previously shown to be required for EutV binding of dual-hairpin substrates (*5*). We tested one mutant, in which these critical residues of the P3 loop were mutated (M1) and a second, in which the same changes were made to the P4 loop (M2). Similar to deletion of P3/P4, these mutant constructs were both induced by EA alone in the *eutX* strain, in contrast to wild-type *eutG-lacZ* (Fig. 3D). Therefore, these data confirm that P3/P4 mutations that prevent EutV binding affect regulation. We also examined the M3 mutation predicted to abrogate AdoCbl binding and cause constitutive inclusion of P3/P4 in EutX (Fig. 2, C and D). As expected, this resulted in uninducible expression (Fig. 3C).

In conclusion, we present evidence of a riboswitch acting in trans, the second described (*13*). In the prior example, a classical sRNA that acts by base-pairing to a target mRNA can be prematurely terminated by a riboswitch. However, EutX acts in a signal transduction pathway that is subject to riboswitch-mediated control within the confines of an sRNA. A few other sRNAs, like EutX, have been shown to affect gene expression by sequestering RNA-binding proteins. The best-studied examples are CsrB and CsrC, which titrate CsrA, a translational inhibitor protein, away from its mRNA targets [reviewed by (*14*)]. EutX demonstrates a mechanism for how protein sequestration can be placed under signal-responsive regulatory control by a riboswitch.

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SUPPLEMENTARY MATERIALS

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RIBOSWITCHES

Sequestration of a two-component response regulator by a riboswitch-regulated noncoding RNA

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Riboswitches are ligand-binding elements contained within the 5′ untranslated regions of bacterial transcripts, which generally regulate expression of downstream open reading frames. Here, we show that in *Listeria monocytogenes*, a riboswitch that binds vitamin B_{12} controls expression of a noncoding regulatory RNA, Rli55. Rli55, in turn, controls expression of the *eut* genes, whose products enable ethanolamine utilization and require B_{12} as a cofactor. Defects in ethanolamine utilization, or in its regulation by Rli55, significantly attenuate *Listeria* virulence in mice. Rli55 functions by sequestering the two-component response regulator EutV by means of a EutV-binding site contained within the RNA. Thus, Rli55 is a riboswitch-regulated member of the small group of regulatory RNAs that function by sequestering a protein and reveals a distinctive mechanism of signal integration in bacterial gene regulation.

Ethanolamine is an abundant molecule in the vertebrate intestine (*1*, *2*), and genes of the ethanolamine utilization pathway (*eut*) are widely conserved in pathogenic bacteria (*3*–*5*). This includes the Gram-positive intracellular human pathogen *Listeria monocytogenes,* in which *eut* expression has been shown to be up-regulated in the intestine during infection of mice (*6*), which suggests that ethanolamine is important for *Listeria* pathogenesis. In *Enterococcus faecalis*, *eut* expression is activated in response to ethanolamine by a two-component response regulator, EutVW (*7*, *8*). In *Salmonella enterica*, ethanolamine utilization requires vitamin B_{12} as a cofactor (*9*), and we noted the presence of a B_{12}-binding riboswitch located upstream of the first gene in the *eut* locus of *L. monocytogenes* (Fig. 1A) (*10*), which suggested that *eut* expression might also be regulated in response to B_{12} availability.

To investigate a role for B_{12}, we examined expression of the *eut* locus in response to B_{12} and ethanolamine by RNA sequencing (RNA-seq) (Fig. 1B) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (fig. S1). We observed expression of the *eutVW* genes under all conditions, albeit at low levels, which suggested that the cell maintains a pool of EutVW to sense and respond to ethanolamine. In contrast, higher-level expression of *eutVW* and expression of other *eut* genes require both B_{12} and ethanolamine (Fig. 1B). These data indicated B_{12} is required to activate *eut* expression and suggested that the B_{12} riboswitch does not prevent transcription of the *eut* locus in the presence of B_{12}, as might be expected for a classical riboswitch (*11*).

To clarify whether the riboswitch has a role in the B_{12}-dependent regulation of *eut* expression, we examined transcription of the riboswitch locus (Fig. 1C) in response to B_{12} and ethanolamine. We were unable to detect any long transcript, which might extend into the downstream *eut* locus. However, we did detect a ~450-nucleotide (nt) transcript, Rli55 (*10*, *12*), in the absence of B_{12} (Fig. 1D), and a smaller (~200-nt) transcript that accumulated in the presence of B_{12} (Fig. 1D). This result suggested that the riboswitch mediates transcription termination of the ~450-nt Rli55 transcript in response to B_{12}, and any remaining long transcript is rapidly degraded (fig. S2A). A strain with a deletion in the B_{12} riboswitch (Δribo) constitutively expressed the long Rli55 transcript under all conditions (Fig. 1E and fig. S2B), which confirmed B_{12}-dependent regulation by the riboswitch. Conversely, neither the long or short Rli55 transcripts were detected in a strain (Δ*rli55*) in which the entire *rli55* locus was deleted (Fig. 1E and fig. S2B). Thus, the B_{12} riboswitch determines whether Rli55 is expressed as a long or short transcript in response to B_{12}.

We reasoned that Rli55 might act as a regulatory RNA controlling expression of the *eut* locus. To test this hypothesis, we examined expression of *eut* genes by qRT-PCR in the wild-type, Δribo, and Δ*rli55* strains. In the wild-type strain, *eut* expression was elevated only when both ethanolamine and B_{12} were present in the media (Fig. 1F and fig. S3, A and B), whereas in the Δribo strain, which constitutively expresses the long Rli55, *eut* genes were never expressed under any conditions. In contrast, in the Δ*rli55* strain, expression of the *eut* genes was high in the presence of ethanolamine alone in addition

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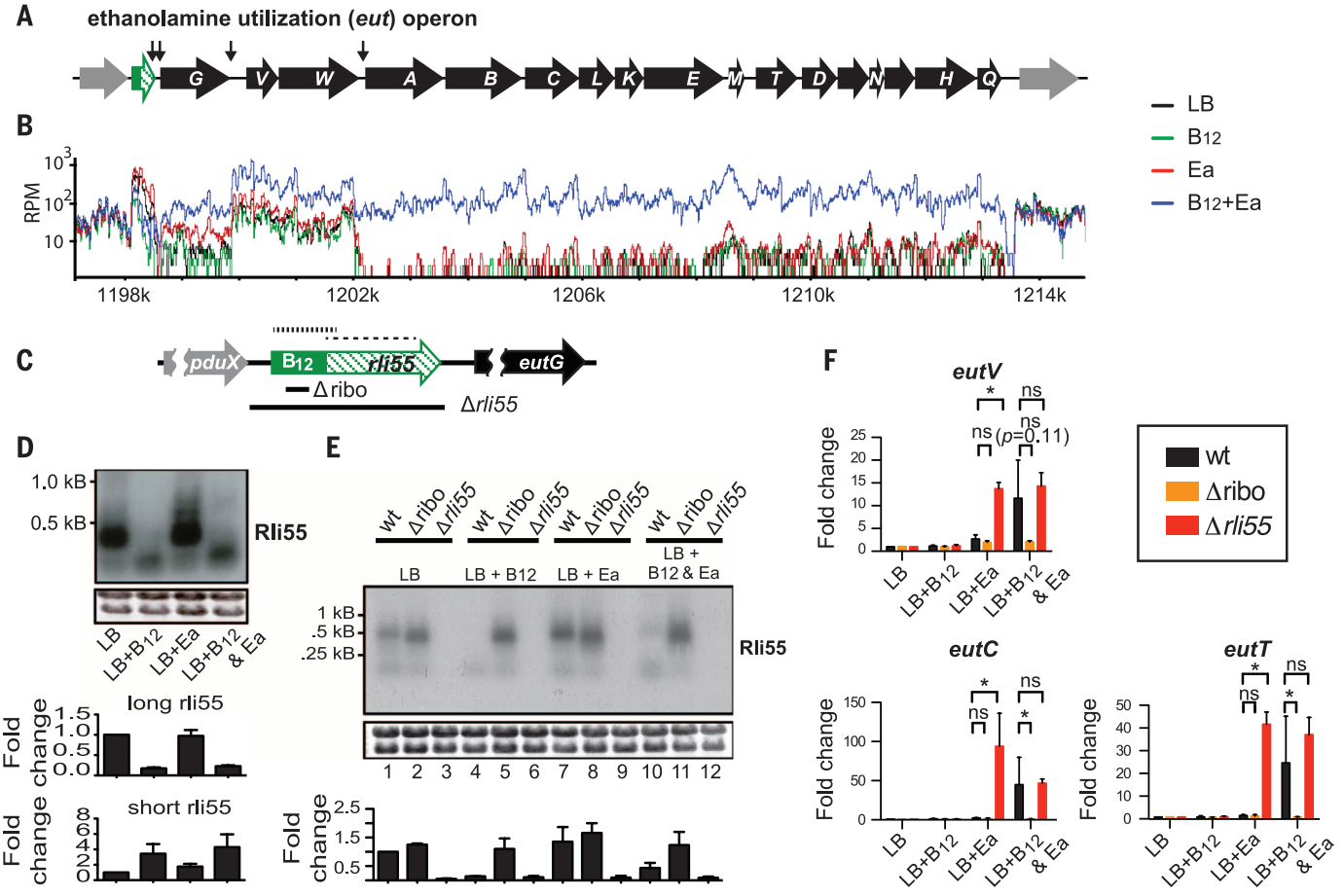

Fig. 1. Control of ethanolamine utilization (*eut*) genes by a B_{12} riboswitch–regulated ncRNA. (**A**) The *eut* locus. Green arrow denotes B_{12} riboswitch. Vertical arrows denote positions of ANTAR elements (*13*) (**B**) RNA-seq coverage of *eut* operon. (**C**) Regions deleted in the Δ*rli55* and Δribo strains are indicated by solid black lines. Northern blot of the *rli55* transcript in the (**D**) wild-type strain with probe indicated by thick dashed line in schematic or (**E**) wild-type (wt), Δribo, or Δ*rli55* strains with probe indicated by thin dashed line in schematic. Ethidium bromide staining of ribosomal RNA is shown as a loading control. Quantification of bands from three experiments is shown below each blot. Expression of the (**F**) *eutV*, *eutC*, and *eutT* genes was evaluated by qRT-PCR in the indicated strains. LB, Luria broth. Values represent means ± SEM, $n = 3$; $^*P < 0.05$; ns, not significant.

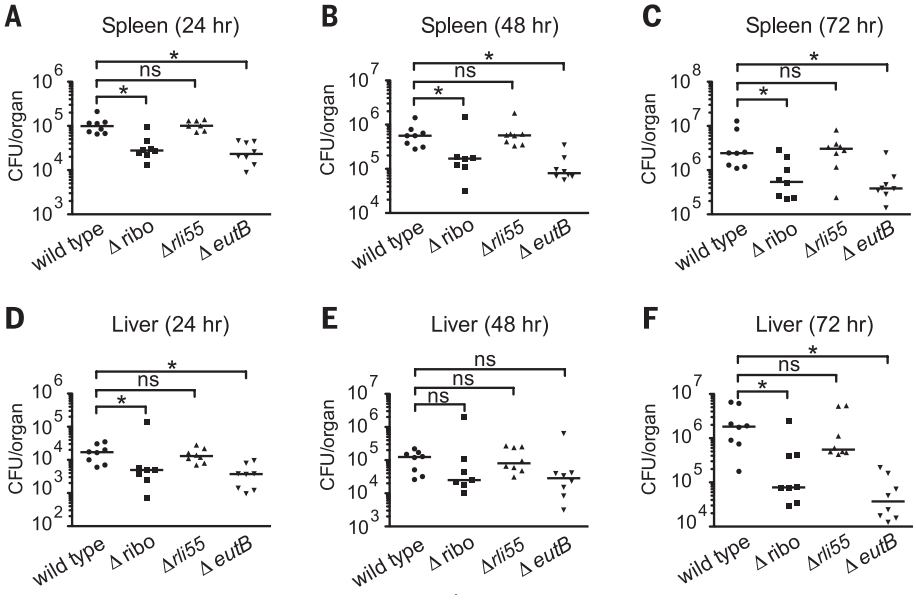

Fig. 2. Ethanolamine utilization and pathogenesis. BALB/c mice were injected intravenously with ~4500 colony-forming units (CFU) of the indicated strain. Mice were killed at (**A**) and (**D**) 24 hours, (**B**) and (**E**) 48 hours, or (**C**) and (**F**) 72 hours, and spleens and livers were removed to assess bacterial load per organ. Results represent two independent experiments with three or four mice per group in each experiment. $^*P < 0.05$, ns, not significant.

to ethanolamine and B_{12} together. Together, these data support a model in which Rli55 prevents the expression of the *eut* locus in the absence of B_{12}, ensuring that the *eut* genes are expressed only in the presence of both ethanolamine (substrate) and B_{12} (cofactor) (fig. S3C).

We tested whether defects in ethanolamine utilization, or its regulation, impacted *L. monocytogenes* virulence by examining the wild-type, Δribo, and Δ*rli55* strains in a mouse intravenous infection model. We also tested a mutant lacking the *eutB* gene encoding an ethanolamine lyase subunit, which is unable to catabolize ethanolamine. The Δribo and Δ*eutB* strains both had significantly reduced bacterial loads at 24 hours after infection compared with the wild-type strain, and these differences increased at 48 hours and 72 hours postinfection (~10- to 50-fold) (Fig. 2, A to F). In contrast, the Δ*rli55* strain, in which *eut* expression is not inhibited, was present in amounts comparable to those in the wild-type strain in the spleen and liver. Thus, defects in ethanolamine utilization or activation of *eut* expression significantly attenuate *L. monocytogenes* virulence.

In *E. faecalis*, ethanolamine is sensed by the sensor-kinase EutW, which subsequently phosphorylates the response regulator EutV (*7*). Phosphorylated EutV in turn binds ANTAR elements (AmiR and NasR transcriptional antiterminator regulator) in the 5′ untranslated regions of actively transcribed *eut* mRNAs, which prevents the formation of a transcription terminator and consequently activates *eut* expression (*13*). In *L. monocytogenes*, ANTAR sites were identified upstream of the *eutA* and *eutV* genes, and a third site was identified in the *rli55* locus upstream of the *eutG* gene and downstream of the B_{12} riboswitch (*13*). We also identified a second ANTAR site in the *rli55* locus (Fig. 3A), which suggested that one or both of these ANTAR elements could be transcribed as part of the 3′ end of Rli55 RNA. Indeed, our RNA-seq data showed that, in the absence of B_{12}, high levels of Rli55 are transcribed as a long transcript encompassing the first ANTAR element (Fig. 3A). In contrast, *rli55* transcription terminates abruptly after the riboswitch in the presence of B_{12}, which indicates that the riboswitch determines whether Rli55 is transcribed with or without an ANTAR element. This notion was supported by the detection of putative Rli55 orthologs in *E. faecalis* and *Streptococcus sanguinis* (fig. S4). In the latter, the riboswitch, in conjunction with a single ANTAR element, has undergone an inversion relative to the adjacent *eutG* gene, which suggests that the riboswitch and the first ANTAR element are functionally linked (fig. S4B) and that this is also the case in *L. monocytogenes*.

To test if the ANTAR elements are involved in Rli55-mediated regulation, we complemented the Δ*rli55* strain with chromosomally integrated *rli55* alleles carrying mutations in the ANTAR elements and examined which ones restored Rli55-mediated regulation of *eut* expression (Fig. 3, B and C, and fig. S5). A strain with an empty

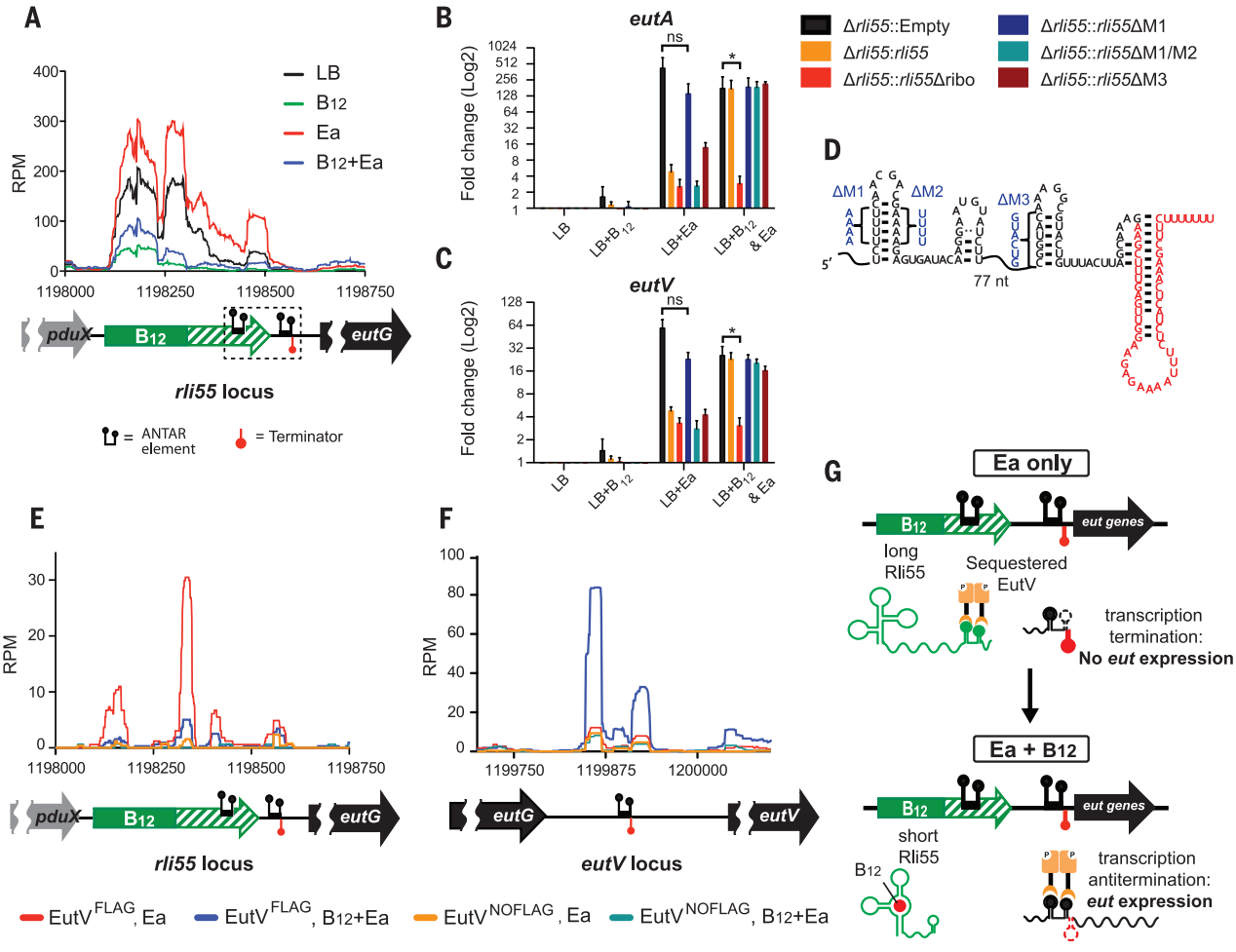

Fig. 3. Rli55 sequesters the two-component response regulator EutV. (**A**) RNA-seq coverage in reads per million (RPM) of the *rli55* locus from bacteria grown as indicated. (**B** and **C**) qRT-PCR of *eutV* and *eutA* genes. Vitamin B_{12} (B_{12}), ethanolamine (Ea). Values represent means ± SEM, $n = 3$. All differences were significantly different (*$P < 0.05$) from the Δ*rli55*::Empty strain in the Ea condition, except where indicated (ns). All differences were not significantly different from the Δ*rli55*::Empty strain in the B_{12} + Ea condition, except where indicated. (**D**) Secondary structure of the ANTAR elements encoded in the boxed region of Fig. 3A. Mutations are shown in blue. (**E** and **F**) RNA-seq coverage in RPM of the *rli55* and *eutV* loci with RNA isolated by coimmunoprecipitation of cell lysates from either $EutV^{FLAG}$ or $EutV^{NOFLAG}$ cultures grown in the presence of ethanolamine (Ea) or ethanolamine and B_{12} together. (**G**) Proposed model of Rli55-mediated regulation of *eut* expression in the presence of ethanolamine (Ea) alone or ethanolamine + B_{12} (Ea + B_{12}).

construct (Δ*rli55*::Empty) could not prevent expression of the *eut* genes in ethanolamine alone (as in the parental Δ*rli55* strain), whereas a strain with a wild-type copy of *rli55* (Δ*rli55*::*rli55*) fully restored Rli55-mediated inhibition. However, a strain with a deletion in the riboswitch (Δ*rli55*::Δribo) inhibited *eut* expression in all conditions, as the riboswitch can no longer terminate *rli55* transcription in response to B_{12}. In strain Δ*rli55*::*rli55*ΔM1, wherein four uridine residues in the first ANTAR site were mutated to adenines (Fig. 3D, ΔM1), inhibition of *eut* expression by Rli55 was abolished in the presence of ethanolamine alone. In contrast, in strain Δ*rli55*::*rli55*ΔM1/M2, where compensatory mutations were made to the opposite side of the ANTAR stem-loop (Fig. 3D, ΔM2), wild-type regulation of *eut* expression was restored. Mutation of the six nucleotides in the stem-loop of the second ANTAR element (Fig. 3D, ΔM3, Δ*rli55*::*rli55*ΔM3) had no significant effect on Rli55-mediated regulation. Thus, the first ANTAR element is necessary and sufficient for Rli55-mediated regulation.

The long form of Rli55 containing an ANTAR element might bind and sequester EutV and so prevent it from activating expression of the *eut* genes in the presence of ethanolamine but absence of B_{12}. When sufficient levels of B_{12} accumulate, B_{12} would bind the riboswitch, producing truncated Rli55 transcripts, which would lack an ANTAR element and be unable to sequester EutV. To examine this hypothesis, we constructed a strain with an additional copy of the *eutV* gene carrying a 2XFLAG-tag ($EutV^{FLAG}$) and first showed that expression of $EutV^{FLAG}$ protein is regulated identically to the native *eutV* gene in response to ethanolamine and B_{12} (fig. S6A). We also constructed a strain with an additional *eutV* gene lacking a FLAG tag ($EutV^{NOFLAG}$). Anti-FLAG immunoprecipitations of cell lysates from these two strains (fig. S6, B and C), followed by RNA-seq analysis (Fig. 3, E and F), showed that Rli55 is enriched by co-immunoprecipitation with $EutV^{FLAG}$ primarily when bacteria are grown in the presence of ethanolamine alone, although we saw no enrichment in a parallel immunoprecipitation with the $EutV^{NOFLAG}$ strain (Fig. 3E). In contrast, the ANTAR element upstream of the *eutV* gene (Fig. 3F) is enriched by coimmunoprecipitation of lysates from $EutV^{FLAG}$ bacteria, but not $EutV^{NOFLAG}$ bacteria, grown in the presence of ethanolamine and B_{12} together but not from lysates of bacteria grown in ethanolamine alone. To a lesser extent, the ANTAR-containing region upstream of *eutA* and the entire *eutA-Q* locus are enriched under the latter condition (fig. S7). These data support a model in which the majority of EutV is bound and sequestered by Rli55 in the presence of ethanolamine alone. Conversely, in the presence of ethanolamine and B_{12}, the riboswitch produces short truncated Rli55 transcripts, which cannot bind EutV, and so allows EutV to bind *eut* mRNAs and to activate *eut* expression (Fig. 3G).

This riboregulatory mechanism coordinates expression of the ethanolamine utilization (*eut*) locus with the availability of B_{12}, the essential cofactor for ethanolamine catabolism. Previously, ethanolamine utilization has been shown to be important after oral infection by *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* (*3*, *4*, *14*); however, the contribution of ethanolamine utilization to *L. monocytogenes* pathogenesis in an intravenous mouse infection model suggests that ethanolamine utilization is important outside of the intestine and possibly in the intracellular environment. This study also extends the role of riboswitches in the regulation of noncoding RNAs (*15*, *16*). Finally, our data show that Rli55 represents a new member of the small family of regulatory RNAs that function by sequestering a protein, which also includes the *6S* and CsrB/C RNAs (*17*), and highlights a distinctive means of signal integration in bacterial gene regulation.

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IMMUNOGENETICS

Chromatin state dynamics during blood formation

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Chromatin modifications are crucial for development, yet little is known about their dynamics during differentiation. Hematopoiesis provides a well-defined model to study chromatin state dynamics; however, technical limitations impede profiling of homogeneous differentiation intermediates. We developed a high-sensitivity indexing-first chromatin immunoprecipitation approach to profile the dynamics of four chromatin modifications across 16 stages of hematopoietic differentiation. We identify 48,415 enhancer regions and characterize their dynamics. We find that lineage commitment involves de novo establishment of 17,035 lineage-specific enhancers. These enhancer repertoire expansions foreshadow transcriptional programs in differentiated cells. Combining our enhancer catalog with gene expression profiles, we elucidate the transcription factor network controlling chromatin dynamics and lineage specification in hematopoiesis. Together, our results provide a comprehensive model of chromatin dynamics during development.

Chromatin plays a major regulatory role in cell-type–specific functions and response (*1*, *2*). The current dogma of cellular differentiation suggests that there is a progressive closing of the regulatory potential of the genome. According to this model, differentiation is a gradual transition from an open chromatin state in multipotent stem cells to a compacted chromatin state in differentiated cells. However, genome-wide histone modification profiling of embryonic stem cells and terminally differentiated cells is not fully compatible with this model

Sequestration of a two-component response regulator by a riboswitch-regulated noncoding RNA

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