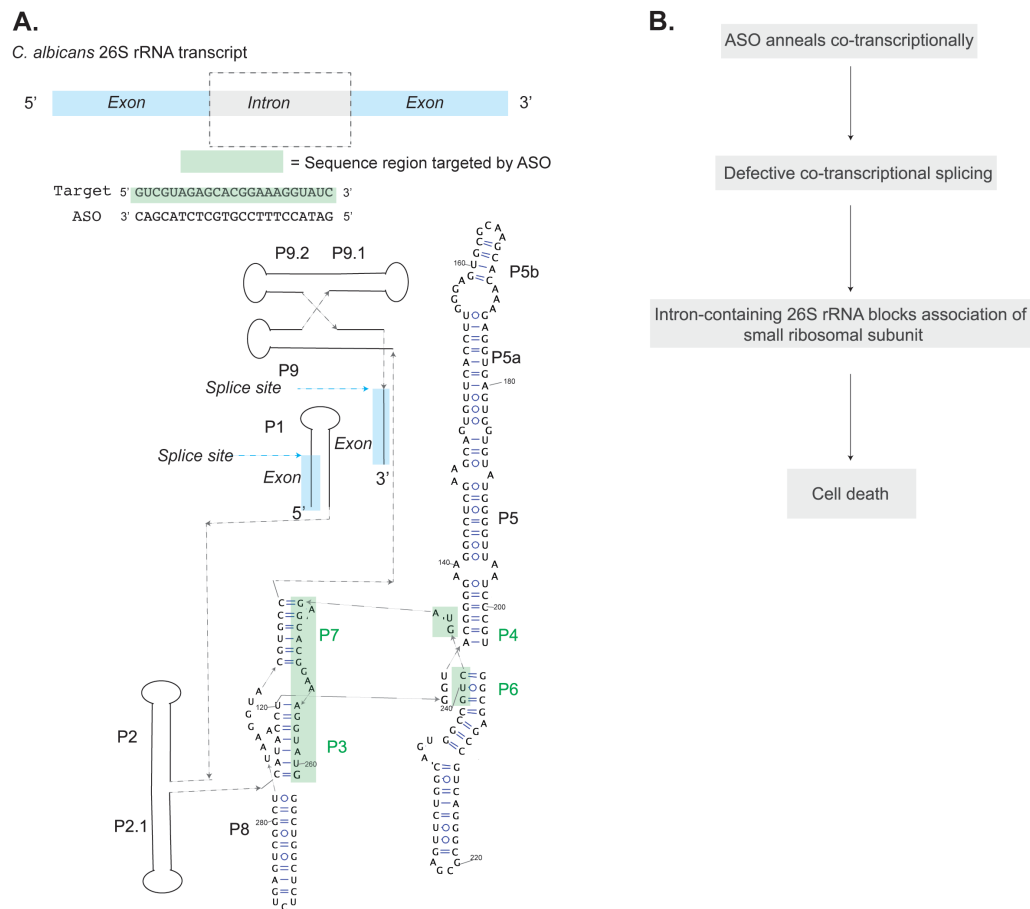


Figure 4.6.

(A) Secondary structure of *C. albicans* 26S rRNA group I intron, with paired regions of the intron core targeted by the ASO labeled in green. The specific stretch of sequence targeted by the ASO is highlighted in green. Splice sites are denoted with blue arrows. (B) Illustration of how the ASO targeting co-transcriptional splicing of the 26S rRNA is lethal to *C. albicans*.

Delivery of ASOs

7.1. Chemical Modifications

Chemical modifications have proven to be one of the most effective strategies for enhancing oligonucleotide drug delivery. Modifications of the nucleic acid backbone, ribose sugar and nucleobase have been used extensively in the design of therapeutic ASOs ¹⁴². Such modifications help improve the pharmacokinetics, pharmacodynamics, and biodistribution of ASOs ¹⁴². A

commonly employed backbone modification is phosphorothioate linkages (PS), where one of the non-bridging oxygen atoms of the inter-nucleotide phosphate group is replaced with sulfur ¹⁴². PS linkages improve an ASO's therapeutic efficacy by providing nuclease resistance and increasing its association with proteins in plasma and within cells ¹⁴². Another common ASO modification is the addition of a functional group at the 2' position of a ribose sugar, with common examples being 2'-*O*-methyl, 2'-*O*-methoxyethyl and 2'-fluoro ¹⁴². These modifications increase resistance to nucleases by substituting the nucleophilic 2'-hydroxyl group, which improves oligonucleotide stability in plasma and increases its half-life in tissues, resulting in extended drug effects ¹⁴². Lastly, rather than using a DNA or RNA-like oligonucleotide, alternative chemistries have been developed, such as phosphorodiamidate morpholino oligonucleotides (PMOs), which are charge-neutral nucleic acids where the five-membered ribose is replaced with a six-membered morpholine ring ¹⁴². The advantage of charge-neutral oligonucleotide chemistries is that they facilitate conjugation to moieties that promote cellular delivery ¹⁴².

7.2. Bioconjugation Strategies

One strategy to deliver ASOs into bacteria is the use of cell-penetrating peptides (CPPs), which are short (generally <30 amino acids) amphipathic or cationic peptide fragments that originate from protein translocation motifs found in nature, or are based on polymers of basic amino acids ¹⁴³. Peptide-PMO (PPMO) conjugates consisting of a CPP covalently linked to a PMO have been identified as a promising antibacterial delivery strategy ¹⁴². For example, PPMO conjugates that target an essential bacterial gene *acpP* in a murine infection model were demonstrated to be bactericidal ¹⁴⁴. A comprehensive list of PPMO conjugates that have been experimentally validated to cross the cell wall across diverse pathogenic bacteria is presented in a review by Xue et al. ¹⁴⁵. Additional strategies that are not based on the covalent modification of

the ASO to the delivery system have attracted interest as alternatives, such as encapsulation in lipid nanoparticles or DNA nanostructures, and are also covered in the review by Xue et al.¹⁴⁵.

Translation of ASO Therapies to the Clinic

There has been significant progress in the translation of oligonucleotide therapies to the clinic, with thirteen ASO-based medicines to treat human diseases approved by the FDA thus far for clinical use⁷⁵. For example, Golodirsen and Eteplirsen are FDA-approved ASOs used to treat Duchenne muscular dystrophy (DMD), which promote the skipping of an exon in the *DMD* gene that would otherwise produce an out-of-frame transcript¹⁴². Generally speaking, ASOs used to treat human disease can operate through many different mechanisms. For example, some are designed to silence disease-causing or disease-modifying genes by promoting RNase H degradation upon DNA oligo annealing to the target mRNA¹⁴². Other ASOs are designed to mask a particular stretch of sequence in an mRNA and interfere with downstream RNA–RNA and/or RNA–protein interactions, with a common application being the modulation of alternative splicing, as described for Golodirsen and Eteplirsen¹⁴².

While there are no FDA-approved antisense antibacterials yet, there have been great strides in their development. Multiple preclinical studies delivered promising results on the use of ASOs as potential antibacterial therapeutics¹⁴⁶. For example, an ASO targeting a gene in *S. aureus* that is critical for replication (*ftsZ*) inhibited the growth of methicillin-resistant *S. aureus* in cell culture, and conferred increased survival to mice infected with the Mu50 strain of *S. aureus*¹⁴⁷. Additionally, in an *S. aureus*-infected cutaneous mouse wound, the use of an ASO targeting the essential *gyrA* mRNA decreased bacterial growth and promoted cutaneous wound healing in the mice¹⁴⁸. Additional efforts to develop ASOs targeting essential genes in pathogenic bacteria are highlighted in the review by Buthelezi et al.¹⁴⁶.

Despite significant advances made in the design of ASO therapeutics, there are many challenges to overcome to achieve their widespread use in the clinic. The effective delivery of ASOs to many tissues is a major hurdle to overcome ¹⁴². For example, injected nucleic acid therapeutics must resist nuclease degradation, bypass renal clearance, and avoid non-productive sequestration by plasma proteins in order to reach the desired target ¹⁴². A combination of chemical modifications and conjugation strategies outlined in Section 7 is typically employed to confer the desired pharmacokinetic and pharmacodynamic properties to an ASO drug ¹⁴², and further advances on these fronts will likely expand the number of conditions that are treated with ASO therapeutics.

Discussion

Widespread antibiotic resistance in pathogenic bacteria is an urgent problem in modern medicine ¹. To combat this problem, riboswitches have emerged as potential targets for new antibiotics, since they are widespread among pathogenic bacteria but absent in human cells ⁴. There have been significant advances in designing small-molecule riboswitch inhibitors, but these strategies still show significant limitations ^{29,37,49}. Antisense oligonucleotides are beginning to attract attention as a new riboswitch inhibition strategy, and several have been designed to specifically target riboswitches while being non-toxic to human cells ²⁷. However, there are limited studies leveraging advances in RNA co-transcriptional folding to design ASO riboswitch inhibitors. The group I intron example (Section 6) demonstrated that regions of RNA that are base-paired in the final folded structure can still be effectively targeted by an ASO. Advances in methodologies to model riboswitch folding intermediates ^{19,100} provide exciting opportunities to expand the search for efficacious ASO inhibitors.

Experimental work on the riboswitches outlined in Section 5 has built foundational principles for riboswitch co-transcriptional folding that can guide the search for novel ASO inhibitors. For example, co-transcriptional SHAPE-seq studies on the *tbpA* riboswitch illustrated how in the absence of the TPP ligand, a strand invasion event disrupts the riboswitch aptamer domain, leading to the riboswitch “on” state. We illustrate how a competitor ASO could prevent effective strand displacement of the riboswitch, biasing the structural ensemble towards the “off” state even in the absence of TPP ligand (Figures 2 and 3). Additionally, biochemical study of TPP riboswitches can provide inspiration for novel ASO design, with an example being ASOs that target key RNA structural transitions in the vicinity of transcriptional pause sites during expression platform folding (Figure 2).

Additionally, there have been exciting advances in targeting dsRNA–ssRNA junctions using ASOs, which can form a triplex adjacent to a duplex at a dsRNA–ssRNA junction ¹⁴⁹. Recent work illustrated how targeting these junctions with an ASO can stimulate ribosomal frameshifting and inhibit Dicer cleavage activity of a pre-miRNA ¹⁴⁹. This work represents an important advance that could inspire the design of ASOs that target riboswitches at dsRNA–ssRNA junctions, further expanding the available sequence space for inhibitor design.

The studies referenced in Sections 7 and 8 demonstrate how ASOs can be modified and/or conjugated to facilitate efficient delivery into the cell ^{142,143,145}. In fact, thirteen ASO-based drugs are already FDA-approved for clinical use, with more in various stages of clinical trial ⁷⁵. Using ASOs to treat infectious diseases, including bacterial infections, is an active area of research with several promising candidates already developed ¹⁴⁶. Taken together, we propose that the methods used to study RNA co-transcriptional folding could be key for designing novel ASOs to target riboswitch RNAs in pathogenic bacteria.

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