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Cross talk between ABC transporter mRNAs via a target mRNA-derived sponge of the GcvB small RNA

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Abstract

There is an expanding list of examples by which one mRNA can posttranscriptionally influence the expression of others. This can involve RNA sponges that sequester regulatory RNAs of mRNAs in the same regulon, but the underlying molecular mechanism of such mRNA cross talk remains little understood. Here, we report sponge-mediated mRNA cross talk in the posttranscriptional network of GcvB, a conserved Hfq-dependent small RNA with one of the largest regulons known in bacteria. We show that mRNA decay from the *gltIJKL* locus encoding an amino acid ABC transporter generates a stable fragment (SroC) that base-pairs with GcvB. This interaction triggers the degradation of GcvB by RNase E, alleviating the GcvB-mediated mRNA repression of other amino acid-related transport and metabolic genes. Intriguingly, since the *gltIJKL* mRNA itself is a target of GcvB, the SroC sponge seems to enable both an internal feed-forward loop to activate its parental mRNA in *cis* and activation of many *trans*-encoded mRNAs in the same pathway. Disabling this mRNA cross talk affects bacterial growth when peptides are the sole carbon and nitrogen sources.

Keywords GcvB; Hfq; noncoding RNA; RNase E; SroC
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Introduction

Messenger RNA (mRNA) has long been seen solely as an intermediate in the transport of genetic information from DNA to the translation apparatus for decoding. In this classical scenario of molecular biology, only the end product of the process—the translated protein—may impact the expression of other genes. Recent work, however, has revealed intriguing cases in which mRNAs cross talk with other mRNAs, and thereby, themselves influence physically unlinked genes.

In eukaryotes, one such type of cross talk is mediated by mRNAs that contain multiple base-pairing sites to 'sponge' microRNAs with complementary sequence, thus minimizing the microRNA-mediated repression of other target mRNAs in the same regulon (Seitz, 2009; Ebert & Sharp, 2010; Rubio-Somoza *et al*, 2011). This mRNA cross-activation mechanism has been generally conceptualized in terms of competitive endogenous mRNAs (ceRNAs) which titrate microRNAs from competing mRNAs by changing their relative concentrations (Salmena *et al*, 2011). However, microRNAs may also be sequestered by other RNAs including a pseudogene transcript (Franco-Zorrilla *et al*, 2007), long noncoding RNAs (Cesana *et al*, 2011), or circular RNAs (Hansen *et al*, 2013; Memczak *et al*, 2013). Moreover, some viruses use noncoding RNA sponges to inactivate host microRNAs (Cazalla *et al*, 2010; Marcinowski *et al*, 2012).

In prokaryotes, mRNA cross talk through competition for small regulatory RNAs (sRNAs) was first reported in the chitosugar utilization pathway of *Salmonella enterica* and *Escherichia coli* (Figueroa-Bossi *et al*, 2009; Overgaard *et al*, 2009). These enteric model bacteria encode the chitosugar-specific porin ChiP whose mRNA is translationally repressed by an abundant Hfq-dependent sRNA, ChiX, under regular growth conditions. However, chitosugars in the environment trigger transcription of a polycistronic chitobiose mRNA (encoding enzymes for chitosugar breakdown) which contains a short complementarity region for the ChiX sRNA (Plumbridge *et al*, 2014). As the ChiX sRNA base-pairs with this site, it gets degraded and its cellular concentration decreases, which boosts the synthesis of the relevant porin for chitosugar uptake.

The sRNAs that associate with Hfq often target dozens of mRNAs (Vogel & Luisi, 2011; Sobrero & Valverde, 2012; De Lay *et al*, 2013) and so constitute nodes in larger posttranscriptional networks that help control many different aspects of bacterial physiology, virulence, and behavior (Storz *et al*, 2011; Papenfort & Vogel, 2014). However, sponges for such global regulators, which would result in cross talk between multiple functionally related bacterial mRNAs as observed in eukaryotic microRNA regulons, have not been described. Here, we report a conserved RNA sponge in the large GcvB-controlled regulatory circuit of bacterial amino acid synthesis and transport.

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GcvB is a widely conserved, ~200 nt Hfq-dependent sRNA and one of the most globally acting posttranscriptional regulators in bacteria, potentially regulating ~1% of all mRNAs in *Salmonella* and *E. coli* (Urbanowski *et al*, 2000; Sharma *et al*, 2007, 2011; Pulvermacher *et al*, 2009; Busi *et al*, 2010; Vanderpool, 2011; Stauffer & Stauffer, 2012; Wright *et al*, 2013; Yang *et al*, 2014). Its regulon (Fig 1A) is highly enriched with transporters of amino acids and short peptides, including the major ABC transporters Dpp and Opp, but also contains amino acid biosynthesis proteins and major transcription factors such as Lrp, PhoP, and CsgD (Modi *et al*, 2011; Sharma *et al*, 2011; Jørgensen *et al*, 2012; Coornaert *et al*, 2013). Since GcvB is highly abundant when bacteria grow rapidly in nutrient-rich media, its major role may be to optimize the energy-expensive import and biosynthesis of amino acids (Sharma *et al*, 2007).

As expected for a regulator of this scope, the cellular level of GcvB is tightly controlled at the level of synthesis; the two transcription factors, GcvA and GcvR, of the glycine cleavage system activate the *gcvB* gene in response to endogenous glycine (Urbanowski *et al*, 2000; Ghrist *et al*, 2001; Heil *et al*, 2002; Stauffer & Stauffer, 2005). However, GcvB also exhibits an exceptionally low cellular RNA half-life of < 2 min (Vogel *et al*, 2003), hinting at additional control on the level of RNA stability. We have now discovered that this cellular lability of GcvB is caused by a conserved RNA sponge that is released from one of GcvB's own target mRNAs.

The new GcvB sponge originates from the polycistronic *gltIJKL* mRNA of a glutamate/aspartate ABC transporter (Willis & Furlong, 1975; Schellenberg & Furlong, 1977; Reitzer & Schneider, 2001), which is a well-studied GcvB target in *Salmonella*. Aided by the Hfq protein, GcvB binds to the 5′ UTR of *gltI* to repress translational initiation on this mRNA; of note, the C/A-rich target site of GcvB also acts as a translation enhancer element in this mRNA (Sharma *et al*, 2007). We show here that this same operon mRNA carries a second GcvB site between *gltI* and *gltJ*; however, this region produces a stable RNA that sponges GcvB and causes mRNA cross talk in the amino acid pathway.

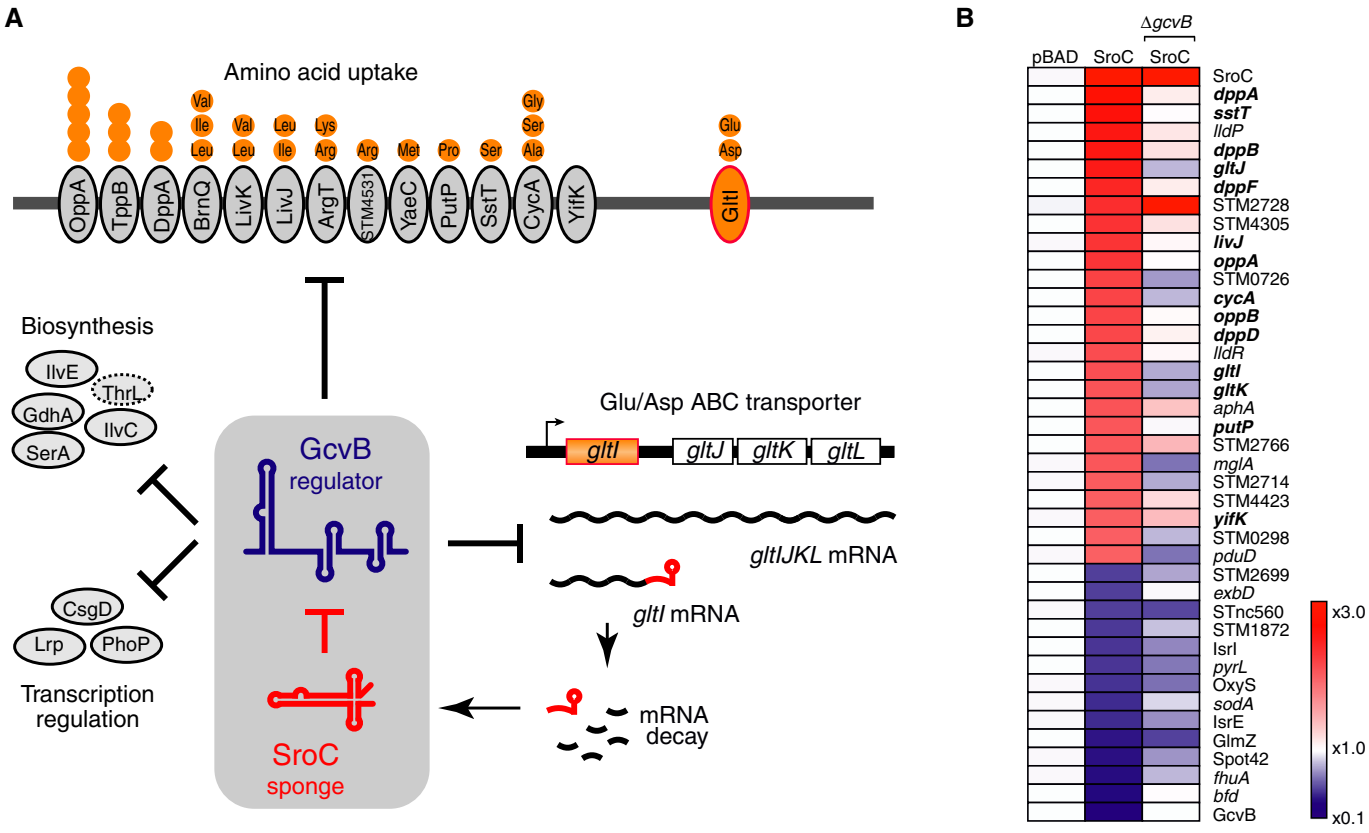

Figure 1. GcvB-SroC regulatory loop affects the GcvB regulon.

A Schematic description of GcvB regulation by SroC as shown in this study. GcvB represses mRNAs of amino acid transporters with different substrate specificity. Orange particles and strands indicate environmental amino acids and peptides, respectively. The GcvB regulon also includes mRNAs of amino acid biosynthetic enzymes and transcriptional regulators. The ThrL leader peptide of the threonine synthesis operon is shown by a dotted oval. One of the GcvB targets, Glu/Asp ABC transporter operon *gltIJKL*, is transcribed in two mRNAs, and the shorter mRNA generates the majority of SroC by processing. Repression of GcvB by SroC results in the derepression of GcvB regulon, including the parental mRNA of SroC.

B Microarray analysis of SroC pulse expression. The *Salmonella* WT (JVS-1574) or Δ*gcvB* (JVS-1044) strains harboring pBAD-ctrl (pKP8-35) or pBAD-SroC (pYC6-4) were induced with 0.2% L-arabinose for 10 min. Gene expression in the WT strain containing pBAD-ctrl was normalized to 1 (left column), and genes that showed > twofold change (*P*-value < 0.1) by SroC overexpression are indicated on the middle column. SroC-induced changes of the genes in Δ*gcvB* background were analyzed by comparing the expression levels in Δ*gcvB* (pBAD-SroC) to those in Δ*gcvB* (pBAD-ctrl) and are shown on the right column. Known GcvB targets are set in boldface. A list of differentially expressed genes and fold changes is included in Supplementary Table S1.

Results

Global mRNA cross talk through depletion of GcvB sRNA

We initially sought to identify a function for SroC, which is a stable ~160 nt RNA fragment encountered in numerous studies of *E. coli* (Vogel *et al*, 2003; Tree *et al*, 2014), *Salmonella* (Sittka *et al*, 2008; Kröger *et al*, 2012, 2013; Ortega *et al*, 2012), and *Klebsiella pneumoniae* (Kim *et al*, 2012). SroC maps to an internal region of the *gltIJKL* operon mRNA, consistent with it being an mRNA breakdown fragment (Vogel *et al*, 2003). However, since SroC associates with Hfq protein *in vivo* (Sittka *et al*, 2008; Chao *et al*, 2012; Tree *et al*, 2014), we speculated that it may regulate other cellular RNAs by base-pairing. Indeed, as shown in Fig 1B, a brief overexpression of SroC in a *Salmonella* wild-type strain altered the levels of 33 mRNAs and seven sRNAs (> twofold change; < 0.1 of *P*-value; Supplementary Table S1).

Whereas computer predictions of potential SroC sites in these regulated genes returned no significant results, we noted that 14 of the 26 SroC-activated mRNAs (Fig 1B) were known targets of GcvB. For example, the *dpp* and *opp* operon mRNAs are repressed by GcvB (Urbanowski *et al*, 2000; Sharma *et al*, 2007) but activated by SroC. Moreover, since GcvB itself was down-regulated (~5.4-fold) by SroC, the observed activation of mRNAs may result from a depletion of their repressor GcvB. Indeed, most of these mRNAs were insensitive to SroC pulse expression in a Δ*gcvB* strain (Fig 1B). In contrast, the mild down-regulation of some sRNAs following SroC pulse expression in the wild-type strain was still observed in the Δ*gcvB* background, suggesting that overexpression of SroC titrates the pool of available Hfq *in vivo* (Papenfort *et al*, 2009; Hussein & Lim, 2011; Moon & Gottesman, 2011), which would destabilize other Hfq-dependent sRNAs.

SroC has been regarded as a stable RNA decay intermediate of the much longer *gltIJKL* operon mRNA (Fig 1A): It carries the characteristic 5′ monophosphate (5′P) of a processed species (Vogel *et al*, 2003) and its 5′ end within the *gltI* coding sequence (Supplementary Fig S1A) is not associated with a transcription start site (Kröger *et al*, 2012). The 3′ end of SroC is likely generated by a conserved ρ-independent transcription terminator in the *gltI-gltJ* intergenic region (Supplementary Fig S1A). Therefore, it may be the expression of the *glt* mRNA of which SroC is a secondary product that normally causes the activation of GcvB targets.

To test this, we constructed a series of mutants in the chromosomal *gltIJKL* locus and evaluated expression changes of GcvB and its representative target OppA. Deletion of *gltIJKL* increased GcvB levels by eightfold and concomitantly reduced the level of OppA by twofold, whereas the deletion of the downstream *gltJKL* genes had no effect (Fig 2A, lanes 1–3). Importantly, the effects of the *gltI* deletion on GcvB and OppA were phenocopied by disrupting only the *sroC* region (lane 4), confirming SroC as the *trans*-activator region of the *gltI* mRNA. Again, SroC acted through GcvB since a Δ*sroC* mutation had no effect on OppA levels in a Δ*gcvB* background (lanes 5–6).

Next, we expressed variants of the *gltI-sroC* region from a plasmid-borne, arabinose-inducible promoter. As in the chromosome, expression of the *gltI-sroC* mRNA decreased GcvB and increased OppA levels (Fig 2B, lane 2). To test whether the GltI protein is involved in the cross-activation of OppA synthesis, we terminated translation of *gltI* at codon 13. Although the levels of SroC from this construct were somewhat lower, we still observed regulation of GcvB and OppA (Fig 2B, lane 3), confirming our hypothesis of RNA-mediated cross talk.

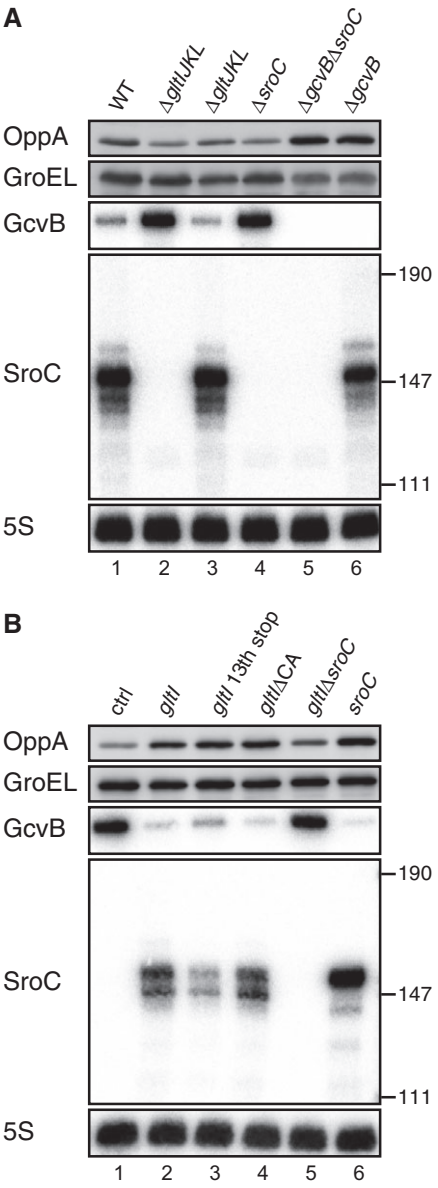

Figure 2. SroC is the effector molecule for GcvB repression.

A Expression changes of OppA and GcvB upon deletion of *gltIJKL* locus. WT (JVS-1574), Δ*gltIJKL* (JVS-5823), Δ*gltJKL* (JVS-10795), Δ*sroC* (JVS-5821), Δ*gcvB*Δ*sroC* (JVS-5822), and Δ*gcvB* (JVS-0236) strains were grown to early stationary phase (OD_{600} of 2.0) in LB medium.

B Expression changes of OppA and GcvB by overexpression of *gltI-sroC*. Δ*gltIJKL* strain (JVS-5823) harboring pBAD-ctrl (lane 1), pBAD-*gltI* (pMM36) derivatives (lanes 2-5), or pBAD-SroC (lane 6) was grown to early stationary phase (OD_{600} of 2.0) in LB medium supplemented with 0.02% L-arabinose.

Data information: Upper two panels: Total protein was analyzed by Western blot to quantify OppA expression. GroEL served as a loading control. Lower three panels: Total RNA was analyzed by Northern blot, and expression of GcvB and SroC was monitored. 5S rRNA served as a loading control. Estimated size from pUC8 marker is indicated on the right.
Source data are available online for this figure.

Interestingly, the *gltI* mRNA itself is a target of GcvB (Fig 1A), raising the possibility that base-pairing at the CA-rich target site in the *gltI* 5′ UTR might contribute to GcvB depletion. However, the same regulation of GcvB and OppA occurred in the presence of a ΔCA mutant plasmid as with the full-length *gltI-sroC* transcript (Fig 2B, lane 4). Lastly, the expression of SroC as a primary transcript was sufficient for both GcvB repression and OppA activation (Fig 2B, lane 6), which independently validated the original microarray results.

A *gltI* mRNA fragment destabilizes the GcvB sRNA

The above results pinpointed SroC as the actual repressor of GcvB but did not reveal on which level GcvB was regulated. Using a transcriptional reporter fusion, we found no significant effect of SroC on *gcvB* transcription (Supplementary Fig S2). To test regulation at the RNA level, we used a two-plasmid system, wherein GcvB was constitutively produced from one plasmid (pP_L-GcvB), and SroC was pulse-expressed from another (pBAD-SroC). Under the conditions used, GcvB levels were ~sixfold higher as compared to chromosomal expression (Fig 3A, lanes 1 and 5). A 10-min pulse of SroC expression depleted the constitutively transcribed GcvB sRNA (Fig 3A, lanes 7 versus 8), indicating that SroC—directly or indirectly—destabilized the GcvB transcript. The Hfq protein which associates with both SroC and GcvB *in vivo* (Sittka *et al*, 2008) is required for this regulation; that is, SroC failed to deplete GcvB in a Δ*hfq* strain (Fig 3A, lane 12).

To prove that SroC targets GcvB on the level of RNA stability, we determined changes in RNA half-life in rifampicin treatment experiments. Figure 3B shows that deletion of either the *gltIJKL* locus or the *sroC* region alone dramatically increased the half-life of GcvB, from ~1.7 min to > 15 min, whereas the GcvB target site in the 5′ UTR of *gltI* had little if any effect on the half-life of GcvB (see ΔCA mutant). Thus, ABC transporter mRNA cross talk is largely determined by SroC acting as a negative regulator of GcvB stability.

RNase E catalyzes both GcvB degradation and SroC biogenesis

Endoribonuclease RNase E is the key enzyme for transcript destabilization in the regulatory network of Hfq-associated RNAs (Massé *et al*, 2003; Morita *et al*, 2005; Saramago *et al*, 2014). To test its involvement in GcvB depletion, we pulse-expressed the *gltI-sroC* region after heat inactivation of the essential RNase E protein using the temperature-sensitive *rne3071* mutant strain (Fig 4A). As expected, SroC depleted GcvB under all conditions in the presence of active RNase E (Fig 4A, lanes 1–6), but GcvB levels remained unchanged upon inactivation of RNase E (lanes 7 versus 8; *rne3071* at 44°C).

A caveat of this experiment was that RNase E also seemed essential to process SroC from the *gltI-sroC* precursor (Fig 4A, lanes 7–8). To overcome this problem, we expressed SroC as a primary transcript, which permitted sufficient SroC accumulation under all RNase E +/− conditions tested (lanes 10, 12, 14, and 16) and helped confirm that RNase E was required for the targeted degradation of GcvB (lanes 16).

The observed requirement of RNase E for the biogenesis of SroC (Fig 4A, lanes 7–8) is consistent with a model in which SroC is an mRNA processing fragment with 5′P (Vogel *et al*, 2003). To confirm

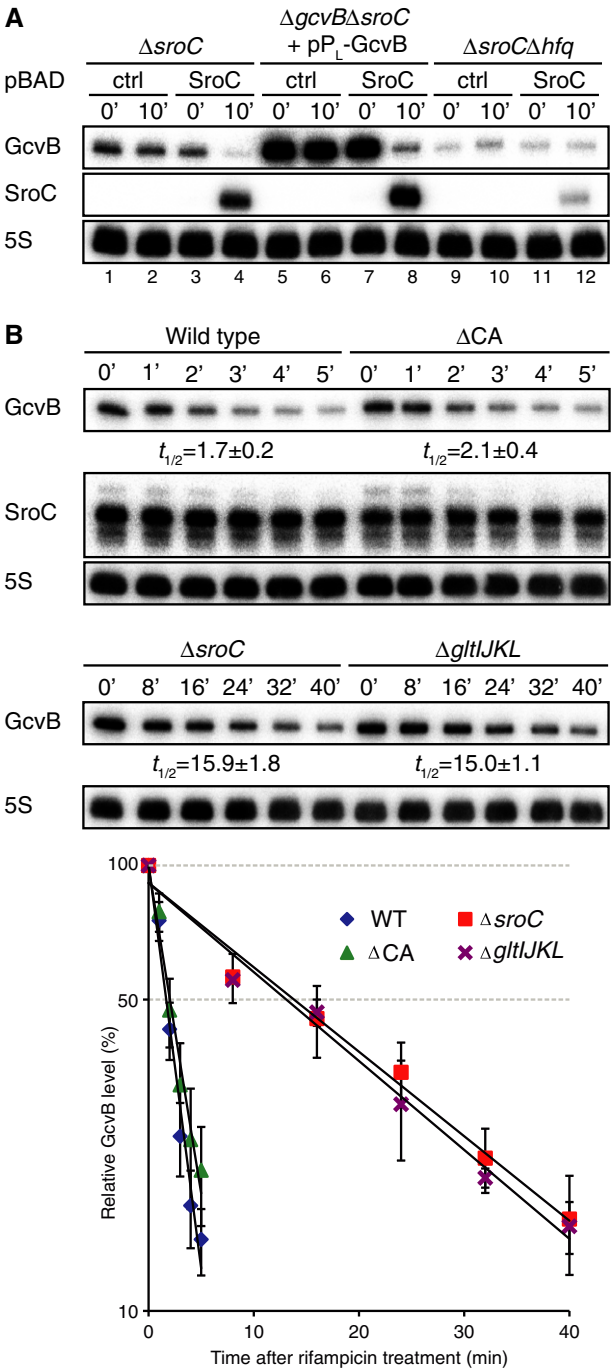

Figure 3. Posttranscriptional regulation of GcvB by SroC.

A *Salmonella* Δ*sroC* (JVS-5821), Δ*gcvB*Δ*sroC* (JVS-5822), and Δ*sroC*Δ*hfq* (JVS-9031) strains were transformed with pBAD-ctrl (pKP8-35) or pBAD-SroC (pYC6-4). Δ*gcvB*Δ*sroC* was cotransformed with pP_L-GcvB (pMM03). Each strain was grown to OD_{600} of 0.5 (0 min) and was further incubated for 10 min in the presence of 0.2% L-arabinose.

B *Salmonella* WT (JVS-1574), Δ*gltIJKL* (JVS-5823), ΔCA (JVS-10741), and Δ*sroC* (JVS-5821) strains were grown to OD_{600} of 2.0 prior to the addition of rifampicin. Total RNA was analyzed by Northern blot to determine decay rates of GcvB. The half-lives were determined from three independent experiments; the standard deviation is indicated.

Source data are available online for this figure.

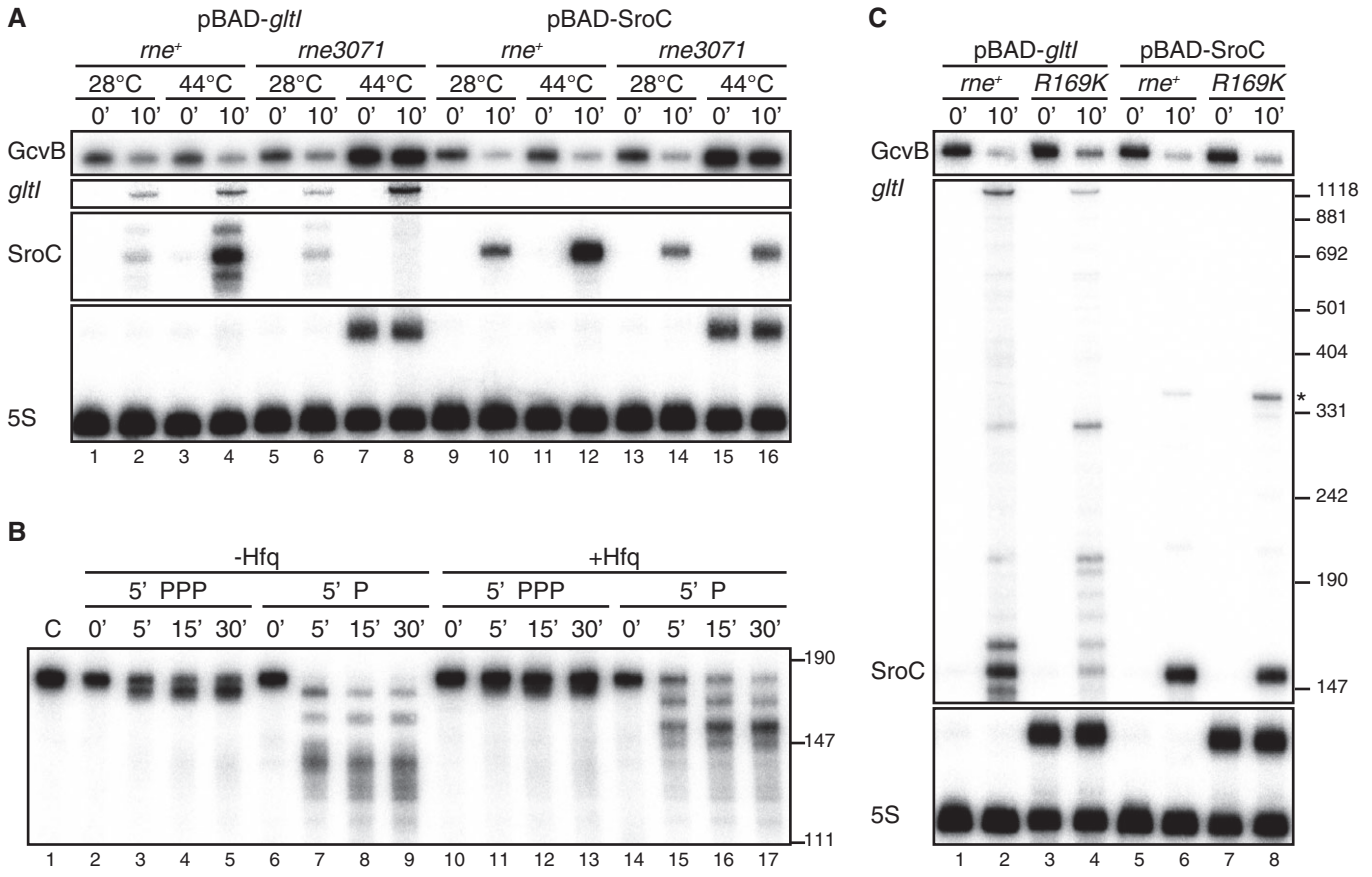

Figure 4. RNase E mediates SroC processing and GcvB degradation in distinct pathways.

A *Salmonella* Δ*gltIJKL rne*$^+$ (JVS-9257) and Δ*gltIJKL rne3071* (JVS-9258) strains transformed with pBAD-*gltI* or pBAD-SroC were grown to OD_{600} of 0.3 at 28°C and further incubated at 44°C for 30 min (OD_{600} of ~0.5, indicated as time point 0). SroC expression was then induced for 10 min in the presence of 0.2% of L-arabinose. Total RNA was analyzed by Northern blots.

B 100 nM of 5′PPP or 5′P *in vitro*-transcribed preSroC was incubated with 100 nM of purified RNase E (1–529) at 30°C for the indicated time in the presence (lanes 10–17) or absence (lanes 2–9) of 100 nM Hfq. The same amount of preSroC was loaded in lane 1 as a control. SroC transcripts were detected using 5′-end-labeled oligonucleotide JVO-2907.

C *Salmonella* Δ*gltIJKL rne*$^+$ (JVS-9257) and Δ*gltIJKL rneR169K* (JVS-11001) strains transformed with pBAD-*gltI* or pBAD-SroC were grown to OD_{600} of 0.5 (0 min) at 37°C and was further incubated for 10 min in the presence of 0.2% L-arabinose. The asterisk indicates transcriptional read-through to the *rrnB* terminator located downstream on the plasmid. Total RNA was prepared from the *Salmonella* strains and subjected to Northern blot analysis. 9S rRNA accumulated in the *rneR169K* strain. Estimated size from pUC8 marker is indicated on the right.

Source data are available online for this figure.

this, we subjected a potential SroC precursor RNA to *in vitro* cleavage with purified RNase E (Fig 4B). Preliminary experiments with serial 5′ truncations of the *gltI-sroC* region *in vivo* (Supplementary Fig S3) helped define SroC as requiring a 20-nucleotide leader as a minimal processing-proficient precursor (preSroC) for the *in vitro* cleavage reactions. Figure 4B shows that *in vitro*-transcribed preSroC containing a 5′P after tobacco acid pyrophosphatase treatment was readily converted to the mature form of SroC by treatment with a catalytic domain (1–529) containing variant of RNase E (lanes 14–17). Correct maturation required the presence of the Hfq protein; without Hfq in the reaction, RNase E generated SroC variants with aberrant 5′ ends (lanes 6–9). Similarly important was the 5′ group of the substrate: preSroC with a 5′ triphosphate (5′PPP), incubated with or without Hfq, was a poor substrate for RNase E (lanes 2–5 and 10–13), suggesting that SroC maturation occurs by 5′-end-dependent mRNA decay initiated in the upstream *gltI* region.

We further explored the observed 5′P dependence in SroC biogenesis by constructing a chromosomal *rneR169K* mutant allele which expresses a variant of RNase E that is defective in 5′P sensing (Callaghan *et al*, 2005; Jourdan & McDowall, 2008; Bandyra *et al*, 2012). The 5′P sensing defect of this *Salmonella* mutant was apparent from the accumulation of the 5S rRNA precursor (Fig 4C), as previously reported in *E. coli* (Garrey *et al*, 2009; Anupama *et al*, 2011). In line with our *in vitro* results, SroC biogenesis from the *gltI-sroC* transcript was dramatically altered in the *rneR169K* strain (Fig 4C, lanes 1–4): Instead of mature SroC, longer processing intermediates accumulated. Intriguingly, however, the lower levels of 5′P-SroC in the *rneR169K* background still permitted GcvB regulation, and this was independently confirmed by the expression of SroC as a primary (5′PPP) transcript in this background (lanes 5–8; note that this construct would also yield 5′P SroC due to enzymatic 5′ pyrophosphate removal *in vivo* (Hui *et al*, 2014)). These results suggest the

involvement of two different mechanisms of RNase E catalysis; that is, SroC is processed by the 5′-end-dependent pathway and GcvB is degraded by the 5′-end-independent pathway of the enzyme. While the molecular details of RNase E action on the GcvB-SroC complex are being investigated, we already note a striking difference to the inactivation of ChiX sRNA on the *chbBC* mRNA where base-pairing changes the 3′ terminator region of ChiX, rendering it susceptible to PNPase (Figueroa-Bossi *et al*, 2009; Plumbridge *et al*, 2014).

Direct interaction between GcvB and SroC

SroC-mediated depletion of GcvB requires the Hfq protein (Fig 3A) whose primary role in the cell is to facilitate RNA interactions (Vogel & Luisi, 2011; Sobrero & Valverde, 2012; De Lay *et al*, 2013). To identify base-pairing regions, we probed the structures of GcvB, SroC, and their hybrid *in vitro* with lead(II) and RNase T1 (Fig 5A). While the cleavage pattern of GcvB alone agreed with previous results (Sharma *et al*, 2007, 2011), the presence of a fivefold excess of SroC suppressed T1 cleavages at guanosine (G) positions 14, 18, and 156 in GcvB; concomitantly, G143 became more susceptible (Fig 5B). SroC also protected stem loop 1 (SL1) of GcvB from backbone cleavage by lead(II). Conversely, GcvB protected SroC from T1 attack at G84, located in a rare accessible region at the tip of SL2 in the otherwise tightly folded SroC RNA. GcvB also protected SL2 of SroC during lead(II) probing, and suppressed T1 cleavage sites at G61 and G65 in the internal bulge of SroC. These results indicate that GcvB and SroC interact by base-pairing at two short complementary regions of 7 or 8 nucleotides. These predicted binding sites, hereafter referred to as BS1 and BS2, are only 14 nucleotides apart in SroC, but located in very distant regions of GcvB (Fig 5B). While the BS1 region of GcvB has not been implicated in base-pairing with other targets before, the BS2 site partially overlaps with the GcvB target region for the *phoP* mRNA in *E. coli* (Coornaert *et al*, 2013).

Alignment of SroC sequences reveals that the GcvB interaction sites are conserved in numerous enteric bacteria that also encode GcvB (Supplementary Fig S1A); the interaction sites in GcvB

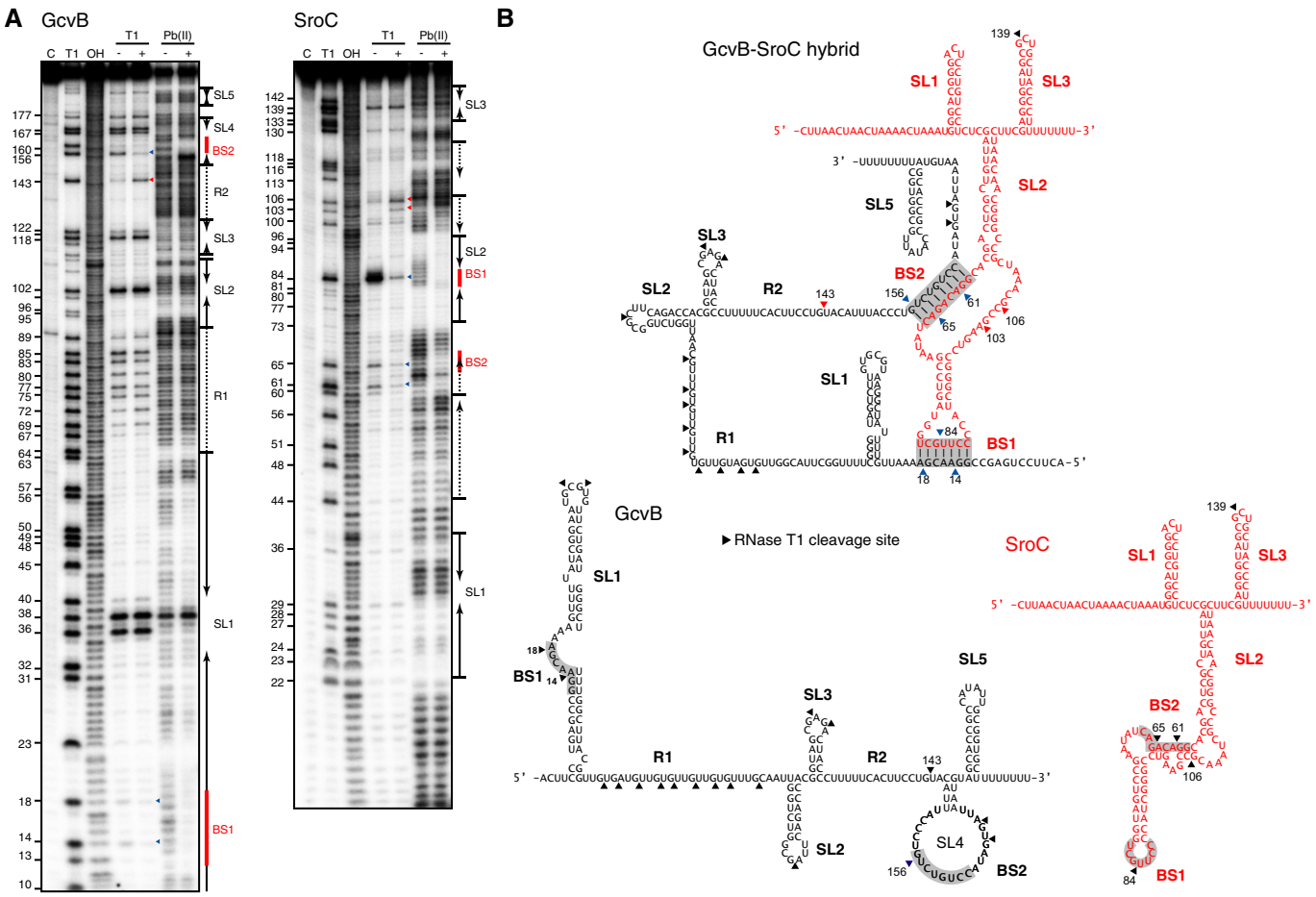

Figure 5. Direct binding between GcvB and SroC sRNAs.

A 20 nM of 5′-end-labeled GcvB and SroC were subjected to RNase T1 and lead(II) cleavage in the absence and presence of 100 nM cold sRNAs, SroC, and GcvB, respectively. Lane C: untreated RNA; lane T1: RNase T1 ladder of denatured RNA; lane OH: alkaline ladder. The position of G residues cleaved by RNase T1 is indicated at the left of picture.

B Secondary structures of GcvB and SroC, paired or alone. RNase T1 cleavage sites are indicated by arrowheads; color indicates those that appeared (red) or disappeared (blue) upon base-pairing. The base pairing sites BS1 and BS2 are shadowed.

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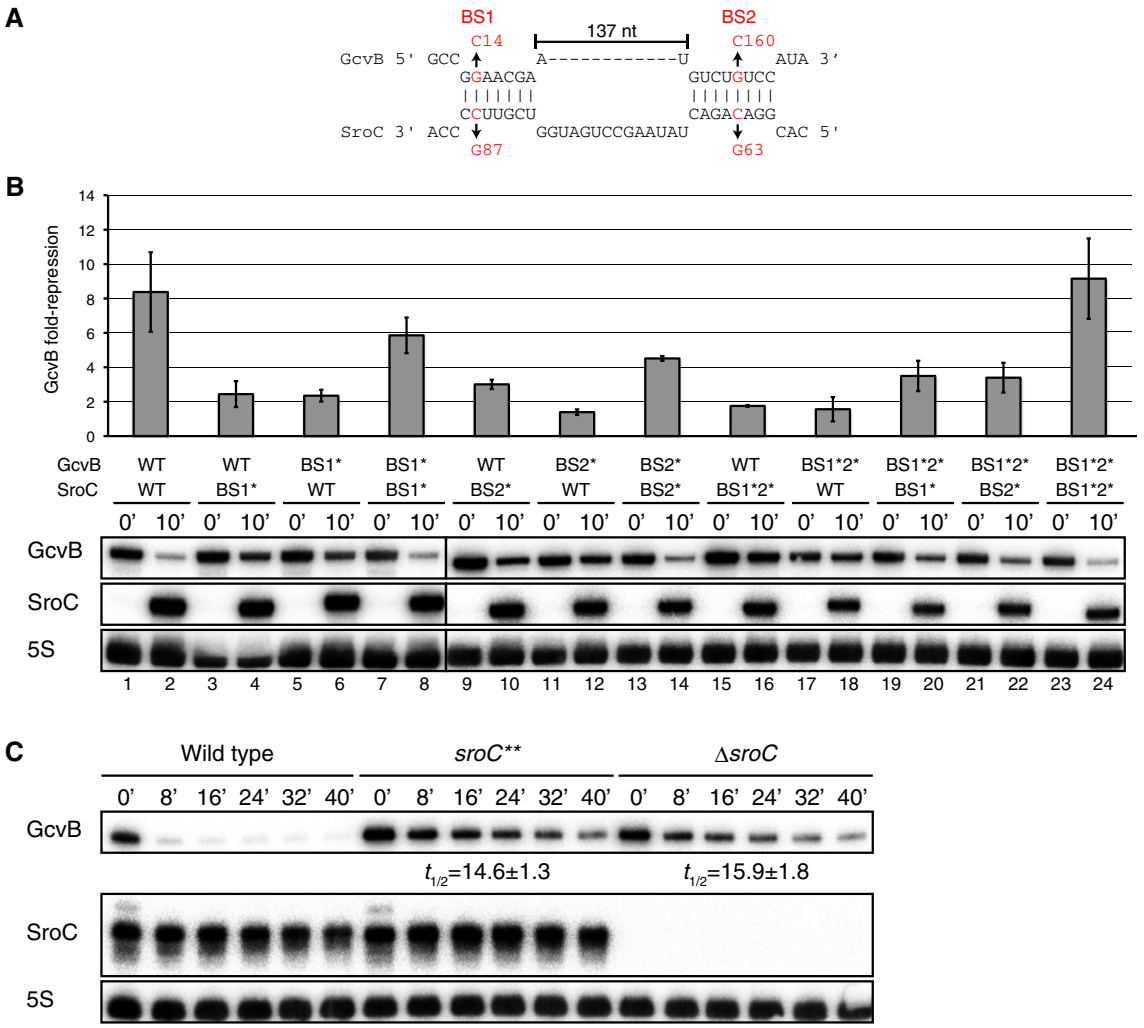

Figure 6. SroC induces GcvB degradation by a base-pairing mechanism.

A GcvB-SroC base-pairing regions. The compensatory base pair changes in GcvB and SroC are indicated in red.

B *Salmonella* Δ*gcvB*Δ*sroC* (JVS-5822) strain was cotransformed with pP_L-GcvB and pBAD-SroC derivatives (see Supplementary Table S4). Each transformant was grown to OD_{600} of 0.5 (0 min) and incubated for another 10 min in the presence of 0.2% L-arabinose. Total RNA was prepared from the cells and subjected to Northern blot analysis. The fold repression of GcvB was determined from three independent replicates; standard deviation is shown.

C The chromosomally modified wild-type *sroC* strain (JVS-10108), *sroC*** mutant (JVS-10111) and *sroC* deletion mutant (JVS-5821) were grown to early stationary phase in LB medium (OD_{600} of 2.0) prior to the addition of rifampicin. Cultures were harvested at the indicated time points after rifampicin treatment. RNA was isolated and analyzed by Northern blot as in Fig 3B.

Source data are available online for this figure.

are also conserved (Supplementary Fig S1B). Using our two-plasmid approach, we confirmed that the GcvB and SroC homologues of *E. coli* are equally functional (Supplementary Fig S1C). In *Yersinia pestis*, however, the BS2 site is not conserved and SL2 containing the other GcvB binding regions is followed by a uridine stretch; as expected, these mutations generate a truncated SroC homologue which is unable to regulate GcvB (Supplementary Fig S1D).

Base-pairing with SroC destabilizes the regulator GcvB

To address the importance of the predicted RNA interaction for GcvB destabilization *in vivo*, point mutations were introduced in the BS1 and BS2 regions of the plasmid-expressed sRNAs (Fig 6A). Either one of two C->G changes at SroC positions 63 (BS1) and 87 (BS2) weakened the repression of GcvB, while the individual compensatory mutations in GcvB (G14C, G160C) rendered GcvB a little less sensitive to SroC (Fig 6B, lanes 4, 6, 10, 12). However, combining the two mutations in GcvB or SroC almost eliminated regulation when either sRNA was paired with its wild-type partner sRNA, but regulation was fully rescued by combination of the double compensatory mutations (GcvB G14C/G160C and SroC C87G/C63G). These *in vivo* results provide strong evidence that SroC destabilizes GcvB through two independent additive RNA interactions. The additive function may be explained by the relatively weak binding strength of each of the two interactions: Their predicted changes in minimal free energy of −13.6 kcal/mol (BS1) and −16.7 kcal/mol (BS2) are

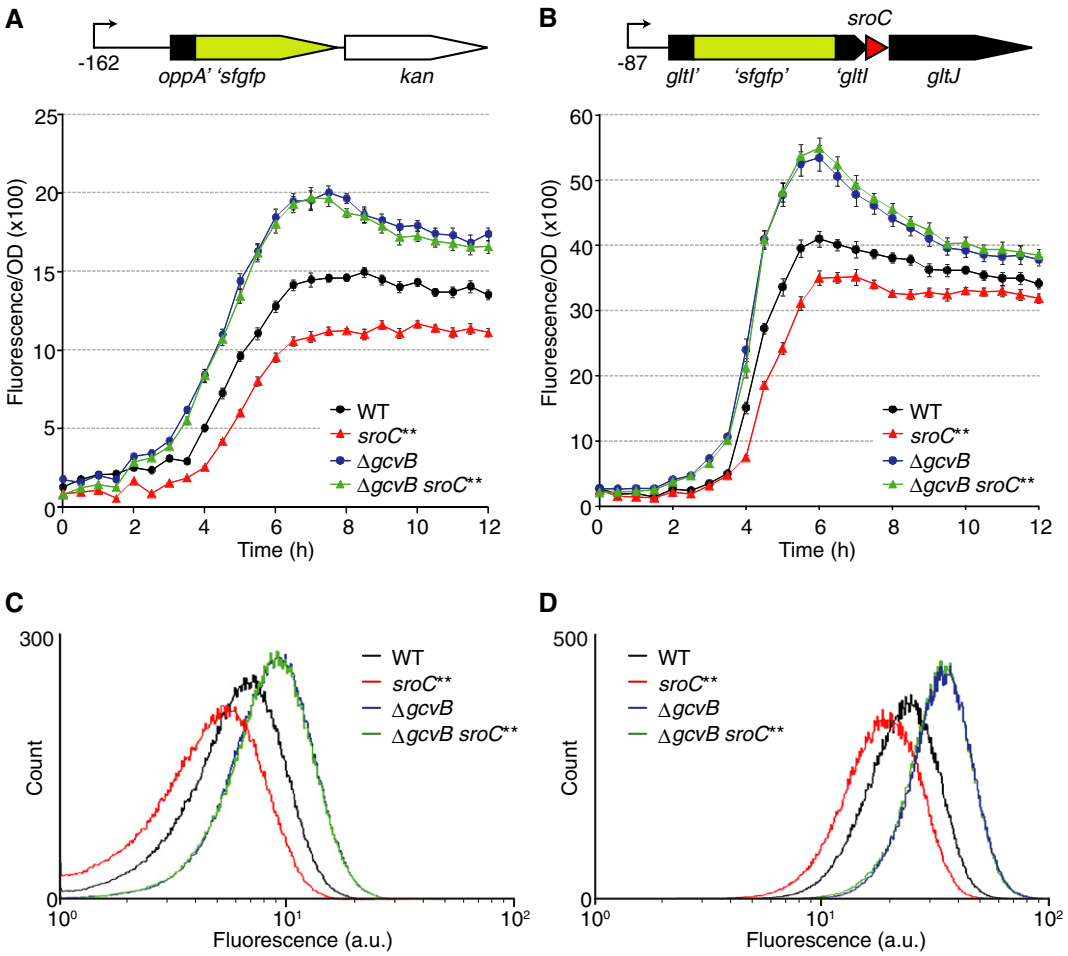

Figure 7. SroC ensures expression of GcvB targets during growth on casamino acids.

A–D *Salmonella* strains with *oppA* (A and C) and *gltI* (B and D) translational fusions to a *sfgfp* reporter gene in WT, *sroC***, Δ*gcvB*, and Δ*gcvB sroC*** background were grown in minimal medium supplemented with 0.4% casamino acids. Fluorescence of bulk culture (A and B) normalized by OD_{595} was measured in 100 µl culture per well on 96-well plate over growth by TECAN plate reader. Fluorescence of 50,000 cells grown to exponential phase (C and D) was monitored by FACS. See also Supplementary Fig S4.

well above the range of, for example, the many mRNA interactions of the RybB sRNA (Papenfort *et al*, 2010).

To quantitatively assess the effect of this bipartite interaction with physiological concentrations of SroC and GcvB, we introduced scar-less point mutations of C63G/C87G (*sroC***) in the chromosomal *gltI-sroC* locus and determined the half-life of GcvB. While SroC itself remained stable, the *sroC*** strain exhibited the same stabilization of GcvB as observed in Δ*sroC* (Fig 6C), validating that base-pairing is required for SroC-mediated GcvB destabilization *in vivo*. Of note, the disruption of GcvB-SroC pairing by point mutations affects only the stability of GcvB, suggesting that SroC does not undergo coupled degradation with its partner RNA by RNase E as previously seen with Hfq-dependent sRNA-mRNA pairs (Massé *et al*, 2003).

SroC ensures expression of its parental mRNA along with other GcvB targets

To investigate how SroC affects the expression of GcvB targets in a chromosomal context, we constructed chromosomal translational superfolder GFP (sfGFP) fusions for *oppA* and *gltI*; the design of the *gltI-sfgfp* fusion maintains wild-type levels of SroC (see Materials and Methods). Using bacterial fluorescence as a proxy, we monitored GcvB- or SroC-dependent expression changes of OppA-sfGFP and GltI-sfGFP during growth in a minimal medium supplemented with 0.4% casamino acids as sole carbon and nitrogen sources (Fig 7A and B). A comparison of wild-type and *sroC*** backgrounds revealed that both fusions require SroC for full expression. However, this difference between the wild-type and *sroC*** strains is eliminated upon the introduction of a Δ*gcvB* mutation. Single-cell measurements revealed largely homogenous expression patterns for each fusion (Fig 7C and D), ruling out the possibility that SroC affects variability of GcvB target expression in the population. These experiments under physiological conditions confirm that mRNA cross talk via the SroC-GcvB axis affects ABC transporter expression both in *cis* and *trans*.

Salmonella and *E. coli* have three major peptide permeases, oligo-/di-peptide ABC transporters OppABCDF/DppABCDF and tripeptide symporter TppB (Hiles & Higgins, 1986), all of which are under the posttranscriptional control of GcvB (Urbanowski *et al*, 2000; Sharma

Table 1. SroC promotes growth on peptides.

Strain	0.4% tryptone[a]	0.4% casamino acids[b]
Wild-type	181 ± 6 min	140 ± 1 min
*sroC***	235 ± 2 min	145 ± 1 min
Δ*gcvB*	172 ± 2 min	134 ± 5 min
Δ*gcvB sroC***	173 ± 4 min	135 ± 6 min

[a]Doubling time for strain grown in the M9 minimal medium supplemented with 0.4% tryptone, calculated from triplicate cultures. "±" denote standard deviation. The associated growth curves are shown in Supplementary Fig S4A and B.
[b]As above but culture in media supplemented with 0.4% casamino acids.

et al, 2007, 2011; Pulvermacher *et al*, 2008). Given that SroC alleviates the repression of these multiple ABC transporters, we expected this sponge to promote growth under peptide-limiting conditions. To address this, we compared the growth of the *sroC* base-pairing mutant strain in two media—tryptone and casamino acids—that both offer carbon and nitrogen for growth but differ in their content of importable peptides (Supplementary Fig S4). As expected, inactivation of SroC increased the doubling time of *Salmonella* during growth on tryptone which is enzymatically digested from cow milk casein and thus abundant in peptides (Table 1). By contrast, we observed no difference between the wild-type and Δ*sroC* strains in casamino acids medium. This latter medium is composed of 19 single amino acids which can be taken up by multiple amino acid transporters (Reitzer, 2005), most of which are not regulated by GcvB. This result indicates that one of the physiological roles of SroC is to fine-tune the expression of GcvB targets according to nutrient availability.

Discussion

By investigating a possible cellular function of a stable mRNA breakdown fragment, we have discovered a mechanism of mRNA cross talk in one of the most global RNA regulons currently known in bacteria. We show that the synthesis of *gltI* mRNA provides both, the template for protein synthesis of a Glu/Asp ABC transporter, and the precursor of an RNA antagonist of the GcvB small RNA which controls a large mRNA regulon of amino acid-related proteins. Through licensing GcvB for degradation by RNase E, the SroC sponge relieves its own parental mRNA from GcvB-mediated repression, resulting in a dual RNA containing feed-forward loop that might facilitate GltIJKL protein synthesis under certain conditions. Concomitantly, the SroC-mediated depletion of GcvB has the potential to cross-activate dozens of *Salmonella* mRNAs with amino acid-related transport and biosynthesis functions. Nucleotide conservation patterns in *gltI* and GcvB argue that this unusual RNA interaction has been maintained by selection and that the *gltI*-SroC-GcvB axis is functional in many enterobacterial species (Supplementary Fig S1). As will be discussed below, SroC may also be seen as a new subtype of the emerging class of 3′ UTR-derived sRNAs whose roles in bacterial physiology are just beginning to be appreciated.

Bacterial mRNA cross talk via antagonists of small RNAs

The GcvB regulon is the first bacterial RNA regulon in which a sponge-mediated cross talk would affect many physically unlinked mRNAs. Nonetheless, although studies of the paradigmatic chitosugar utilization pathway have focused on cross-activation of a single porin mRNA upon the degradation of ChiX sRNA (Figueroa-Bossi *et al*, 2009; Overgaard *et al*, 2009), we speculate that other ChiX targets such as the DpiA/B two-component system (Mandin & Gottesman, 2009b) are also likely to be affected. Likewise, it has been speculated that the RprA sRNA is trapped by the abundant *csgD* mRNA under certain growth conditions; this should affect the synthesis of additional RprA targets, foremost the *rpoS* mRNA (Mika *et al*, 2012).

In both the above scenarios, ChiX or RprA would be sequestered by intact mRNAs, whereas the primary GcvB antagonist is SroC and not its parental mRNA species. That is, SroC exceeds the *glt* transcripts with respect to both, cellular abundance and enrichment by Hfq (Chao *et al*, 2012). Nonetheless, the outcomes of this competition for mRNA cross talk will depend on how good a competitor SroC is compared to other targets; this will be additionally influenced by the transcription rate of the *gltI* operon synthesis, the net SroC levels, and the affinity and binding kinetics of GcvB for SroC as compared to the binding of other GcvB targets. While these parameters remain to be determined, we speculate that this novel mRNA-sRNA-sRNA-mRNA scheme of cross talk may be advantageous for two reasons: (i) Unlike the high molecular weight *glt* mRNAs, SroC is not expected to be diffusion-limited, increasing its chance to interact with GcvB in the cell; (ii) the activity of SroC may be subject to independent further posttranscriptional control, allowing for the integration of extra input signals in this complex RNA-based circuit.

Enriched stable mRNA fragments such as SroC have been observed in Hfq coIP experiments in diverse organisms (Zhang *et al*, 2003; Sonnleitner *et al*, 2008; Berghoff *et al*, 2011; Chao *et al*, 2012; Möller *et al*, 2014). Given that the concentration of Hfq is limiting *in vivo* (Fender *et al*, 2010; Moon & Gottesman, 2011), we predict that some of these stable mRNA fragments function as sRNA sponges akin to SroC.

A lesson for experimental target prediction

Examples of positive regulation by sRNAs were for many years considered to be rare exceptions to target repression, but they have recently received increased attention following reports of new direct and indirect mechanisms of mRNA activation (Sonnleitner *et al*, 2011; Fröhlich *et al*, 2013; Göpel *et al*, 2013; Jørgensen *et al*, 2013; Papenfort *et al*, 2013; Salvail *et al*, 2013). Nonetheless, the activation of a large number of apparent targets by SroC (Fig 1B) was unprecedented. We can now explain this by a high rate of false positives, caused by titration of another sRNA. Thus, SroC holds an important lesson for future experimental target predictions as we enter the era of systematic screening of sRNA functions (Papenfort *et al*, 2008; Mandin & Gottesman, 2009a; Nichols *et al*, 2011; De Lay & Gottesman, 2012; Brochado & Typas, 2013). Curated sRNA target databases (Cao *et al*, 2010) will be needed to identify obvious false positives among target activation patterns.

SroC as a new type of 3′ UTR-derived small RNA

RNA species such as SroC pose a challenge to functional transcript annotation. From a genetic point of view that may ignore the underlying mechanism, it is the *gltI* mRNA that cross talks with other ABC transporter mRNAs via GcvB. On the molecular level, however,

SroC is an Hfq-associated sRNA produced from the 3′ UTR of the *gltI* mRNA. While there has been much effort to predict and characterize conserved freestanding sRNA genes, there is increasing evidence for widespread functions of conserved 3′ UTR-embedded sRNAs. Such transcripts usually share the terminator with their 'host' mRNA and fall into two classes according to their biogenesis (Chao *et al*, 2012). The first class includes the Hfq-dependent DapZ or MicL sRNAs, which are independently expressed from mRNA gene internal promoters; both DapZ and MicL act in pathways unrelated to their respective mRNA loci (Chao *et al*, 2012; Guo *et al*, 2014). Members of the other class, for example, s-SodF sRNA of *Streptomyces coelicolor* (Kim *et al*, 2014), are strictly generated by mRNA processing, for lack of a gene internal promoter. Interestingly, similar to SroC regulating (indirectly) amino acid-related mRNAs, the s-SodF sRNA mediates cross talk of two superoxide dismutase-encoding mRNAs (Kim *et al*, 2014). Clearly, more examples are needed to address whether biogenesis type and target choice of 3′ UTR-embedded sRNAs are correlated.

SroC is a previously unknown variation of the theme of 3′ UTR-derived sRNAs whose biogenesis warrants deeper investigation. The single *gltIJKL* promoter produces both, the full-length *gltIJKL* operon mRNA to make the entire Glu/Asp ABC transport system and the shorter mRNA of the periplasmic Glu/Asp-binding protein, GltI, alone (Kröger *et al*, 2013). The periplasmic high-affinity binding protein recaptures endogenous compounds leaking from the cell (Stirling *et al*, 1989) and is therefore made in much higher stoichiometry than the other components (Boos & Lucht, 1996). This is commonly achieved by gene order (first position in ABC transporter operons) and selective stabilization of a monocistronic mRNA from 3′ to 5′ exonucleolytic decay. By contrast, the monocistronic *gltI* mRNA and its SroC derivative are generated by a leaky Rho-independent terminator. We note that the SroC region harbors additional conserved nucleotides outside the GcvB contact regions, which raises the possibility that a *cis*-acting element or a *trans*-acting factor regulates transcription termination in the *gltIJ* intergenic region and, thus, the release of SroC and with it the levels of GcvB.

Regulating the regulator GcvB

The cellular level of GcvB has been known to be tightly controlled at the level of transcription, through the activity of the GcvA/GcvR transcription factors that respond to glycine availability (Urbanowski *et al*, 2000; Ghrist *et al*, 2001; Heil *et al*, 2002; Stauffer & Stauffer, 2005). Such tight transcriptional control reflects the situation of many other sRNAs, some of which possess the most highly regulated promoters in their respective regulons (Pfeiffer *et al*, 2007; Mutalik *et al*, 2009). However, GcvB is also a paradigm for a very unstable sRNA, displaying the shortest (< 2 min) half-life of twenty sRNAs in the first systematic evaluation of sRNA stability (Vogel *et al*, 2003). The molecular cause of its lability remained elusive; GcvB possesses terminal structures (Sharma *et al*, 2007) that should protect it from rapid degradation.

At first, coupled degradation whereby sRNAs are degraded as they base-pair with targets (Massé *et al*, 2003) seemed a straightforward explanation for this lability, given the extensive GcvB target regulon (Pulvermacher *et al*, 2009; Modi *et al*, 2011; Sharma *et al*, 2011; Coornaert *et al*, 2013; Wright *et al*, 2013). Our study, however, now reveals a singular factor—the SroC sponge—to account for much of the instability of GcvB (Figs 3B and 6C) demonstrating that GcvB is regulated at both the level of synthesis and decay, with consequences for the whole GcvB regulon (Fig 1B). Comparison of intracellular copy numbers suggests that SroC targets GcvB in a near-stoichiometric manner. Stationary-phase *Salmonella* grown in rich medium expresses ~50 copies of SroC per cell (Supplementary Fig S5); if SroC is genomically depleted, the steady-state level of GcvB increases by ~fourfold and the half-life by ~eightfold (Fig 3B). When the level of SroC is reduced by half, GcvB levels increase by only ~threefold (Supplementary Fig S5). We consider SroC itself as a true sponge as under no condition, GcvB affects its steady-state levels.

Others recently reported the discovery of a prophage-specific anti-GcvB sRNA, named AgvB, in a strain of enterohemorrhagic *E. coli* (Tree *et al*, 2014). Physiological levels of AgvB when it acts remain unknown, but an overexpression experiment suggests that it can antagonize GcvB without affecting sRNA stability. Importantly, unlike SroC which binds GcvB outside the established mRNA binding sites, the AgvB sRNA may directly compete with mRNA regulation by mimicking a C/A-rich target site complementary to the R1 seed region of GcvB. Clearly, although these two GcvB antagonists differ in their molecular mechanism, their existence argues that control of the global regulator GcvB at the RNA level equally benefits endogenous gene expression and horizontally acquired genetic elements. It will also be interesting to see whether functionally analogous riboregulators of amino acid-related mRNAs, for example, the enterobacterial DapZ sRNA, the *Staphylococcus* RsaE sRNA, or the α-proteobacterial AbcR sRNAs (Geissmann *et al*, 2009; Bohn *et al*, 2010; Caswell *et al*, 2012; Chao *et al*, 2012; Torres-Quesada *et al*, 2013; Overlöper *et al*, 2014), are kept in check by similar types of RNA sponges.

Outlook

An important physiological question to answer in the future is why the *gltIJKL* locus evolved to express such a potent GcvB sponge. This operon encodes a Glu/Asp ABC transporter, which together with the GltP and GltS proteins supplies bacteria with Glu or Asp (Schellenberg & Furlong, 1977; Reitzer, 2004). This could be relevant for *Salmonella* infection of epithelial cells, for glutamate is limiting in the *Salmonella*-containing vacuole (Bowden *et al*, 2010). However, a review of published *Salmonella* mutagenesis data sets (Supplementary Table S2) has so far failed to suggest a suitable system to study SroC effects in the nutrient-poor environment of bacterial hosts. Under some conditions, the SroC-GcvB axis may also affect the mRNAs of the global transcriptional regulators, Lrp, CsgD, and PhoP (Modi *et al*, 2011; Sharma *et al*, 2011; Coornaert *et al*, 2013; Wright *et al*, 2013). Lrp alone affects the expression of ~10% of all genes (Cho *et al*, 2008, 2011), which include prominent GcvB targets such as *oppABCDF*, *dppABCDF*, *tppB*, *livJ-livKHMGF*, *brnQ-proY*, *cycA*, *thrLABC*, and *gdhA* genes. This would suggest that a plethora of coherent or incoherent feed-forward loops in response to leucine and glycine levels alters gene expression dynamics (Beisel & Storz, 2010).

A combination of approaches profiling both gene expression and metabolic changes at high resolution will be needed to address how global mRNA cross talk through SroC affects the expression kinetics and hierarchy of GcvB targets. In addition, we do not rule out the

possibility that SroC regulates other mRNAs independent of GcvB, for example, the phage-derived STM2728 mRNA. Last but not least, its exceptional intracellular stability (Vogel *et al*, 2003) asks the question whether SroC itself is turned over in a controlled manner, perhaps through adaptor-mediated recruitment of RNase E as recently shown for other sRNAs (Suzuki *et al*, 2006; Göpel *et al*, 2013).

Materials and Methods

Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium strain SL1344 (JVS-1574) was used as a wild-type strain. The strains used in this study are listed in Supplementary Table S3. Bacterial cells were grown at 37°C with shaking at 220 rpm in LB broth (Lennox) medium or minimal medium (the same as M9 but without NH_4Cl; 12.8 g Na_2HPO_4 $7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl per liter). Minimal medium was supplemented with tryptone or casamino acids at a final concentration of 0.4% as a sole carbon and nitrogen source. Solutions of tryptone and casamino acids (BD Biosciences) were sterilized by filtration. Where appropriate, media were supplemented with antibiotics at the following concentrations: 100 μg/ml ampicillin (Ap), 50 μg/ml kanamycin (Km), 20 μg/ml chloramphenicol (Cm) and 500 μg/ml rifampicin.

Strain construction

Deletion strains were constructed by the lambda Red system (Datsenko & Wanner, 2000). *sroC*, *gltIJKL,* and *gltJLK* were deleted using pKD4 as a template and primer pairs, JVO-0303/JVO-0304, JVO-5007/JVO-5008, and JVO-11569/JVO-5008, respectively. The resulting Km-resistant strains were confirmed by PCR, and the mutant loci were transduced into appropriate genetic backgrounds by P22 phage. To eliminate the resistance genes from the chromosome, strains were transformed with the temperature-sensitive plasmid pCP20 expressing FLP recombinase (Datsenko & Wanner, 2000).

Chromosomal mutant strains, *sroC* (JVS-10108, JVS-10111) and *gltI*ΔCA (JVS-10741), were constructed by scar-less mutagenesis using a two-step lambda Red system (Blank *et al*, 2011). DNA fragments containing a Cm^R resistance marker and a I-SceI recognition site amplified with primer pairs JVO-0303/JVO-0304 and JVO-7448/JVO-11078 from the template plasmid pWRG100 were integrated into the chromosomal *sroC* and *gltIp* region, respectively, by lambda Red recombinase expressed from pKD46 (Datsenko & Wanner, 2000). The resultant mutant was transformed by pWRG99, and the mutant allele amplified from pBAD-SroC or pBAD-*gltI* derivatives (Supplementary Table S4) with JVO-9194/JVO-9195 or JVO-7451/JVO-9611 was integrated by the lambda Red recombinase expressed from pWRG99, which subsequently expressed I-SceI endonuclease by supplementation of 2 μg/ml of anhydrotetracyclin to select the resultant recombinant in which the Cm^R I-SceI allele was eliminated. Successful recombinants were confirmed by Cm sensitivity, PCR, and sequencing.

To integrate *oppA* and *gltI* translational fusions into *Salmonella* chromosome, *oppA*^{16aa}-*sfgfp* and *gltI*^{16aa}-*sfgfp* were amplified with primer pairs JVO-10629/JVO-9762 and JVO-10631/JVO-10688 using pDP124 (pCBP derivative replaced with *sfgfp*) (Wahl *et al*, 2009) as a template and were recombined by lambda Red system (Datsenko & Wanner, 2000). To omit the downstream terminator sequence and Km resistance gene, the C-terminal 23 aa of the *gltI* sequence was fused with *sfgfp* by scar-less mutagenesis (Blank *et al*, 2011). A PCR fragment amplified with JVO-10927/JVO-10668 from pWRG100 was first integrated into the *gltI-sfgfp* Km^R strain, and next, the Cm^R I-SceI allele was replaced by an 80-mer dsDNA of JVO-10966/JVO-10967 using pWRG99.

Salmonella rne3071 mutant (JVS-7000) and its parental strain (JVS-6999) were kindly provided by L. Bossi. Mutation in the 5′-end sensor of RNase E (R169K) was introduced into the chromosomal *rne* gene by a previously described procedure in Figueroa-Bossi *et al* (2009). A DNA fragment was amplified with the JVO-10059 (the same as ppC66) and JVO-11002 primers from genomic DNA of strain JVS-6999 (contains the Cm resistance gene in the IGR between *rluC* and *rne*) and integrated into the wild-type strain by lambda Red recombinase expressed from pKD46 (Datsenko & Wanner, 2000). The mutant (JVS-10999) was selected for Cm resistance and small colony formation and was confirmed by sequencing; it also showed the expected accumulation of 9S rRNA.

Plasmid construction

A complete list of all plasmids used in this study can be found in Supplementary Table S4. GcvB expression plasmid pMM03 and its control pMM01 were constructed by replacing the XhoI-AvrII fragment of pTP11 and pTP09 (Sharma *et al*, 2007) with that containing a Cm resistance marker and a p15A *ori* from pZA31-luc (Lutz & Bujard, 1997), respectively. To overexpress SroC and *gltI-sroC* mRNA under the control of arabinose-inducible promoter, *sroC* and *gltI-sroC* fragments were amplified with JVO-4625/JVO-4626 and JVO-7775/JVO-0306 and cloned into a pBAD backbone by the procedure described previously in Papenfort *et al* (2006), to yield pYC6-4 and pMM36, respectively. pMM44 and pMM45 expressing *gltI*ΔCA and preSroC were generated by PCR with JVO-1973/JVO-1974 and JVO-7997/JVO-4531 using pMM36 as a template and self-ligation of the PCR fragments. The plasmid-borne *gcvB* and *sroC* were mutated by site-directed mutagenesis using overlapping PCR with primer pairs, JVO-9090/JVO-9091 (*gcvB* G14C), JVO-9214/JVO-9215 (*gcvB* G160C), JVO-7578/JVO-9020 (*sroC* C87G), and JVO-9216/JVO-9217 (*sroC* C63G), respectively.

Microarray analysis

Microarray analysis of SroC overexpression from P_{BAD} promoter was performed as described (Papenfort *et al*, 2006; Sharma *et al*, 2011). The wild-type (JVS-1574) or Δ*gcvB* (JVS-1044) strains were transformed with pKP8-35 (pBAD-ctrl) or pYC6-4 (pBAD-SroC) and were grown in LB medium. At optical density (OD_{600}) of 1.5, L-arabinose was added to cultures at a final concentration of 0.2% to induced SroC expression for 10 min, and total RNA was prepared with SV total RNA isolation system (Promega). The gene expression in WT strain containing pBAD-ctrl was normalized (to 1) and used as a standard for differential gene expression analysis. SroC-induced changes in Δ*gcvB* were analyzed by comparing the expression levels in Δ*gcvB* (pBAD-SroC) to those in Δ*gcvB* (pBAD-ctrl) and plotted in Fig 1B. A list of differentially expressed genes and fold changes can be found in Supplementary Table S1.

Northern blot analysis

Bacterial culture was immediately frozen at an appropriate condition by the addition of 0.2 vol/vol of stop solution (95% ethanol and 5% phenol). Total RNA was isolated using the TRIzol reagent (Invitrogen). Five μg of total RNA was denatured at 95°C for 5 min in RNA loading buffer (95% v/v formamide, 10 mM EDTA, 0.1% w/v xylene cyanole, 0.1% w/v bromophenol blue) and separated by gel electrophoresis on 4% polyacrylamide/7 M urea gels in 1× TBE buffer. RNA was transferred from the gel onto Hybond-XL nylon membrane (GE Healthcare) by electroblotting. The membrane was cross-linked by UV light, and after prehybridization in Roti-Hybri-Quick buffer (Roth), a [^{32}P]-labeled probe was hybridized at 42°C. Membrane was washed in three subsequent 15-min steps in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.5× SSC/0.1% SDS buffers at 42°C. To detect GcvB, SroC, and 5S rRNA, oligonucleotides JVO-0750, JVO-2907, and JVO-0322 were 5′-end-labeled with [^{32}P]-γ-ATP by T4 polynucleotide kinase (Fermentas) and purified over G25 columns (GE Healthcare). Signals were visualized on Typhoon FLA 7000 (GE Healthcare) and quantified using AIDA software (Raytest).

Western blot analysis

Western blot was performed following a previously published protocol (Urban & Vogel, 2007). Briefly, 1 OD of bacteria culture was collected by centrifugation for 2 min at 16,100 *g* at 4°C, and the pellet was dissolved in 100 μl of 1× protein loading buffer. After heating for 5 min at 95°C, 2 μl of samples were separated on 10% SDS–PAGE. OppA and GroEL were detected as described previously in Sharma *et al* (2007).

In vitro structure probing

In vitro transcripts of *Salmonella* GcvB and SroC were generated with the T7 Megascript Kit (Ambion) using DNA templates amplified with oligonucleotides JVO-0941/JVO-8375 and JVO-7588/JVO-8374, respectively. A total of 20 pmol of RNA was 5′-end-labeled and purified as described previously in Papenfort *et al* (2006). Structure probing was performed using 0.1 pmol of RNA in 10 μl reactions as previously described in Sharma *et al* (2007). 5′-end-labeled RNA was denatured for 1 min at 95°C followed by incubation on ice for 5 min and hybridized with fivefold excess of cold RNA for 10 min at 37°C in the presence of 1 μg of yeast tRNA and 1× structure buffer (10 mM Tris, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$; Ambion). The RNA mixture was digested with a final concentration of 5 mM lead(II) (Fluka) or 0.005 units of RNase T1 (Ambion) for 1.5 or 3 min at 37°C. RNase III cleavage reaction was conducted with 2 units of ShortCut RNase III (NEB) in the presence of 1 mM DTT for 5 min at 37°C. RNase T1 ladder was generated by incubating 0.2 pmol of denatured RNA with 0.1 units of RNase T1 in 1× sequencing buffer (Ambion) for 5 min at 37°C. OH ladder was obtained by incubating 0.2 pmol of RNA in alkaline hydrolysis buffer (Ambion) for 5 min at 95°C. Reactions were stopped by adding 12 μl loading buffer II (95% v/v formamide, 18 mM EDTA, 0.025% SDS, xylene cyanole, bromophenol blue; Ambion). Samples were denatured for 3 min at 95°C and run on 6% polyacrylamide/7 M urea sequencing gels in 1× TBE buffer at 40 W for 90 min. Gels were dried and analyzed using Typhoon FLA 7000 (GE Healthcare) and AIDA software (Raytest).

In vitro RNase E cleavage assay

In vitro transcripts of preSroC were generated with the T7 Megascript Kit (Ambion) using a DNA template generated with oligonucleotides JVO-8373/JVO-8374 and were hydrolyzed by tobacco acid pyrophosphatase (Epicentre). In a 10 μl reaction buffer (25 mM Tris–HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DDT), 100 nM of 5′PPP or 5′P preSroC were bound with 100 nM Hfq for 10 min at 30°C and incubated with 100 nM of RNase E (1–529). Reactions were stopped by the addition of equal volume of RNA loading buffer (95% v/v formamide, 10 mM EDTA, 0.1% w/v xylene cyanole, 0.1% w/v bromophenol blue) and heating for 5 min at 95°C. Processing products were separated by gel electrophoresis on 4% polyacrylamide/7 M urea gels and were visualized by Northern blot as described above.

Fluorescence monitoring during growth

Salmonella strains were precultured in 2 ml of 0.4% casamino acids medium overnight. On a 96-well plate, 100 μl of 0.4% tryptone or casamino acids medium was inoculated by 1/100 of preculture. The plate was incubated at 37°C with shaking at 3-mm amplitude with monitoring GFP fluorescence (excitation at 485 ± 20 nm, emission at 535 ± 25 nm) and optical density (absorbance at 595 ± 10 nm) every 15 min using an Infinite 200 Pro machine (Tecan). GFP fluorescence was normalized by OD_{595}, and that of GFP-negative cells grown under the same condition was subtracted.

Flow cytometry

Salmonella translational fusion strains were grown in 0.4% casamino acids medium to exponential phase, and cells were fixed with 4% paraformaldehyde and resuspended in 1× PBS buffer (pH 7.4). GFP fluorescense intensity was quantified for 50,000 events by flow cytometry using FACSCalibur (BD Biosciences). Data were analyzed by Cyflogic software (CyFlo Ltd).

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

MM and JV designed the study; MM and YC performed the experiments; MM, YC, and JV analyzed the data; MM and JV wrote the paper with input from YC.

Conflict of interest

The authors declare that they have no conflict of interest.

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