In Brief
Chao et al. discover that the essential bacterial RNase E cleaves numerous transcripts at preferred sites by sensing uridine as a 2-nt ruler. RNase E processing of various precursor RNAs produces many small regulatory RNAs, constituting a major small-RNA biogenesis pathway in bacteria.

Highlights
- TIER-seq precisely maps ~22,000 endogenous RNase E cleavage sites in Salmonella
- Consensus motif of RNase E reveals a 2-nt uridine ruler-and-cut mechanism
- RNase E is a central component in both maturation and degradation of small RNAs
- There is a general small-RNA biogenesis pathway requiring RNase E and Hfq

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In Vivo Cleavage Map Illuminates the Central Role of RNase E in Coding and Non-coding RNA Pathways

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SUMMARY

Understanding RNA processing and turnover requires knowledge of cleavages by major endoribonucleases within a living cell. We have employed TIER-seq (transiently inactivating an endoribonuclease followed by RNA-seq) to profile cleavage products of the essential endoribonuclease RNase E in Salmonella enterica. A dominating cleavage signature is the location of a uridine two nucleotides downstream in a single-stranded segment, which we rationalize structurally as a key recognition determinant that may favor RNase E catalysis. Our results suggest a prominent biogenesis pathway for bacterial regulatory small RNAs whereby RNase E acts together with the RNA chaperone Hfq to liberate stable 3’ fragments from various precursor RNAs. Recapitulating this process in vitro, Hfq guides RNase E cleavage of a representative small-RNA precursor for interaction with a mRNA target. In vivo, the processing is required for target regulation. Our findings reveal a general maturation mechanism for a major class of post-transcriptional regulators.

INTRODUCTION

Small, non-coding RNAs (sRNAs) that associate with the RNA chaperone Hfq constitute the largest class of post-transcriptional regulators in Gram-negative bacteria (De Lay et al., 2013; Storz et al., 2011; Vogel and Luisi, 2011; Wagner and Romby, 2015). Initially defined as a class in non-pathogenic Escherichia coli (Zhang et al., 2003), Hfq-dependent sRNAs have been globally mapped in numerous important human pathogens (Barquist and Vogel, 2015; Holmqvist et al., 2016; Koo et al., 2011; Melamed et al., 2016; Tree et al., 2014). These sRNAs generally act as multi-target repressors and activators through seed pairing interactions with the 5’ untranslated region (UTR) of mRNAs (Desnoyers et al., 2013; Feng et al., 2015; Papenfort and Vanderpool, 2015). A full understanding of these sRNA-mediated networks requires knowledge of how their RNA constituents are synthesized and turned over.

Many of the bacterial sRNAs characterized to date are transcribed from non-coding intergenic regions and operate as full-length, primary transcripts capped with a 5’ triphosphate (5’PPP). However, some primary sRNAs such as ArcZ and RprA are converted into shorter stable species that retain the seed region for target mRNA recognition (Mandin and Gottesman, 2010; Papenfort et al., 2009, 2015). It is currently unclear whether such processing generates the active sRNAs, as is the case with eukaryotic microRNAs (Kim, 2005). Moreover, several recent studies reported sRNAs that are produced from the 3’ region of mRNA genes (Miyakoshi et al., 2015b), only a subset of which are the result of gene-internal promoters (Chao et al., 2012; Guo et al., 2014), while many others appear to originate from mRNA processing. These 3’-derived sRNAs are likely to be functional, since they abundantly associate with Hfq (Chao et al., 2012), whose cellular concentration is limited (Wagner, 2013). Their physiological importance is further supported by established roles of the 3’-mRNA-derived sRNAs CpxQ and SroC in the envelope stress response or amino acid pathways, respectively (Chao and Vogel, 2016; Miyakoshi et al., 2015a). Furthermore, 3’ fragments of E. coli tRNA precursors function as molecular sponges of conserved sRNAs (Lalaouna et al., 2015). Collectively, these findings suggest that sRNA processing is a prevalent event; however, both its functional relevance and the major responsible nuclease(s) remain to be established.

Of several candidate nucleases involved in sRNA processing and turnover, the conserved and essential endoribonuclease E (RNase E) is the likely central player (Mackie, 2013; Massé et al., 2003; Saramago et al., 2014). It can be inferred, from transcript accumulation upon its inactivation, that RNase E drives the
decay of most mRNAs in E. coli (Bernstein et al., 2004; Clarke et al., 2014), and in Salmonella it processes the mRNA 3’ end-
derived CpxQ and SroC sRNAs (Chao and Vogel, 2016; Miyakoshi et al., 2015a). RNase E also degrades several sRNAs in the
absence of Hfq or upon base pairing with target mRNAs (Bandrya et al., 2012; Massé et al., 2003; Moll et al., 2003). Conversely, some sRNAs activate gene expression by blocking RNase E cleavage sites in target mRNAs (Fröhlich et al., 2013; Papenfort et al., 2013). In addition, RNase E is known to engage in rRNA and tRNA precursor processing (Aprión and Lassar, 1978; Bessarab et al., 1998; Kime et al., 2014; Li and Deutscher, 2002; Ow and Kushner, 2002).

Despite the importance of RNase E in post-transcriptional control, its activity toward most non-coding RNAs is not known. Previous studies have characterized major RNase E cleavage sites in a few abundant transcripts (e.g., Aprión and Lassar, 1978; Delvillani et al., 2011; Ehretsmann et al., 1992; Jackie, 1991; Ow and Kushner, 2002; Patel and Dunn, 1992; Régnier and Hajnsdorf, 1991; Roy and Aprión, 1983) and concluded that the enzyme preferentially cleaves AU-rich regions in single-stranded RNA (Arraiano et al., 2010; Huang et al., 1998; McDowall et al., 1994, 1995). Here, to achieve a systems-level understanding of RNase E activity, we have analyzed in depth the in vivo RNase E cleavage events in Salmonella typhimurium, a close relative of E. coli and a pathogenic model organism to study post-transcriptional regulation (Westermann et al., 2016). Our genome-wide capture of tens of thousands of endogenous cleavage sites reveals a minimal consensus sequence and a 2 3 nt uridine ruler-and-cut structural mechanism for this major endoribonuclease. Intriguingly, RNase E employs this mechanism to cleave many coding and non-coding transcripts at the 3’ end and releases stable, Hfq-bound RNA fragments, indicating that sRNA biogenesis through endonucleolytic processing is widespread. Searches for these predicted critical uridines in sRNAs enabled us to show that maturation by RNase E is essential for target regulation by the ArcZ sRNA. Moreover, our data reveal a high frequency of RNase-E-mediated cleavages in Hfq-dependent sRNAs, supporting the functional link between RNase E and Hfq for the first time on a global level.

RESULTS

A Transcriptome-wide Map of RNase E Cleavage Sites In Vivo

To globally map RNase E cleavage events in vivo, we profiled 5’ ends of cellular transcripts by comparative RNA-seq before and 30 min after programmed activation of the enzyme using a temperature-sensitive meTS mutant (me-3071) (Aprión and Lassar, 1978; Figueroa-Bossi et al., 2009). We refer to this approach, which builds upon work by Clarke and colleagues (Clarke et al., 2014) as transient inactivation of endoribonuclease followed by RNA-seq (TIER-Seq; see Figure 1A). At the permissive temperature (28°C), Salmonella wild-type (WT) me and mutant meTS strains both exhibit full RNase E activity, whereas upon shift to 44°C, only WT RNase E retains its activity to process RNA. To achieve a comprehensive RNase-E-specific “degradome” analysis at single-nucleotide resolution (Figure 1A), we analyzed biological duplicates of all four of the above strains and conditions in the early stationary growth phase (OD600 of 2) by RNA-seq, obtaining ~130 million reads (Figure S1A). In agreement with previous work showing that RNase E cleaves AU-rich sequences (McDowall et al., 1994, 1995), the inactivation of RNase E leads to a ~5% reduction of cDNA reads with 5’-A/T bases (Figure S1B).

To pinpoint cleavage sites, we aligned all reads to the Salmonella genome, mapping a total of ~500,000 unique 5’ ends (Figures 1B and 1C). WT and meTS samples from growth at 28°C gave nearly identical 5’ end profiles (R² = 0.98; Figures 1B and S1C), confirming that the mutant RNase E is fully functional at the permissive temperature, whereas at the non-permissive temperature (44°C), many positions were selectively depleted in the meTS cDNA libraries (Figure 1C). Since Salmonella has no 5’→3’ exoribonuclease (Hui et al., 2014), we interpret these depleted positions as RNase E cleavage sites (Figure 1A). This classification is supported by the capture of many previously known E. coli RNase E cleavage sites (Figure 1D)—for example, in the rpoS, csPE, uncC/atpC, and glmUS mRNAs (Delvillani et al., 2011; Joanny et al., 2007; Patel and Dunn, 1992; Régnier and Hajnsdorf, 1991), in the 9S precursor of 5S rRNA (Roy and Aprión, 1983), and near the 3’ end of tRNAs (Ow and Kushner, 2002). Applying a threshold of >3-fold as significant depletion (p < 0.05, FDR < 0.05) in the meTS samples at 44°C, we assigned 22,033 RNase-E-mediated cleavages in the Salmonella transcriptome, expanding by several orders of magnitude the database of in vivo target sites for this ribonuclease. The full list of cleavage sites is available in Table S1.

A Systems-Level View on RNase E Activity in RNA Metabolism

Systematic analysis of the 22,033 RNase E cleavage sites revealed their distribution in coding and non-coding transcripts from the Salmonella chromosome and virulence plasmids (Figure 2A): ~80% occurred in mRNAs, primarily in the coding sequence (CDS), indicating that a major activity of RNase E is to degrade mRNAs in addition to processing housekeeping RNAs. Altogether, we detected a total of 2,557 mRNAs cleaved by RNase E, with a different number of cleavage sites per transcript (Figure 2B); these represent 78% of 3,286 Salmonella mRNAs that are well expressed (RPKM > 10, Table S2) in the early stationary phase. Notably, the assay captured many essential genes and virulence genes required for intracellular growth (Table S3), which provide insights into the processing of transcripts from indispensable genes and the roles of RNase E in Salmonella pathogenesis (Viegas et al., 2013), respectively. Longer transcripts generally tend to contain a higher number of cleavage sites to gene length, RNase E cleavage frequency in these genes (RPKM > 10) ranges from 0 to >30 sites per kilobase, with a median value at ~5.7 cleavages per kilobase, or one site every ~175 nt of mRNA (Figure 2C). This non-saturating cleavage pattern might suggest that most sites in mRNAs are inaccessible, perhaps due to structural constraints or protein binding.

The position of an RNase E site within a transcript may provide information about the function of the cleavage. For example, RNase E auto-regulates its synthesis by cutting in the 5’ UTR

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of its own mRNA (Jain and Belasco, 1995); our analysis readily captured this critical site (Figure S2A). As another example, we detect the RNase E site in the 5′ UTR of cfa mRNA (Figure S2A) that becomes protected by the trans-acting RydC sRNA, with the consequence that the transcript is stabilized (Dimastrogiovanni et al., 2014; Fröhlich et al., 2013). Thus, our candidate list of ~1,300 RNase E cleavage sites identified in the 5′ UTRs of 548 genes (Table S4) provides a resource to predict sites for post-transcriptional control by sRNAs and/or RNA-binding proteins.

Figure 1. Global Mapping of Endogenous RNase E Cleavage Sites in _Salmonella_ using TIER-Seq
(A) Schema of the TIER-seq approach. Endogenous cleavage sites were identified by analyzing the 5′ ends of RNase E cleavage products (purple) in the WT and _rneTS_ strains at the non-permissive temperature (44°C). Total RNA from WT and _rneTS_ was converted to cDNAs and sequenced; the 5′ ends depleted in the _rneTS_ libraries at 44°C indicate the RNase E cleavage sites (e.g., purple U).
(B and C) Global analysis of 5′ end profile at the permissive temperature 28°C (B) and non-permissive temperature 44°C (C). The plots show the read counts for every 5′ base in WT samples and the relative fold change compared to _rneTS_ samples. Candidate RNase E cleavage sites that show >3-fold depletion in _rneTS_ samples (p < 0.05, FDR < 0.05) are colored in red.
(D) TIER-seq captures known RNase E cleavage sites with single-nucleotide resolution. _TS_ indicates the _rneTS_ samples. R1 and R2 are two biological replicates. The major RNase E sites are marked by red arrowheads and bold lettering; secondary cleavage sites are indicated by open arrowheads. The ORF or mature RNAs are shadowed by gray boxes. See also Figures S1 and S2.
A Specific Sequence Motif Recognized by RNase E

Even seemingly non-specific nucleases often exhibit a certain degree of sequence or structural preference. To understand the substrate determinants of RNase E activity, we analyzed the primary sequences and putative secondary structures around all of the 22,033 cleavage sites. At the cleavage site we observed an overall increase in the calculated folding energy ($\Delta G$), indicating little secondary structure (Figure 2D), and a spike of AU-rich sequences (Figure 2E), both of which agree with previously studied individual RNase E sites (McDowall et al., 1994, 1995). Importantly, sequence alignment of all 22,033 sites predicts a minimal RNase E consensus sequence (Figure 2F) with a marked preference for uridine at the +2 position in the 5 nt "RN\^Y\^WUU" core motif (with R as G/A, W as A/U, and N as any nucleotide). This RNase E motif, based entirely on global in vivo data, fully recapitulates...
preferences previously documented with model substrates in vitro (Ehretsmann et al., 1992; Kaberdin, 2003; Mackie, 1991) and with cell-derived RNA (Del Campo et al., 2015), while it clearly differs from recognition motifs of other major bacterial endoribonucleases such as tRNA-processing RNase P (McClain et al., 1987) or RNase III, which cleaves double-stranded RNA (Gan et al., 2005).

**RNase E Cleavages Underlie sRNA Biogenesis from 3′ UTRs**

In analyzing cleavage-site distributions relative to mRNA start or stop codons (Figures 3A and 3B), we observed that, on average, 5′ UTRs and the coding regions showed similar cleavage frequencies. Translation initiation regions were slightly counter-selected, perhaps because the prominent Shine-Dalgarno sequence (GGAGGA) is devoid of RNase E cleavage motifs. In contrast, RNase E sites were enriched around mRNA stop codons (Figure 3B); the high AU-rich content and/or translation termination may favor this enrichment. Since bacterial 3′ UTRs are generally short (Belasco, 2010), many of these stop codon sites may represent the most downstream sites, leaving 3′ fragments for degradation by 3′ → 5′ exoribonucleases. Interestingly, approximately one-third of these mRNAs carry protective r-independent terminators (Arraiano et al., 2010) that can, in principle, interact with the sRNA chaperone Hfq (Otaka et al., 2011; Sauer and Weichenrieder, 2011). These data point to the
possibility that stable 3’ UTR fragments accumulate with functional consequence in the guise of regulatory sRNAs (Table S5; Chao et al., 2012; Miyakoshi et al., 2015b). Indeed, we have detected the mRNA 3’ UTR processing sites that produce the CpxQ and SroC sRNAs (Figure S2B). Northern blot probing of several selected candidates revealed distinct RNA species from mRNA 3’ ends, the generation of which required both active RNase E and the presence of Hfq (Figures 3C and S2C). Most of these 3’-derived sRNAs co-accumulate with their parental mRNA transcripts and possess potential seed regions (Figure S3), suggesting that they are bona fide regulatory sRNAs with conserved targets and functions. In addition, the cleavage sites in these sRNAs resemble the “RNWUU” sequence (Figure S2D), supporting the recognition of this consensus by RNase E (Figure 2F).

to the vicinity of the seed region (Figure S4D), as exemplified by their clustering in the well-characterized seed of SgrS and RybB (Figures S4E and S4F). These data suggest that RNase E may inactivate sRNAs by removing the seed region; this is in agreement with previous results for MicC (Bandyra et al., 2012) and RyhB (Massé et al., 2003; Moll et al., 2003). Both MicC and RyhB are turned over by RNase E through seed cleavage if the target is absent, and this could provide a surveillance mechanism for accurate seed matching (Bandyra et al., 2012).

Another group of sRNAs is spared from immediate degradation following RNase E cleavage; instead, these RNAs appear to be processed by the enzyme. The highly conserved ArcZ and RprA sRNAs, which each regulate a number of targets, including rpoS (Majdalani et al., 2001; Mandin and Gottesman,
The crucial roles of U₁₂ and Hfq in RNase E cleavage were also evident for the RprA sRNA (Figure S5). Full-length RprA precursor (pre-RprA) was processed by RNase E in vitro at its internal seed sequence (GA₃U₁₁U₁₂U₁₃), producing mature RprA only in the presence of Hfq. Mutating U₁₂ alone significantly reduced the maturation of RprA by RNase E, which was fully abolished by changing both U₁₁U₁₂ to non-preferred guanines. The essentiality of U₁₂ in RprA processing could also be demonstrated in vivo (Figure S5C), as well as in directing the cleavage of the cfa mRNA (Figure S2E). Together, these mutational studies further validate our TIER-seq-based prediction of U₁₂ as a key nucleotide for specific RNase E cleavage of cellular transcripts.

RNAse-E-Dependent sRNA Maturation Is Essential for Target Regulation

To consider RNase E as an sRNA maturation factor with functional consequences requires that its processing activity is essential for sRNA function. Demonstrating such a property requires first the development of a system in which processing of an sRNA precursor can be impeded without changing or losing the seed region. The ArcZ sRNA offers such a system: exploiting our finding that the crucial U₁₂ in the RNase E motif of ArcZ abolished cleavage enabled us to produce pre-ArcZ with diminished amounts of 3’ ArcZ in vivo (Figure 5). We examined the ability of the pre-ArcZ to repress the synthesis of Tpx (Figure 5E), whose mRNA is targeted by the conserved seed region of ArcZ (Papenfort et al., 2009; Figures 6A and S6). While a 10 min expression of WT ArcZ downregulated the tpx mRNA by 7-fold, the U₁₁G₁₂ mutant (variant GAUGU) achieved only 3-fold repression despite the higher levels of precursor (Figure 5E). Additional mutation of an adjacent uridine (variants GAGGU or GAUGU) fully inhibited 3’ ArcZ production and abrogated tpx regulation despite higher levels of the precursor, strongly suggesting that only the mature 3’ ArcZ is the functional regulator.

According to previous work (Papenfort et al., 2009), the U₁₁U₁₃ residues in the RNase E site of ArcZ may not engage in base paring with tpx (Figure 6A). If they do at all, they might extend the duplex by two additional A:U pairs; this could be disrupted by the non-functional, locked GAUGU variant of pre-ArcZ. To rule out that the failure of the GAAGU variant (ArcZ-GG) to repress tpx was simply due to insufficient base pairing, we introduced a compensatory AU → CC mutation in the tpx-GFP fusion (Tpx-CC), but again no regulation by the ArcZ-GG variant was observed (Figure 6B). Likewise, the processing-deficient ArcZ-GG variant also failed to regulate the sdaC mRNA target either...
in its WT form or with a duplex-extending CC mutation (Figure S6B). Thus, RNase E cleavage is essential for the production of functional ArcZ.

A likely explanation for ArcZ maturation to be essential for regulation is that the ArcZ seed may only become available for target pairing upon RNase E cleavage. To test this, we examined sRNA duplex formation with tpx mRNA in vitro. Electrophoretic mobility shift assays with radiolabeled sRNA showed that the mature 3' ArcZ binds to the target region of tpx mRNA (a 216 nt fragment containing 5' UTR and early CDS) with very high affinity ($K_D = 15$ nM; Figure 6C); by contrast, an ~500-fold excess of pre-ArcZ over target was insufficient for full duplex formation (Figure 6D), similar to the low affinity observed for pre-ArcZ binding to the rpoS mRNA (Soper et al., 2010). In addition, Hfq promotes formation of the sRNA target duplex in the case of mature ArcZ, but less so for pre-ArcZ (Figures S6C and S6D). These results were further confirmed by reciprocal experiments with labeled tpx mRNA. Again, mature ArcZ readily bound to the target and formed a stable ArcZ-tpx-Hfq ternary complex (Figure 6E), whereas excess of the pre-ArcZ RNA only competed with the tpx-Hfq complex formation and released free tpx mRNA. These combined in vivo and in vitro results show that pre-ArcZ undergoes an RNase-E-dependent maturation to activate ArcZ for...
repression of tpx and perhaps other targets. This demonstrates for the first time that RNase E cleavage is required to activate an Hfq-dependent sRNA.

DISCUSSION

Bacterial transcripts are generally short lived (Bernstein et al., 2002; Chen et al., 2015) and subject to rapid turnover by cellular ribonucleases (Hui et al., 2014; Mackie, 2013). Gene expression and regulation typically take place at the level of primary transcripts bearing the native 5' PPP end. This is fundamentally different from higher eukaryotes, where nearly all types of regulatory transcripts undergo processing and maturation as a prerequisite for function. Our identification of numerous conserved regulatory sRNAs that result from RNase E cleavage (Figures 3 and S3) illustrates the complexity of the bacterial “RNA degradome.” These increasing numbers of processing-derived RNA species (Chao and Vogel, 2016; Davis and Waldor, 2007; Detcheva et al., 2011; Guo et al., 2014; Miyakoshi et al., 2015a) contrast with the general perception that cleaved bacterial transcripts are usually labile species of little biological relevance.

TIER-seq offers a generic approach both for global analysis of processed transcripts and cleavage sites in living cells with single-nucleotide resolution and for mechanistic understanding of ribonuclease activities at a systems level. We have here employed a temperature-sensitive strain to transiently inactivate the endogenous RNase E, which minimizes the potentially confounding effects of “non-native” conditions used in previous degradome studies where the nucleases were genetically deleted (Linder et al., 2014), ectopically overexpressed (Schifano et al., 2014), or supplemented in vitro (Clarke et al., 2014). To circumvent the need for a thermosensitive mutant and temperature-induced transcriptomic changes (Table S7), future TIER-seq studies may benefit from using alternative means of transient nuclease inactivation such as small molecules (Kime et al., 2015), small inhibitory proteins (Kim et al., 2008; Lee et al., 2003), target-specific proteases (Cameron and Collins, 2014), or conditionally spliced inteins (Zeidler et al., 2004).

RNase-E-Dependent sRNA Biogenesis and Maturation in Bacteria

We identify RNase E as a key factor both for the biogenesis of many 3’ UTR-derived sRNAs and for the maturation of active sRNAs from their non-coding precursors. This establishes RNase E cleavage as a second major pathway for the biogenesis of Hfq-dependent sRNAs (Figure 7A). As compared to the canonical pathway of de novo transcription, this cleavage-based biogenesis may confer several advantages. RNase E can generate sRNAs from diverse origins, including essentially all existing transcripts (Figure 7A), greatly expanding the sRNA repertoire in the cell. This pathway could reduce regulatory overhead during evolution of new genes (Mattice, 2004), using the existing regulatory elements of the parental transcripts to control the expression of 3’-derived sRNAs. Activating an internal seed sequence by sRNA precursor cleavage, as shown here for ArcZ, offers an additional layer of control in post-transcriptional regulation—for example, via an adaptor protein such as RapZ.

Figure 6. Maturation of ArcZ sRNA Is Essential for Target Regulation

(A) Established base pair interactions between ArcZ and tpx mRNA (Papenfort et al., 2009). The major cleavage site in ArcZ is indicated.

(B) Western blot detection of GFP levels. GFP was fused with tpx 5’ UTR; the introduced mutations are shown in (A). “WT” refers to WT full-length ArcZ, “mat” refers to mature ArcZ, and “GG” refers to the GAUGG variant of ArcZ. GroEL served as loading control.

(C) Direct interaction of tpx with mature ArcZ by EMSA. Radiolabeled mature ArcZ was incubated with increasing concentration of tpx mRNA in the presence of Hfq (40 nM). The gel was resized; see Figure S6.

(D) Direct interaction of tpx with pre-ArcZ by EMSA. Mature ArcZ was co-shifted with tpx mRNA.

(E) Mature ArcZ was co-shifted with tpx mRNA. Radiolabeled tpx mRNA was incubated with increasing concentration of pre-ArcZ or mature ArcZ (0, 6, 25, 100, 400, and 2,000 nM) in the presence of 40 nM Hfq. See also Figure S6.
respect, the Hfq-RNase E complex in bacteria could have a dual function: it processes precursor transcripts to stable, mature sRNA and guides the mature sRNA for target regulation.

**A U+2 Ruler-and-Cut Mechanism Mediates Specific RNase E Cleavage**

The hallmark of the RNase E consensus motif inferred from our in vivo map (Figure 2) is a predominant uridine at 2 nt downstream of the cleavage sites (U+2), and we provide in vivo and in vitro evidence that the U+2 is crucial for RNase E cleavage. Analysis of the available crystal structure of an RNase E-RNA complex shows that the enzyme interacts with RNA at +2 nt via a stable stacking interaction of the nucleobase with Phe67 and Lys112 (Callaghan et al., 2005; Mackie, 2013). However, this structure contains a non-cognate substrate with G+2, representing a stable RNA-binding conformation trapped at the pre-cleavage state. Why is a uridine at this position preferred for cleavage? A molecular dynamics simulation analysis in which G+2 is substituted for U in silico suggests that the RNase E-RNA complex undergoes a conformational change favored by the presence of U+2; this allows us to propose a new model (Figure 7B) whereby RNase E mediates specific cleavage using a U+2 ruler-and-cut mechanism. Simulations of the pre-cleavage state show that U+2 was tightly bound in a crevice of the protein formed by the backbone of the Lys112Gly113Ala114Ala115 loop and the Lys112 side chain, resulting in a binding pocket that favors uracil in this position (e.g., Phe67 and Lys112) indicates that this may be a conserved mechanism for the RNase E protein family.

The uridine ruler-and-cut mechanism is also employed by other endoribonucleases, including the unrelated human nuclease RNase L. RNase L recognizes uridine in single-stranded RNAs and cleaves 2 nt downstream (Han et al., 2014), whereas RNase E cuts 2 nt upstream due to different dimeric structure arrangements. Interestingly, a fraction of RNase E sites contain C+2 (Figure 2F), indicating that RNase E displays a certain degree of flexibility by accepting a cytosine in the absence of other specificity signals. Indeed, in vitro experiments with poly(A) RNA demonstrate that C+2 can serve as a cleavage signal (Kaberdis, 2003), which further suggests that RNase E may distinguish the smaller pyrimidine from purine bases by steric hindrance (Figures 7B, S7A, and S7B). Nevertheless, U+2 is the preferred signal (Figure 2F), likely because its C4 oxygen possesses hydrogen bonding potential with RNase E (Figure S7B). In addition, some flexibility of RNase E is reflected near the cleavage sites, as RNase E frequently cuts 1 nt upstream or downstream of the determined cleavage site. To

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**Figure 7. Mechanism of RNase E Cleavage and an Alternative sRNA Biogenesis Pathway**

(A) RNase E cleavage constitutes a major sRNA biogenesis pathway in bacteria. (B) Proposed model for the +2 uridine ruler-and-cut mechanism of specific RNase E cleavage. The scissile phosphate is attacked hydrolytically by a water molecule (not shown) that is coordinated by the magnesium ion bound by the carboxylates of D346 and D303. Stacking interactions (between F67 and K112) form a stable RNA-binding conformation trapped at the pre-cleavage state. Why is a uridine at this position preferred for cleavage? A molecular dynamics simulation analysis in which G+2 is substituted for U in silico suggests that the RNase E-RNA complex undergoes a conformational change favored by the presence of U+2; this allows us to propose a new model (Figure 7B) whereby RNase E mediates specific cleavage using a U+2 ruler-and-cut mechanism. Simulations of the pre-cleavage state show that U+2 was tightly bound in a crevice of the protein formed by the backbone of the Lys112Gly113Ala114Ala115 loop and the Lys112 side chain, resulting in a binding pocket that favors uracil (e.g., Phe67 and Lys112) indicates that this may be a conserved mechanism for the RNase E protein family.

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compensate for this, short stretches of uridines (1–4 U) are often found at the +2 positions, which may serve to reinforce RNase E recognition and cleavage (e.g., ArcZ; Figure S5).

Our identification of crucial U+2 residues for RNase-E-specific cleavage enables straightforward mutations of individual cleavage sites of interest instead of global inactivation of the enzyme. This will aid the molecular investigation of 3′ UTR-derived sRNAs and of RNase-E-mediated, post-transcriptional regulations, not only in the Hfq region but also for the recently discovered class of ProQ-associated sRNAs (Smirnov et al., 2016)—many of which might be RNase E targets, too. This information may also help design novel CRISPR-Cas or antisense-RNA-based synthetic tools to activate gene expression by specifically blocking a cleavage site, as well as helping to engineer stable mRNAs for better gene expression.

**EXPERIMENTAL PROCEDURES**

Full methods are described in the Supplemental Experimental Procedures; so are details of bacterial strains, plasmids, and oligonucleotides.

**Transient Inactivation of RNase E**

The Salmonella me376 strains refer to me-3071 and its isogenic WT control previously established in (Figueroa-Bossi et al., 2009). Bacteria were grown in Lennox LB medium at 28°C to an OD600 of 2, then shifted to 44°C for 30 min to inactivate RNase E.

**RNA-Seq and Data Analysis**

cDNA libraries were constructed following a standard protocol (Chao et al., 2012; Westermann et al., 2016). Briefly, RNA was polyadenylated at 3′ end and ligated to an adapter at 5′ end after treatment with tobacco acid pyrophosphatase. First-strand cDNA was synthesized using oligo(dT)-adaptor and M-MLV reverse transcriptase. The linear amplified cDNAs were multi-plexed and sequenced using Illumina HiSeq. Reads were mapped to Salmonella genome using READemption; 5′ end coverage was visualized in IGB. The RNase E sites, which are depleted 5′ ends in the me376 samples relative to WT at 44°C, were identified using DESeq2.

**ACCESSION NUMBERS**

The sequencing data have been deposited in the GEO database under GEO: GSE81869.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.11.002.

**AUTHOR CONTRIBUTIONS**

Y.C. and J.V. conceived the research; Y.C., N.S., C.C., M.S., and K.P. conducted experiments; Y.C., L.L., K.U.F., and B.F.L. analyzed data; D.G. and H.-J.W. performed MD simulations; R.R. performed RNA-seq; Y.C., B.F.L., and J.V. wrote the manuscript.

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