RESEARCH ARTICLE

Figures and figure supplements

Gene autoregulation by 3′ UTR-derived bacterial small RNAs

Mona Hoyos *et al*

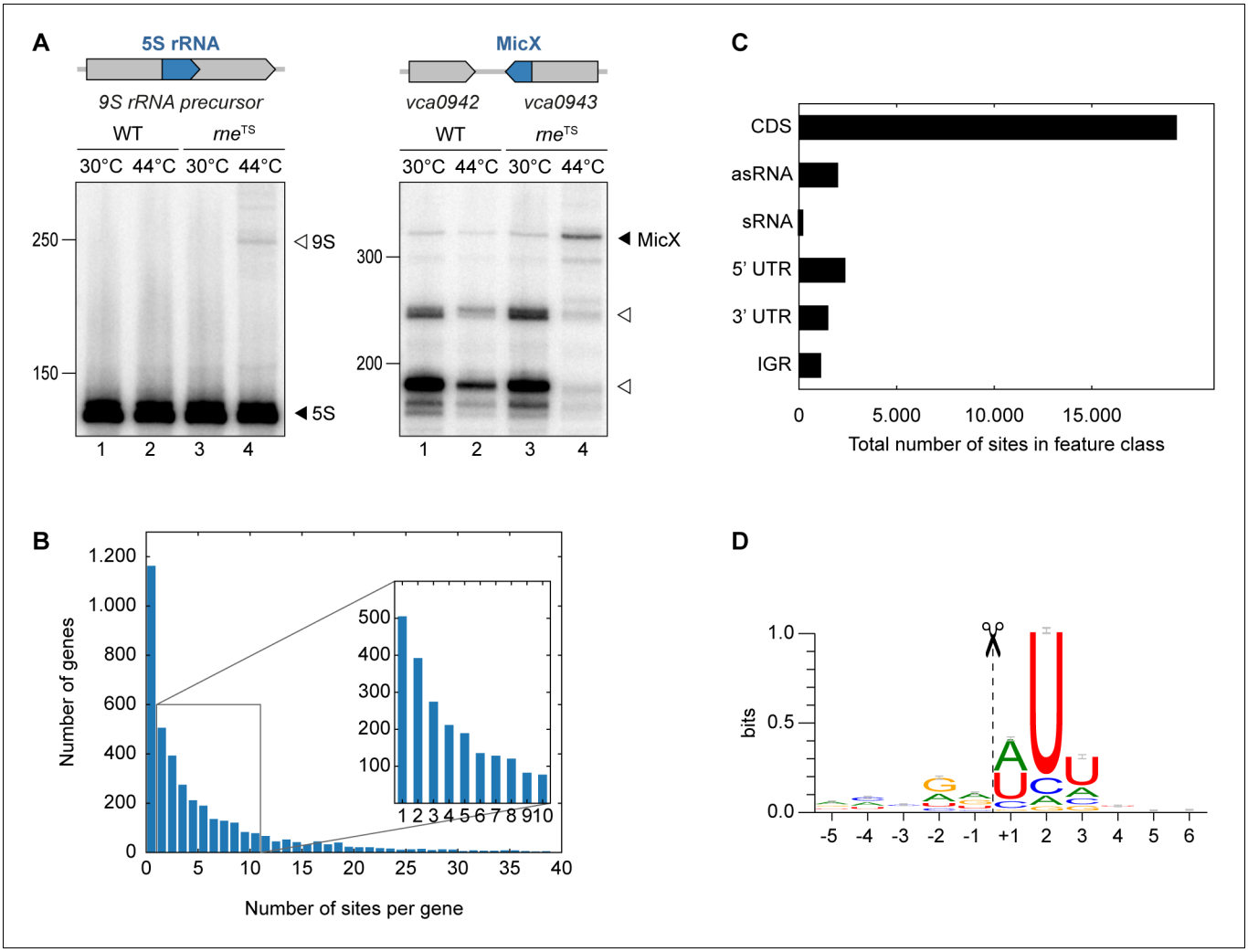

Figure 1. TIER-seq analysis of *V. cholerae*. (**A**) *V. cholerae* wild-type and *rne*^{TS} strains were grown at 30˚C to stationary phase (OD_{600} of 2.0). Cultures were divided in half and continuously grown at either 30˚C or 44˚C for 60 min. Cleavage patterns of 5S rRNA and 3' UTR-derived MicX were analyzed on Northern blots. Closed triangles indicate mature 5S or full-length MicX, open triangles indicate the 9S precursor or MicX processing products. (**B, C, D**) Biological triplicates of *V. cholerae* wild-type and *rne*^{TS} strains were grown at 30˚C to late exponential phase (OD_{600} of 1.0). Cultures were divided in half and continuously grown at either 30˚C or 44˚C for 60 min. Isolated RNA was subjected to RNA-seq and RNase E cleavage sites were determined as described in the materials and methods section. (**B**) Number of cleavage sites detected per gene. (**C**) Classification of RNase E sites by their genomic location. (**D**) The RNase E consensus motif based on all detected cleavage sites. The total height of the error bar is twice the small sample correction.

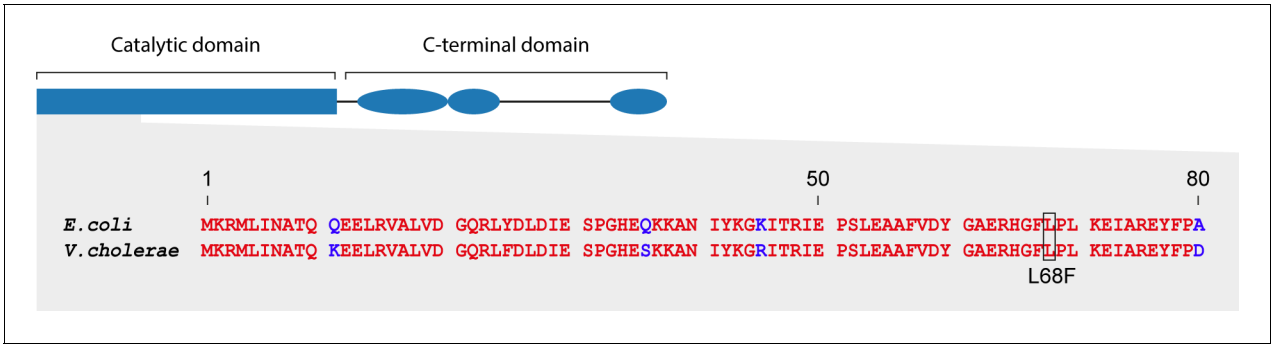

Figure 1—figure supplement 1. Conservation of RNase E between *E. coli* and *V. cholerae*. Sequence alignment of the first 80 N-terminal amino acids of RNase E from *E. coli* and *V. cholerae*. The temperature-sensitive *rne-3071* mutation changing a leucine to phenylalanine at position 68 is indicated.

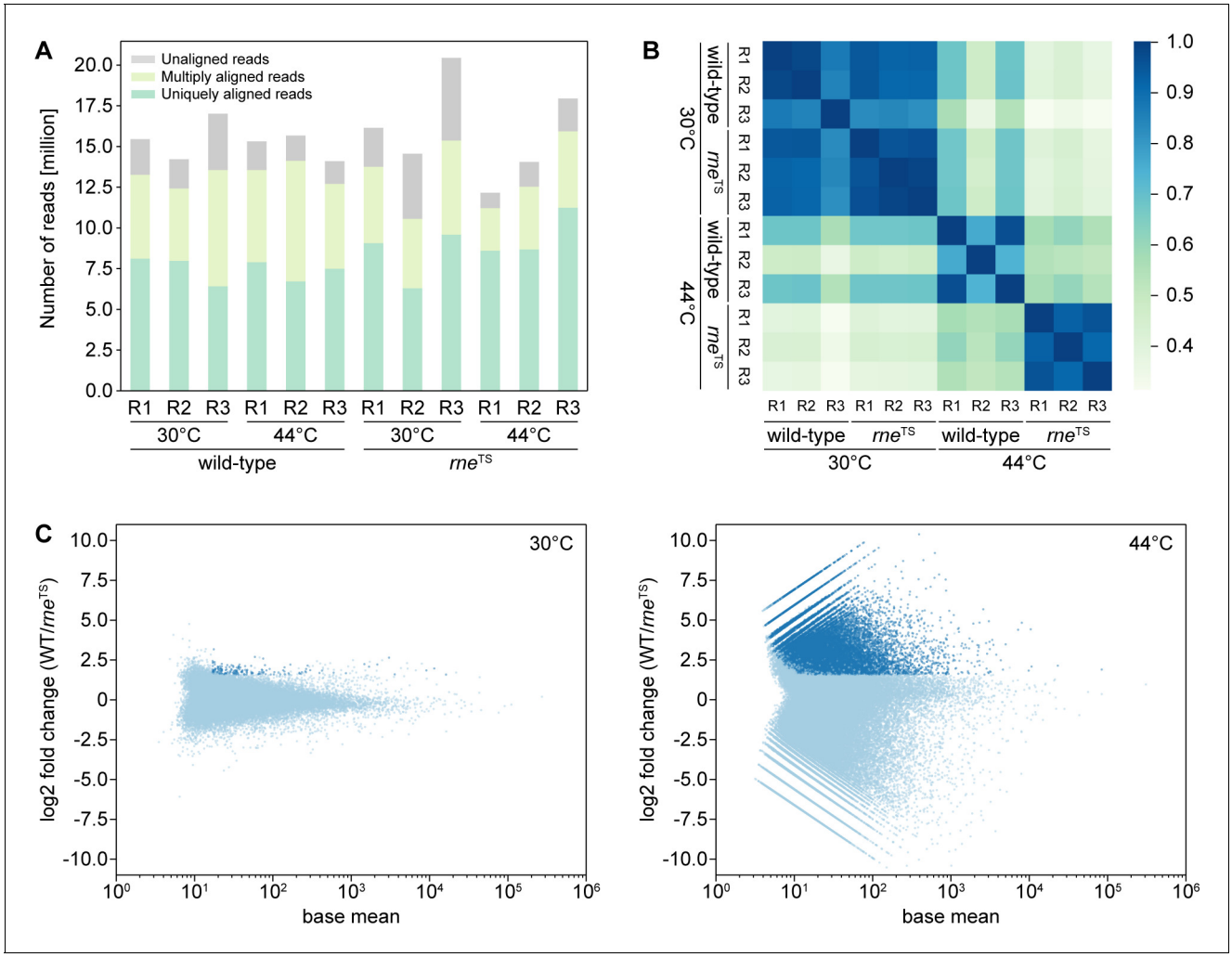

Figure 1—figure supplement 2. TIER-Seq read mapping statistics. TIER-seq was performed as described in ***Figure 1***. (**A**) Total number of raw cDNA reads obtained for all samples, showing the fractions of uniquely aligned reads (dark green), multiply aligned reads (light green) or unaligned reads (grey). R1-R3 indicate the biological triplicates. (**B**) Similarity of 5′ ends profiles of uniquely aligned reads, obtained by comparison of all detected 5′ end positions between the respective cDNA libraries. Colored rectangles show the Pearson correlation coefficient corresponding to the scale bar on the right. (**C**) Global analysis of 5′ profiles at the permissive (30°C, left) and non-permissive temperature (44°C, right). Plots show average coverage levels of 5′ read ends and the respective $\log_2$ fold change in wild-type samples compared to *rne*^{TS} samples. Candidate RNase E cleavage sites were determined as positions enriched ≥3 fold in the wild-type (p-value<0.05) and are shown in dark blue.

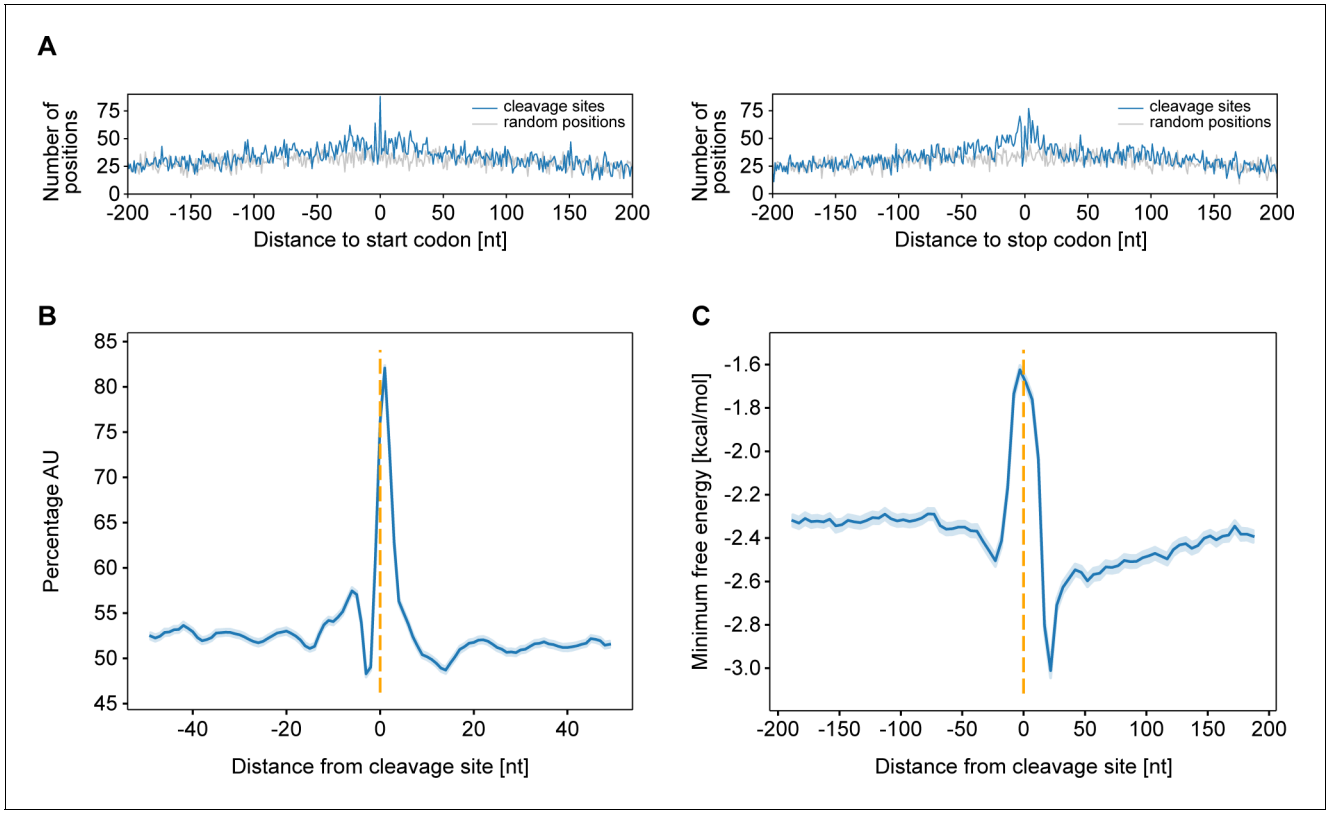

Figure 1—figure supplement 3. Position and characteristics of RNase E cleavage sites. TIER-seq was performed as described in ***Figure 1***. (**A**) Frequency of RNase E sites or the same number of randomly selected genome positions dependent on their relative position to start codons (left) and stop codons (right). (**B**) AU content around the RNase E cleavage sites. The 95% confidence interval is indicated in light blue. (**C**) Degree of RNA structure around RNase E cleavage sites. Minimal folding energy (MFE) was calculated in five nt steps for each 25 nt window. The 95% confidence interval is indicated in light blue.

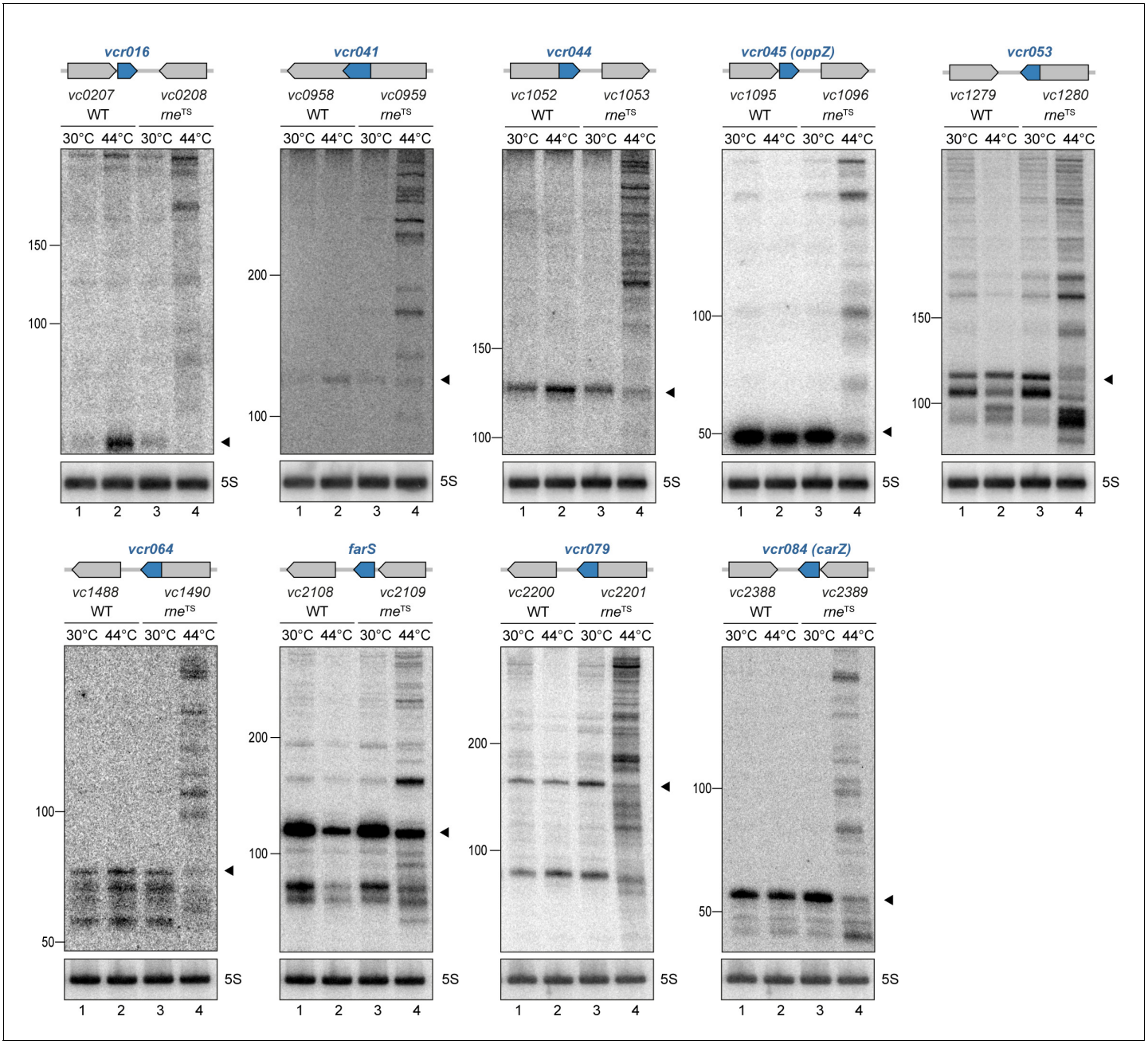

Figure 1—figure supplement 4. RNase E-mediated maturation of sRNAs from 3′ UTRs. *V. cholerae* wild-type and *rne*^{TS} strains were grown at 30˚C to stationary phase (OD_{600} of 2.0). Cultures were divided in half and continuously grown at either 30˚C or 44˚C for 30 min. Cleavage patterns of 3′ UTR-derived sRNAs were monitored on Northern blots. The genomic locations and relative orientations are shown above the gels. Genes are shown in gray; sRNAs are shown in blue. Filled triangles indicate the size of mature sRNAs. 5S rRNA served as loading control.

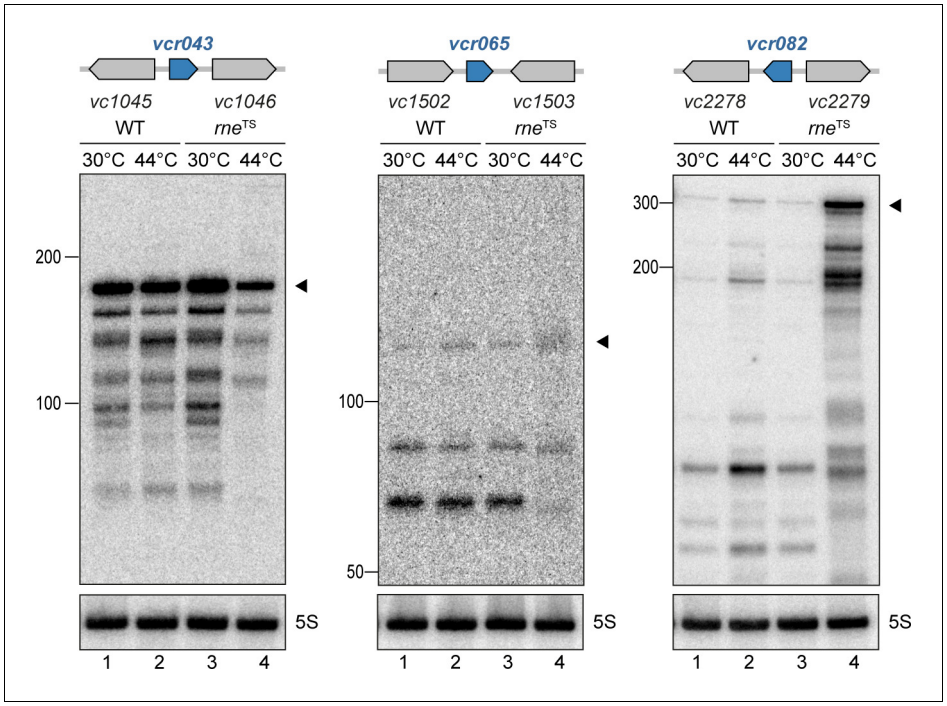

Figure 1—figure supplement 5. RNase E-mediated maturation of sRNAs from IGRs. *V. cholerae* wild-type and *rne*^{TS} strains were grown at 30˚C to stationary phase (OD_{600} of 2.0). Cultures were divided in half and continuously grown at either 30˚C or 44˚C for 30 min. Cleavage patterns of intergenic sRNAs were monitored on Northern blots. The genomic locations and relative orientations are shown above the gels. Genes are shown in gray; sRNAs are shown in blue. Filled triangles indicate the size of unprocessed sRNAs. 5S rRNA served as loading control.

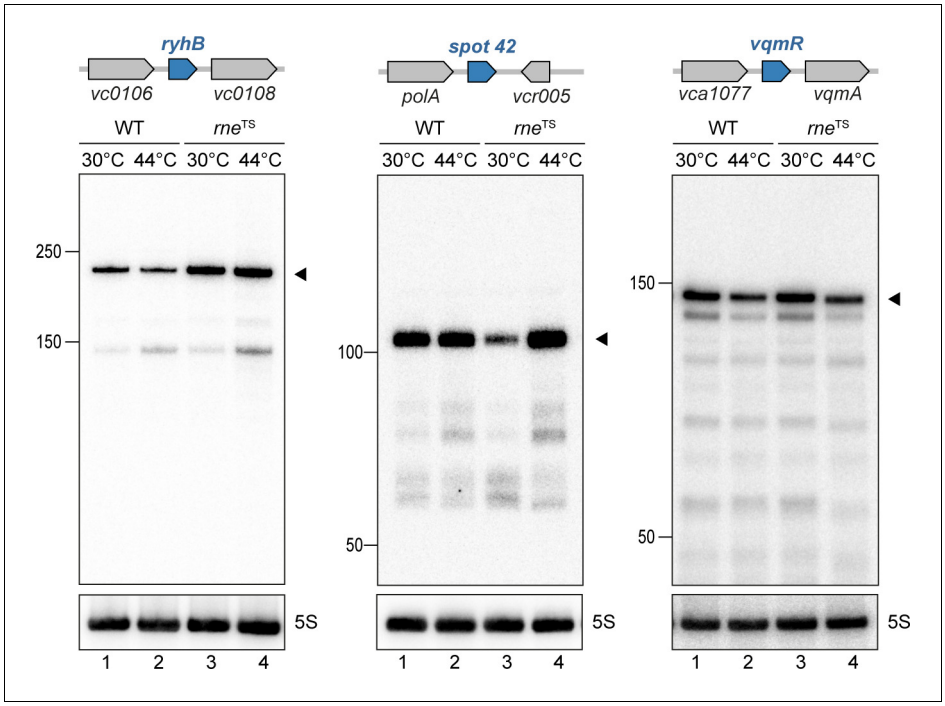

Figure 1—figure supplement 6. Expression of RNase E-independent sRNAs. *V. cholerae* wild-type and *rne*^{TS} strains were grown at 30˚C to early exponential phase (OD_{600} of 0.2; RyhB and Spot 42) or to stationary phase (OD_{600} of 2.0; VqmR). Cultures were divided in half and continuously grown at either 30°C or 44°C for 30 min. Cleavage patterns of sRNAs without detectable RNase E cleavage sites were monitored on Northern blots. The genomic locations and relative orientations are shown above the gels. Genes are shown in gray; sRNAs are shown in blue. Filled triangles indicate the size of full-length sRNAs. 5S rRNA served as loading control.

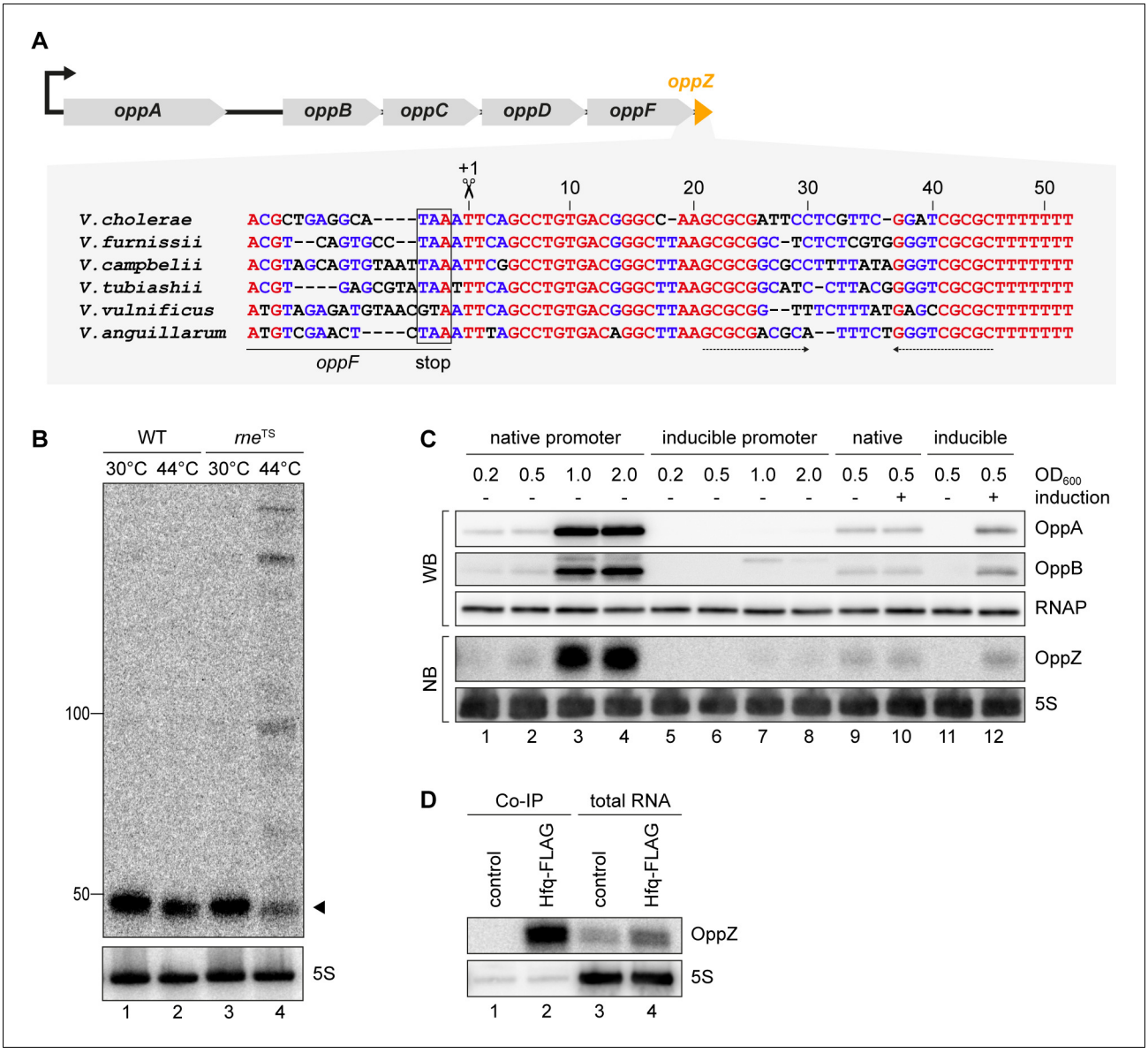

Figure 2. OppZ is produced from the *oppABCDF* 3' end. (**A**) Top: Genomic organization of *oppABCDF* and *oppZ*. Bottom: Alignment of *oppZ* sequences, including the last codons of *oppF*, from various *Vibrio* species. The *oppF* stop codon, the RNase E cleavage site and the Rho-independent terminator are indicated. (**B**) *V. cholerae* wild-type and rne^{TS} strains were grown at 30°C to stationary phase (OD_{600} of 2.0). Cultures were divided in half and continuously grown at either 30°C or 44°C for 30 min. OppZ synthesis was analyzed by Northern blot with 5S rRNA as loading control. The triangle indicates the size of mature OppZ. (**C**) Protein and RNA samples were obtained from *V. cholerae oppA*::3XFLAG *oppB*::3XFLAG strains carrying either the native *oppA* promoter or the inducible pBAD promoter upstream of *oppA*. Samples were collected at the indicated OD_{600} and tested for OppA and OppB production by Western blot and for OppZ expression by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively. Lanes 1–8: Growth without L-arabinose. Lanes 9–12: Growth with either H_2O (-) or L-arabinose (+) (0.2% final conc.). (**D**) *V. cholerae* wild-type (control) and *hfq*::3XFLAG (Hfq-FLAG) strains were grown to stationary phase (OD_{600} of 2.0), lysed, and subjected to immunoprecipitation using the anti-FLAG antibody. RNA samples of lysate (total RNA) and co-immunoprecipitated fractions were analyzed on Northern blots. 5S rRNA served as loading control.

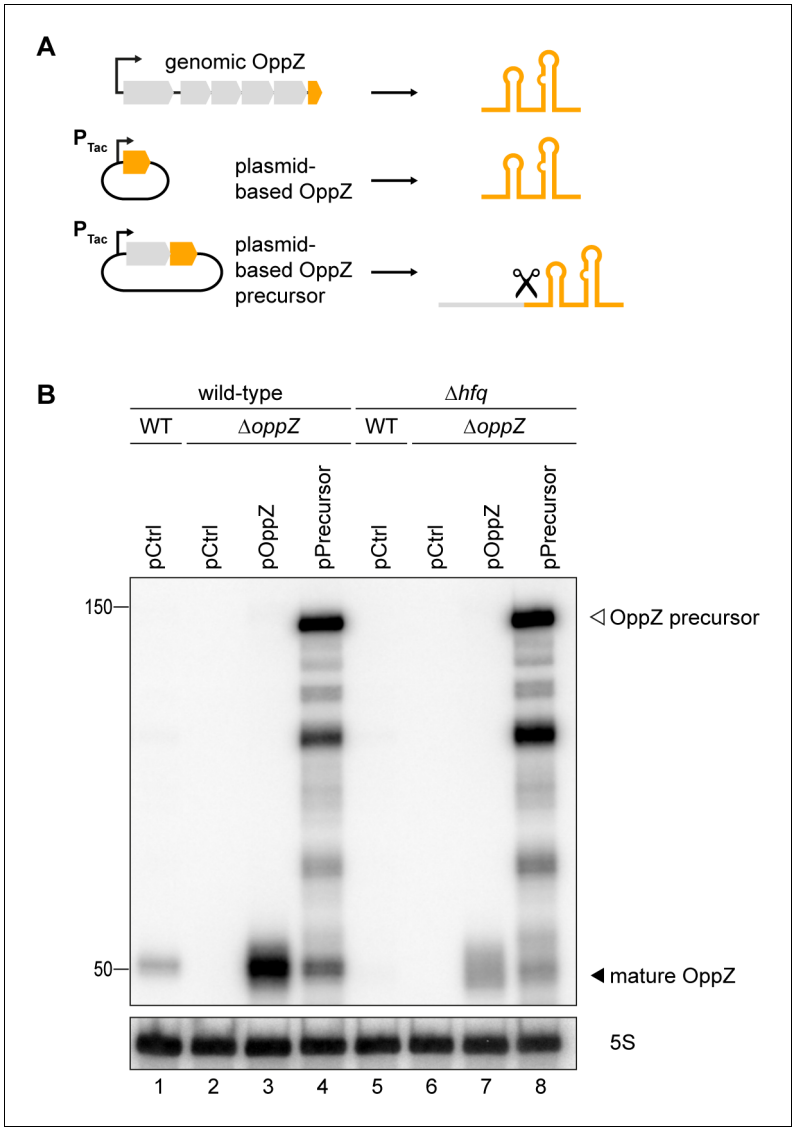

Figure 2—figure supplement 1. Hfq dependence of OppZ processing. (**A**) Schematic description of the analyzed OppZ variants. OppZ was produced natively from the genomic *opp* locus, expressed as mature sRNA from a plasmid (pOppZ) or cleaved from a plasmid-encoded precursor transcript including the 3' end of *oppF* (pPrecursor). Expression of both plasmid-based *oppZ* variants was driven by a constitutive promoter. (**B**) *V. cholerae* wild-type, Δ*oppZ*, Δ*hfq* or Δ*hfq* Δ*oppZ* strains carrying *oppA*::3XFLAG *oppB*::3XFLAG genes and a control plasmid or the indicated OppZ expression plasmid were grown to stationary phase (OD_{600} of 2.0). RNA samples were collected and OppZ processing was analyzed by Northern blot. 5S rRNA served as loading control.

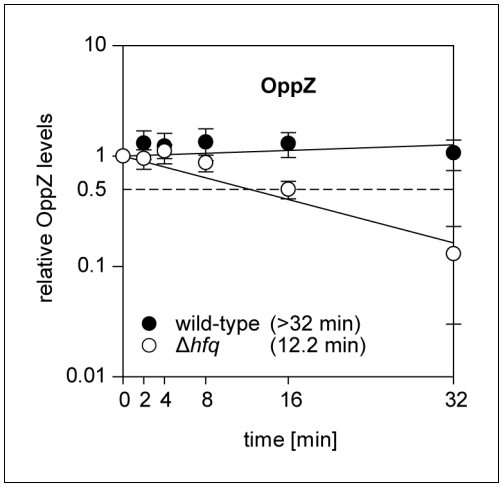

Figure 2—figure supplement 2. Hfq dependence of OppZ stability. *V. cholerae* wild-type and Δ*hfq* strains were grown to early stationary phase (OD_{600} of 1.5) and treated with rifampicin to terminate transcription. RNA samples were obtained at the indicated time points and OppZ transcript levels were monitored by Northern blot and normalized to 5S rRNA levels as loading control. Error bars represent the SD of three biological replicates.

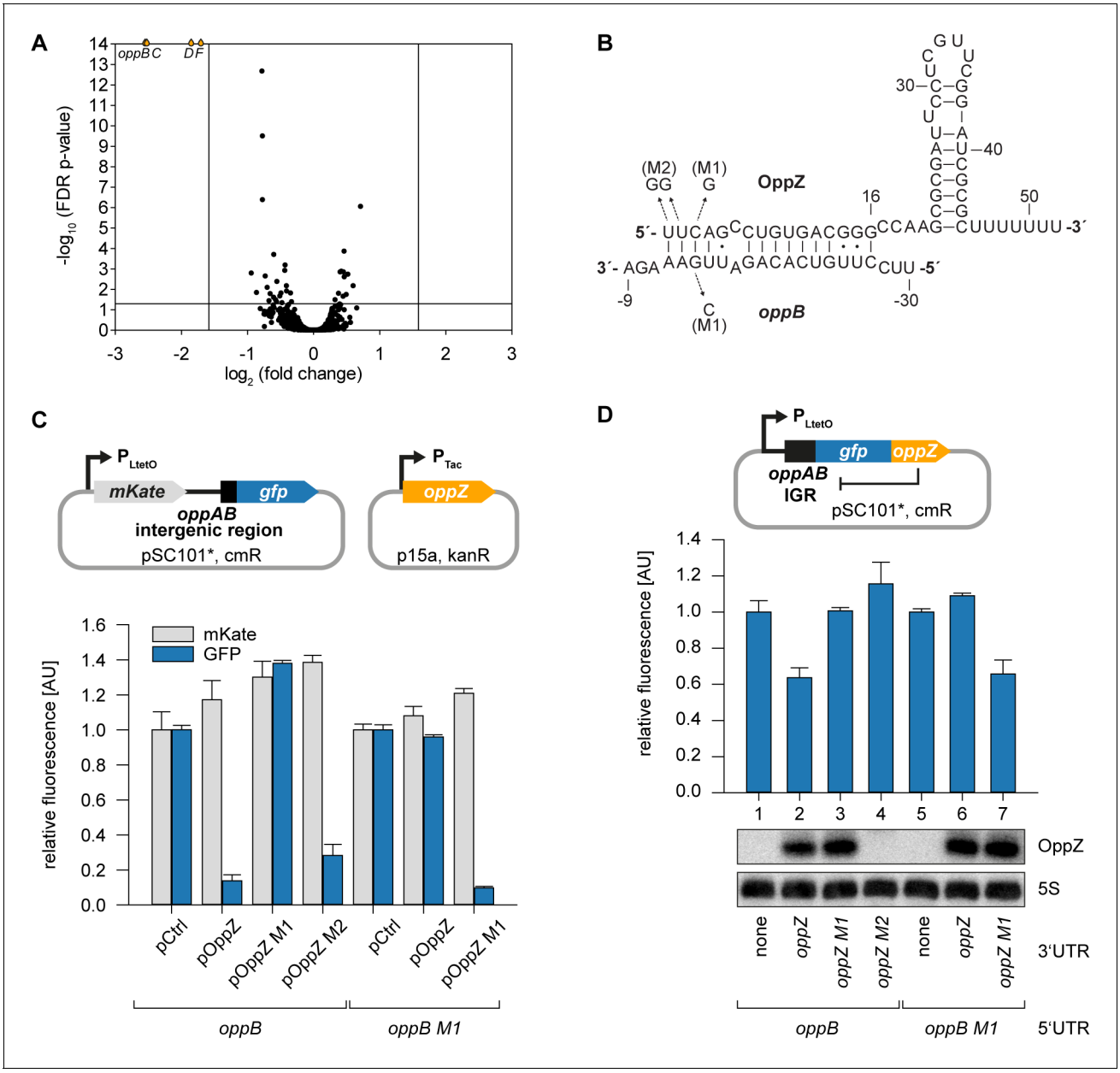

Figure 3. Feedback autoregulation at the suboperonic level. (**A**) Volcano plot of genome-wide transcript changes in response to inducible OppZ over-expression. Lines indicate cut-offs for differentially regulated genes at 3-fold regulation and FDR-adjusted p-value≤0.05. Genes with an FDR-adjusted p-value<10^{-14} are indicated as droplets at the top border of the graph. (**B**) Predicted OppZ secondary structure and base-pairing to *oppB*. Arrows indicate the mutations tested in (**C**) and (**D**). (**C**) *E. coli* strains carrying a translational reporter plasmid with the *oppAB* intergenic region placed between *mKate2* and *gfp* were co-transformed with a control plasmid or the indicated OppZ expression plasmids. Transcription of the reporter and *oppZ* were driven by constitutive promoters. Cells were grown to OD_{600} = 1.0 and fluorophore production was measured. mKate and GFP levels of strains carrying the control plasmid were set to 1. Error bars represent the SD of three biological replicates. (**D**) Single-plasmid regulation was measured by inserting the indicated *oppZ* variant into the 3' UTR of a translational *oppB::gfp* fusion. Expression was driven from a constitutive promoter. *E. coli* strains carrying the respective plasmids were grown to OD_{600} = 1.0 and GFP production was measured. Fluorophore levels from control fusions without an sRNA gene were set to one and error bars represent the SD of three biological replicates. OppZ expression was tested by Northern blot; 5S rRNA served as loading control.

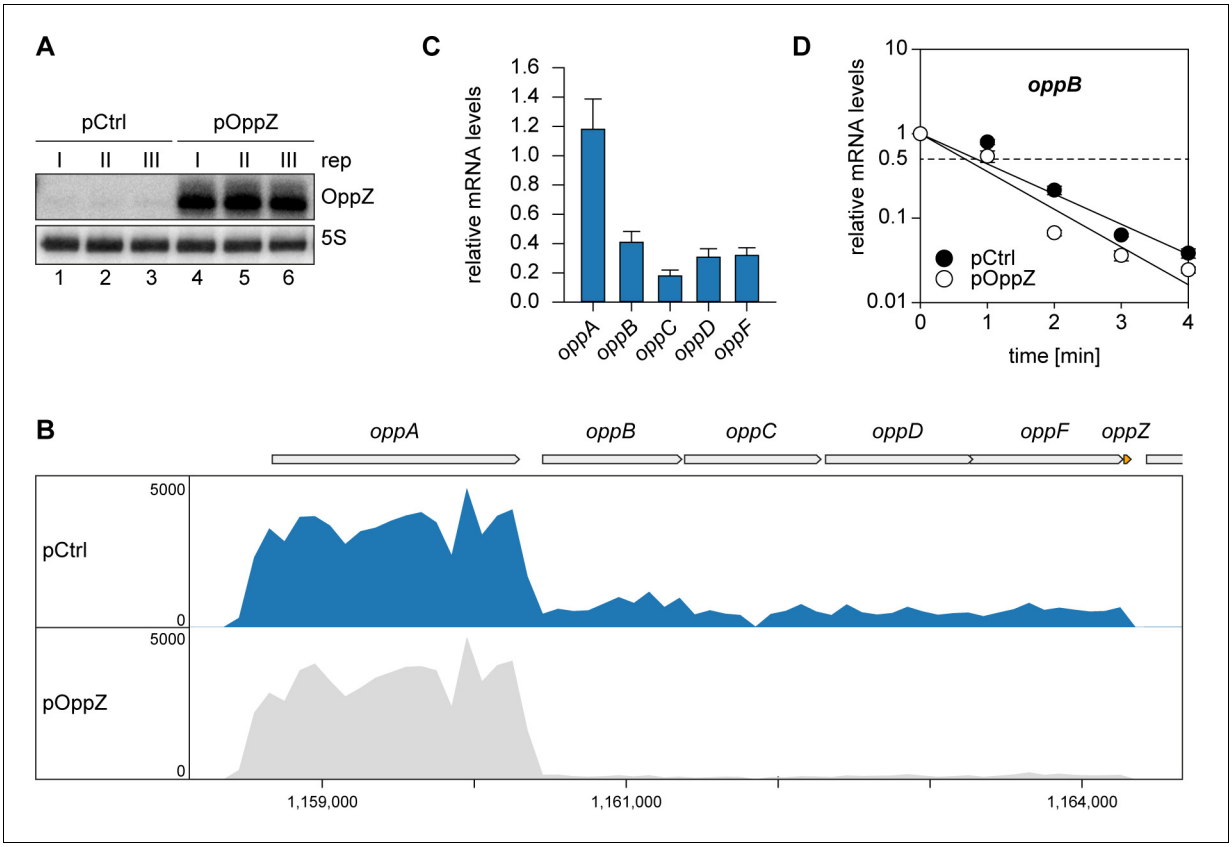

Figure 3—figure supplement 1. Pulse expression of OppZ reduces *oppBCDF* transcript levels. (**A**) *V. cholerae* carrying pBAD1K-*oppZ* (pOppZ) or a control plasmid (pCtrl) were grown in biological triplicates to exponential phase (OD_{600} of 0.5) and *oppZ* expression was induced by L-arabinose (0.2% final conc.). RNA samples were collected after 15 min and analyzed for OppZ levels by Northern blot; 5S rRNA served as loading control. (**B**) Samples from (**A**) were subjected to RNA-seq and average coverage of the *opp* operon is shown for one representative replicate. (**C**) *V. cholerae* Δ*oppZ* carrying pBAD1K-*oppZ* or a control plasmid were grown to late exponential phase (OD_{600} of 1.0) and *oppZ* expression was induced by L-arabinose (0.2% final conc.) for 15 min. mRNA levels of *oppABCDF* were analyzed by qRT-PCR. Bars show mRNA levels upon OppZ induction compared to the control; error bars represent the SD of three biological replicates. (**D**) *V. cholerae* Δ*oppZ* strains carrying either pBAD1K-ctrl (pCtrl) or pBAD1K-*oppZ* (pOppZ) were grown to late exponential phase (OD_{600} of 1.0) and treated with L-arabinose (0.2% final conc.) to induce sRNA expression. After 15 min of induction, rifampicin was added to terminate transcription. RNA samples were obtained at the indicated time points and *oppB* transcript levels were monitored by qRT-PCR. Error bars represent the SD of three biological replicates.

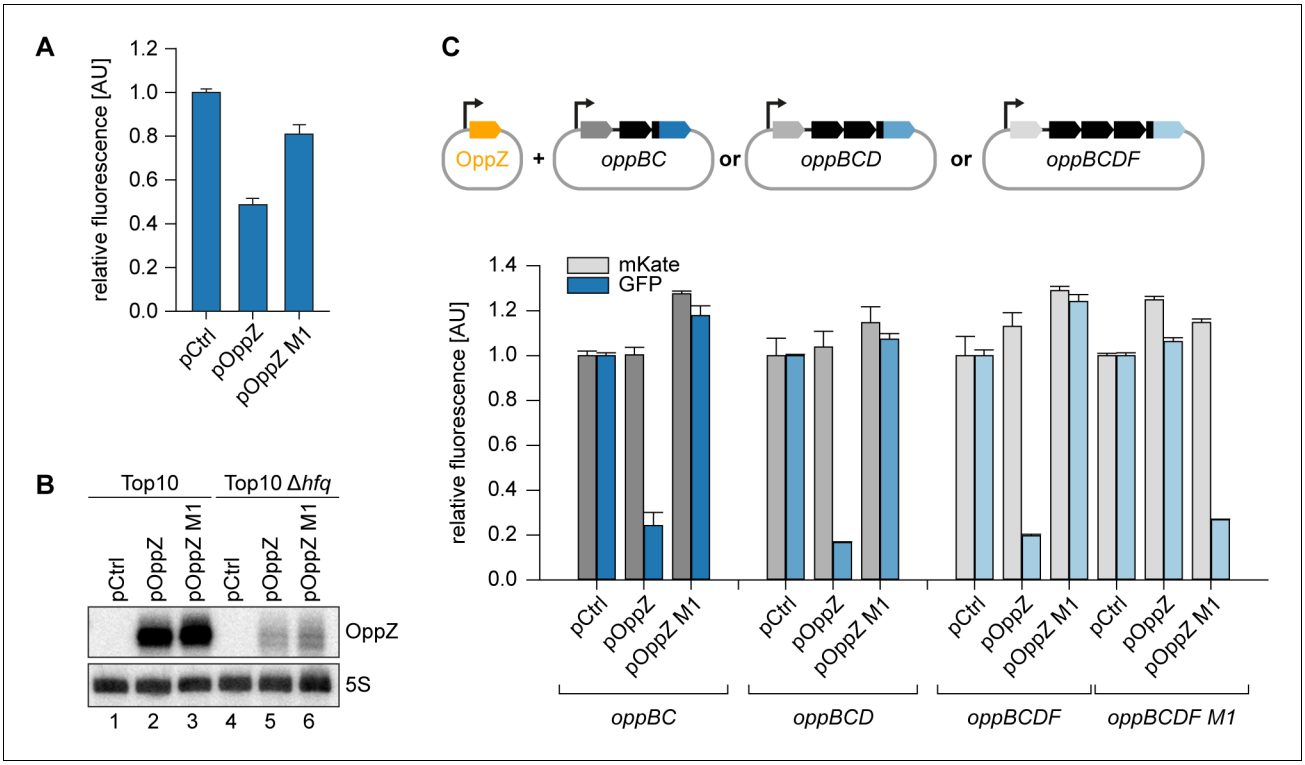

Figure 3—figure supplement 2. Hfq-dependent, post-transcriptional repression of OppBCDF by OppZ. (**A**) *E. coli* Δ*hfq* strains carrying the translational *oppB-gfp* reporter plasmid and either a control plasmid or the indicated OppZ expression plasmids were grown to OD_{600} = 1.0 and fluorophore production was measured. GFP levels of the control strain were set to 1. Error bars represent the SD of three biological replicates. (**B**) *E. coli* wild-type or Δ*hfq* strains carrying the translational *oppB-gfp* reporter plasmid and either a control plasmid or the indicated OppZ expression plasmids were grown to OD_{600} = 1.0. RNA samples were analyzed for OppZ levels by Northern blot; 5S rRNA served as loading control. (**C**) *E. coli* strains carrying translational reporter plasmids with the indicated parts of the *opp* operon placed between *mKate2* and *gfp* were co-transformed with a control plasmid or the respective OppZ expression plasmids. Transcription of the reporter and *oppZ* were driven by constitutive promoters. Cells were grown to OD_{600} = 1.0 and fluorophore production was measured. mKate and GFP levels of strains carrying the control plasmid were set to 1. Error bars represent the SD of three biological replicates.

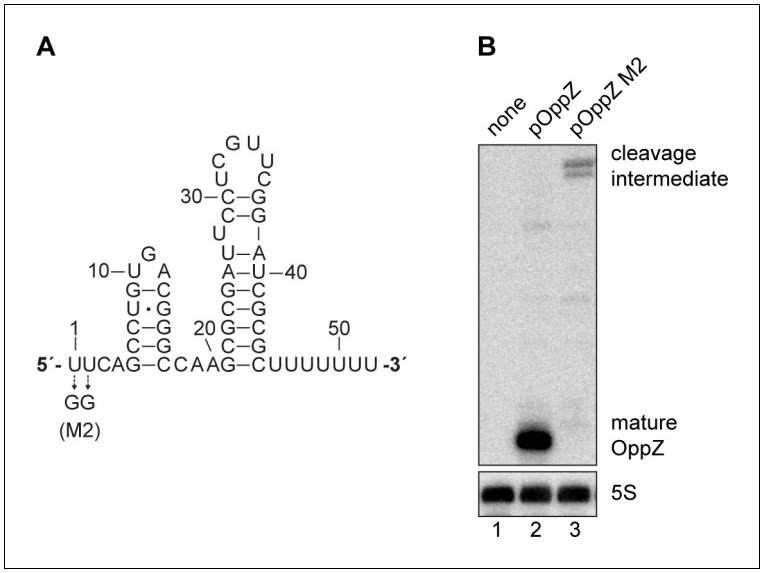

Figure 3—figure supplement 3. Mutational analysis of the RNase E site in *oppZ*. (**A**) Predicted structure of the OppZ sRNA. The M2 mutation blocking cleavage by RNase E is indicated. (**B**) *E. coli* strains carrying the empty pXG10-SF plasmid or derivatives with the indicated *oppZ* gene in the 3' UTR of *gfp* were grown to OD_{600} = 1.0. RNA samples were analyzed for OppZ processing by Northern blot; 5S rRNA served as loading control.

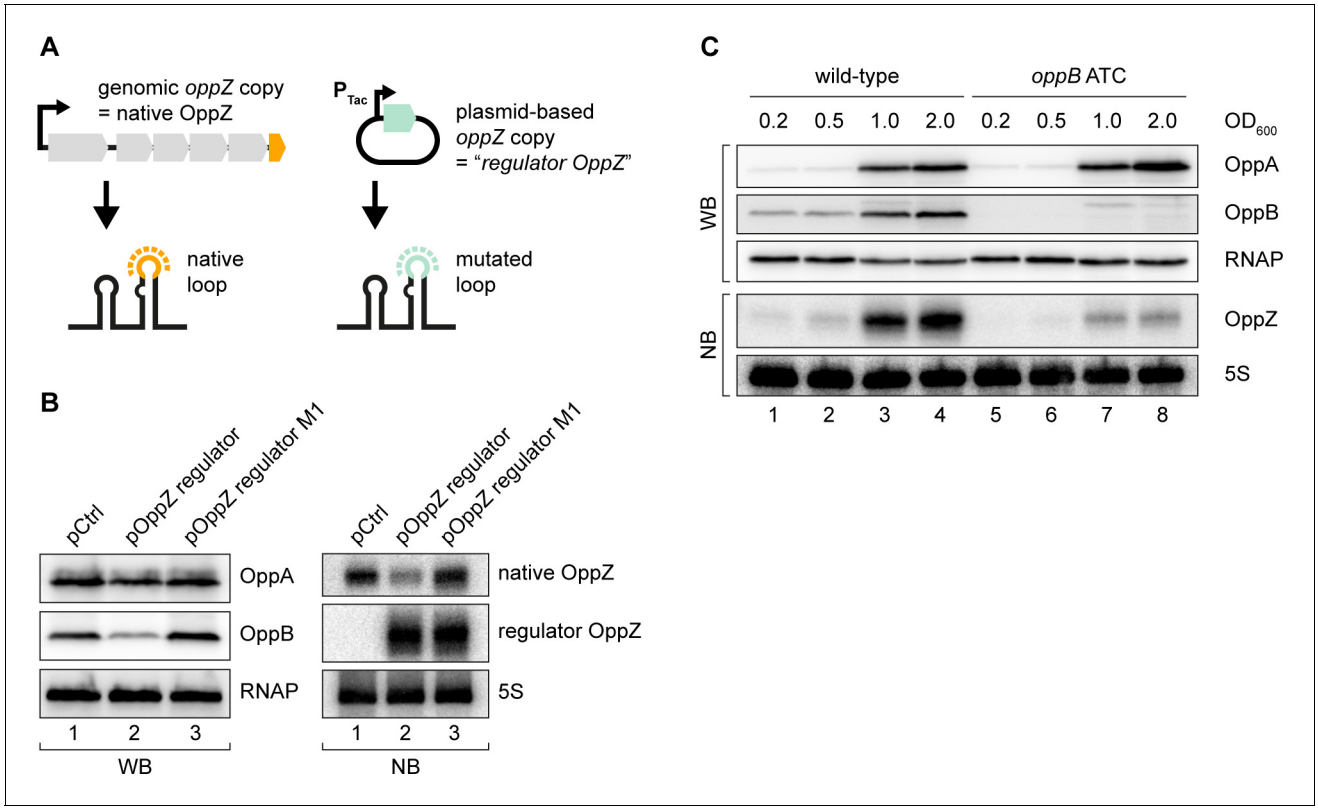

Figure 4. Translational control of OppZ synthesis. (**A**) Schematic of the analyzed OppZ variants containing the native stem loop sequence (produced from the genomic *oppZ* locus) or a mutated stem loop sequence ('*regulator OppZ*' produced from a plasmid-based constitutive promoter). (**B**) *V. cholerae oppA*::3XFLAG *oppB*::3XFLAG carrying a control plasmid (pCMW-1) or a plasmid expressing *regulator OppZ* (pMD194, pMD195) were grown to stationary phase (OD_{600} of 2.0). OppA and OppB production were tested by Western blot and expression of native OppZ and regulator OppZ was monitored on Northern blot using oligonucleotides binding to the respective loop sequence variants. RNAP and 5S rRNA served as loading controls for Western blot and Northern blot, respectively. (**C**) The *oppB* start codon was mutated to ATC in an *oppA*::3XFLAG *oppB*::3XFLAG background. *V. cholerae* strains with wild-type or mutated *oppB* start codon were grown in LB medium. Protein and RNA samples were collected at the indicated OD_{600} and tested for OppA and OppB production by Western blot and for OppZ expression by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively.

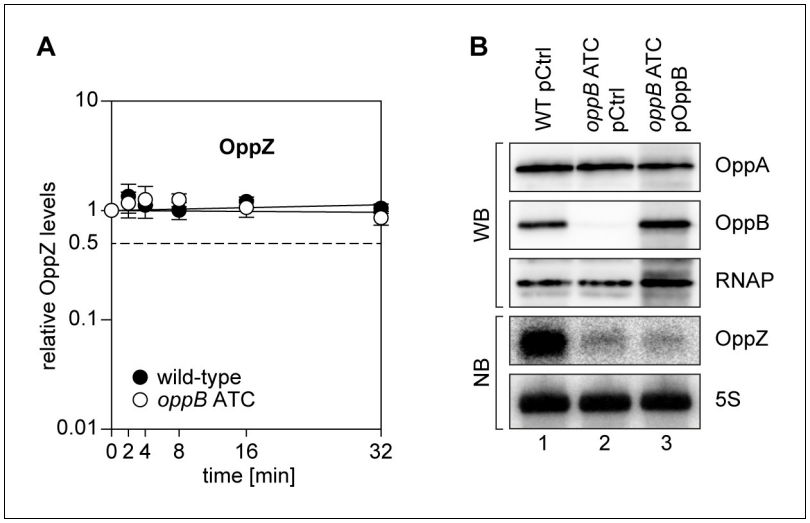

Figure 4—figure supplement 1. Translational control of OppZ synthesis. (**A**) *V. cholerae* wild-type and *oppB* ATC strains were grown to stationary phase (OD_{600} of 2.0) and treated with rifampicin to terminate transcription. RNA samples were obtained at the indicated time points and OppZ transcript levels were monitored by Northern blot and normalized to 5S rRNA levels as loading control. Error bars represent the SD of three biological replicates. (**B**) *V. cholerae* wild-type and *oppB* ATC strains carrying either a control plasmid (pCtrl) or an inducible *oppB* complementation plasmid (pOppB) were grown to late exponential phase (OD_{600} of 1.0) and *oppB* expression was induced by the addition of L-arabinose (0.2% final conc.). Protein and RNA samples were obtained after 60 min and tested for OppA and OppB production by Western blot and for OppZ expression by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively.

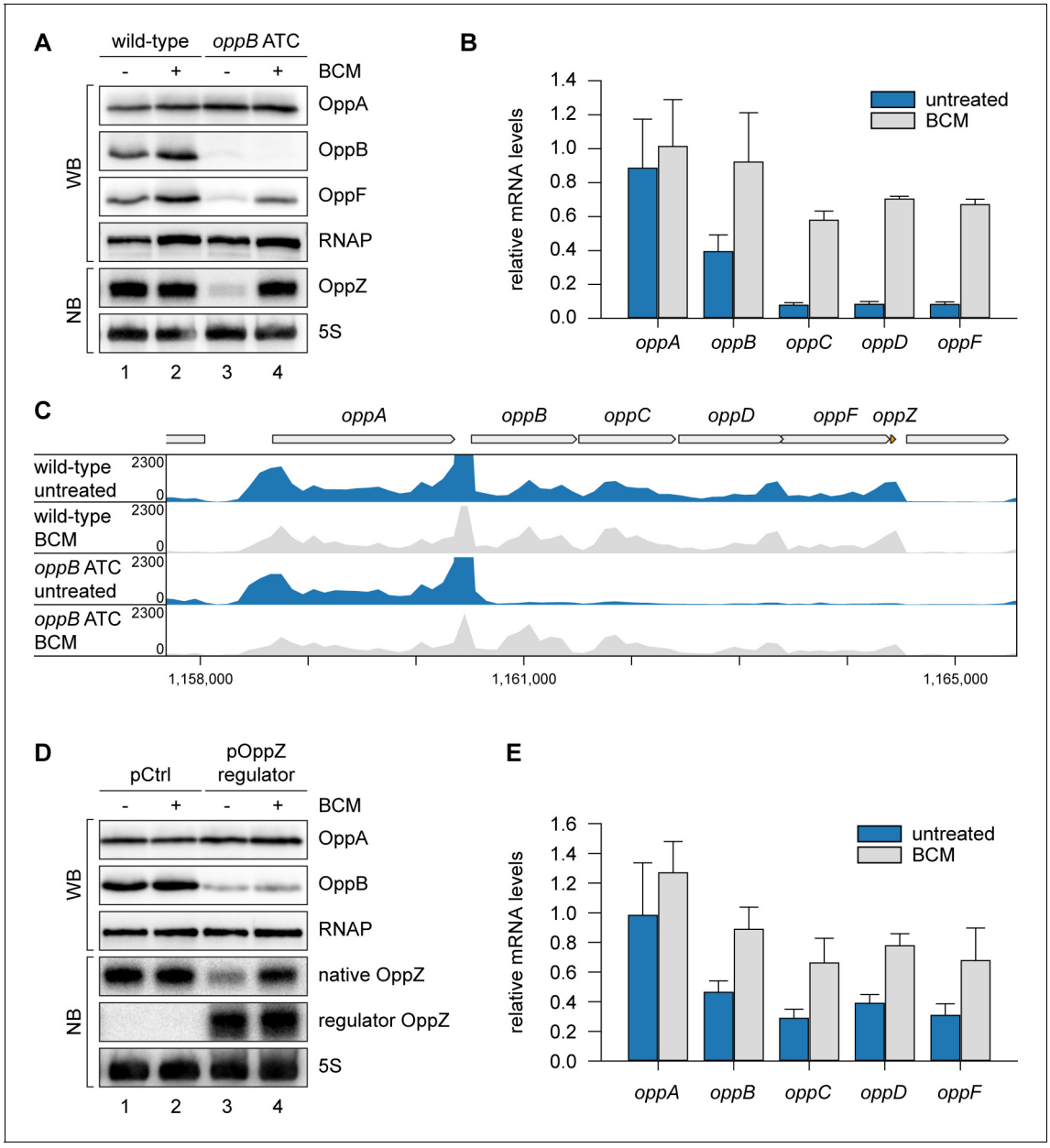

Figure 5. OppZ promotes transcription termination through Rho. (**A**) *V. cholerae oppA*::3XFLAG *oppB*::3XFLAG *oppF*::3XFLAG strains with wild-type or mutated *oppB* start codon were grown to early stationary phase (OD_{600} of 1.5). Cultures were divided in half and treated with either H_2O or BCM (25 µg/ml final conc.) for 2 hr before protein and RNA samples were collected. OppA, OppB and OppF production were tested by Western blot and OppZ expression was monitored by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively. (**B**) Biological triplicates of *V. cholerae oppA*::3XFLAG *oppB*::3XFLAG strains with wild-type or mutated *oppB* start codon were treated with BCM as described in (**A**). *oppABCDF* expression in the *oppB* start codon mutant compared to the wild-type control was analyzed by qRT-PCR. Error bars represent the SD of three biological replicates. (**C**) Triplicate samples from (**B**) were subjected to Term-seq and average coverage of the *opp* operon is shown for one representative replicate. The coverage cut-off was set at the maximum coverage of annotated genes. (**D**) *V. cholerae oppA*::3XFLAG *oppB*::3XFLAG strains carrying a control plasmid (pMD397) or a plasmid expressing *regulator OppZ* (pMD398) were treated with BCM as described in (**A**). OppA and OppB production were tested by Western blot and expression of native OppZ and regulator OppZ was monitored on Northern blot using oligonucleotides binding to the respective loop sequence variants. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively. (**E**) Levels of *oppABCDF* in the experiment described in (**D**) were analyzed by qRT-PCR. Error bars represent the SD of three biological replicates.

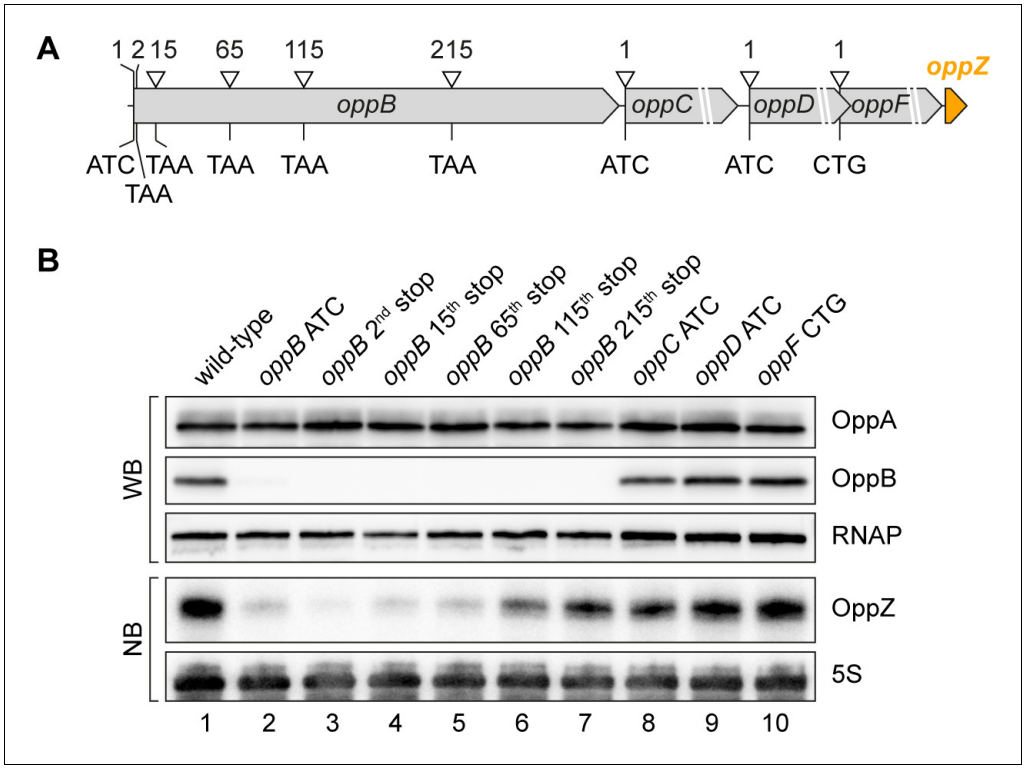

Figure 6. Influence of OppBCDF translation on OppZ expression. (**A**) The depicted mutations were individually inserted into the *opp* locus to inactivate the start codons of *oppB*, *oppC*, *oppD* or *oppF* or to insert STOP codons at the positions 2, 15, 65, 115 or 215 of *oppB*. (**B**) *V. cholerae oppA*::3XFLAG *oppB*::3XFLAG strains with the described *opp* mutations were grown: wild-type (lane 1), the *oppB* start codon mutated (lane 2), a STOP codon inserted at the 2nd, 15th, 65th, 115th or 215th codon of *oppB* (lanes 3–7) or mutated start codons of *oppC*, *oppD* or *oppF* (lanes 8–10). At stationary phase (OD_{600} of 2.0), protein and RNA samples were collected and tested for OppA and OppB production by Western blot and for OppZ expression by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively.

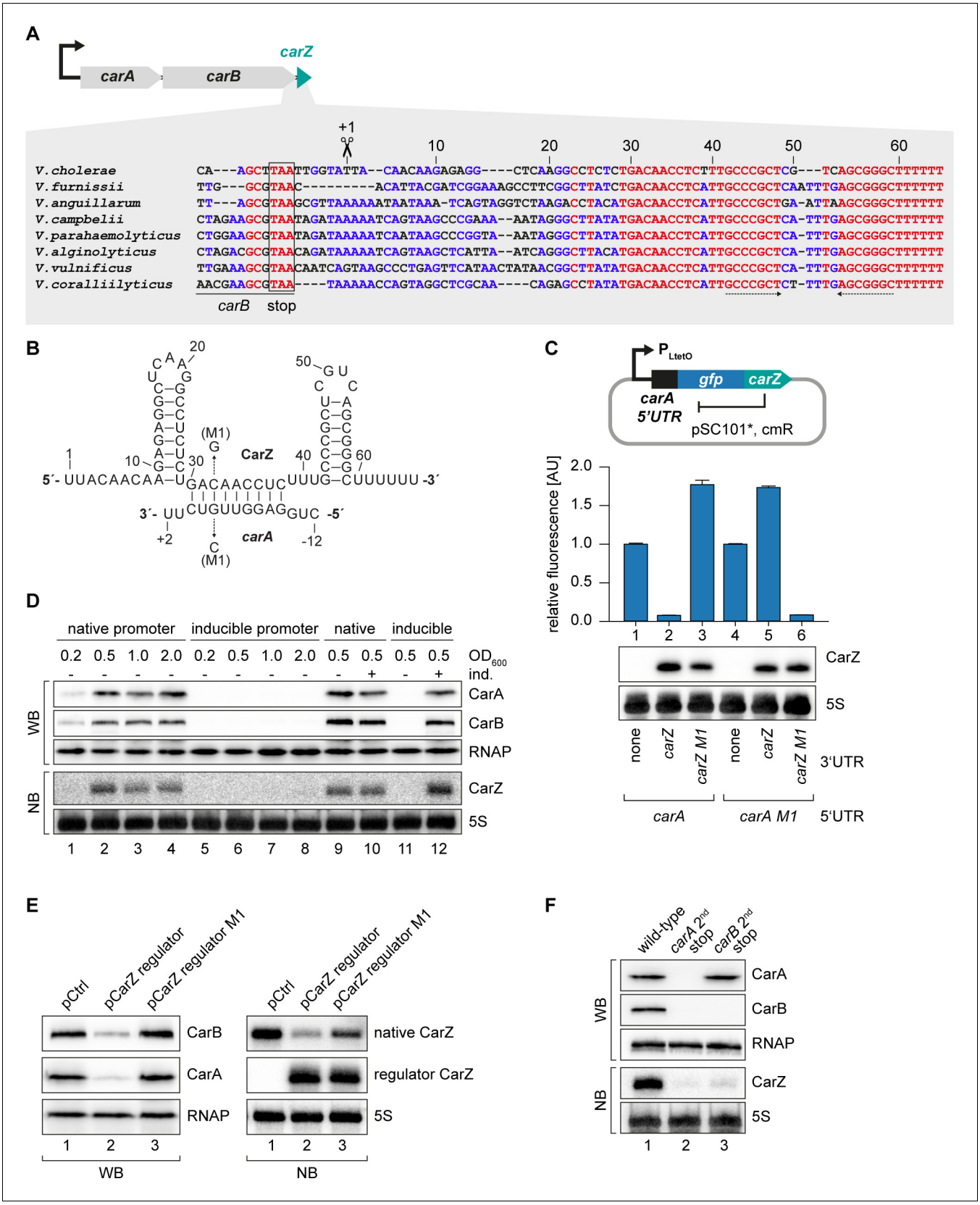

Figure 7. CarZ is another autoregulatory sRNA from *V. cholerae*. (**A**) Top: Genomic context of *carAB* and *carZ*. Bottom: Alignment of *carZ* sequences, including the last codons of *carB*, from various *Vibrio* species. The *carB* stop codon, the RNase E cleavage site and the Rho-independent terminator are

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Figure 7 continued

indicated. (**B**) Predicted CarZ secondary structure and base-pairing to *carA*. Arrows indicate the single nucleotide mutations tested in (C). (**C**) Single-plasmid feedback regulation of *carA* by CarZ was measured by inserting the indicated *carZ* variant into the 3' UTR of a translational *carA::gfp* fusion. Expression was driven from a constitutive promoter. *E. coli* strains carrying the respective plasmids were grown to OD_{600} = 1.0 and GFP production was measured. Fluorophore levels from control fusions without an sRNA gene were set to one and error bars represent the SD of three biological replicates. CarZ expression was tested by Northern blot; 5S rRNA served as loading control. (**D**) Protein and RNA samples were obtained from *V. cholerae carA*::3XFLAG *carB*::3XFLAG carrying either the native *carA* promoter or the inducible pBAD promoter upstream of *carA*. Samples were collected at the indicated OD_{600} and tested for CarA and CarB production by Western blot and for CarZ expression by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively. Lanes 1–8: Growth without L-arabinose. Lanes 9–12: Growth with either H_2O (-) or L-arabinose (+) (0.2% final conc.). (**E**) *V. cholerae carA*::3XFLAG *carB*::3XFLAG strains carrying a control plasmid or a plasmid expressing a CarZ variant with a mutated stem loop (*regulator CarZ*) were grown to late exponential phase (OD_{600} of 1.0). CarA and CarB production were tested by Western blot and expression of native CarZ or regulator CarZ was monitored on Northern blot using oligonucleotides binding to the respective loop sequence variants. RNAP and 5S rRNA served as loading controls for Western blot and Northern blot, respectively. (**F**) *V. cholerae carA*::3XFLAG *carB*::3XFLAG strains with the following *carA* or *carB* mutations were grown: wild-type (lane 1) or a STOP codon inserted at the 2nd codon of *carA* (lane 2) or *carB* (lane 3), respectively. At late exponential phase (OD_{600} of 1.0), protein and RNA samples were collected and tested for CarA and CarB production by Western blot and for CarZ expression by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively.

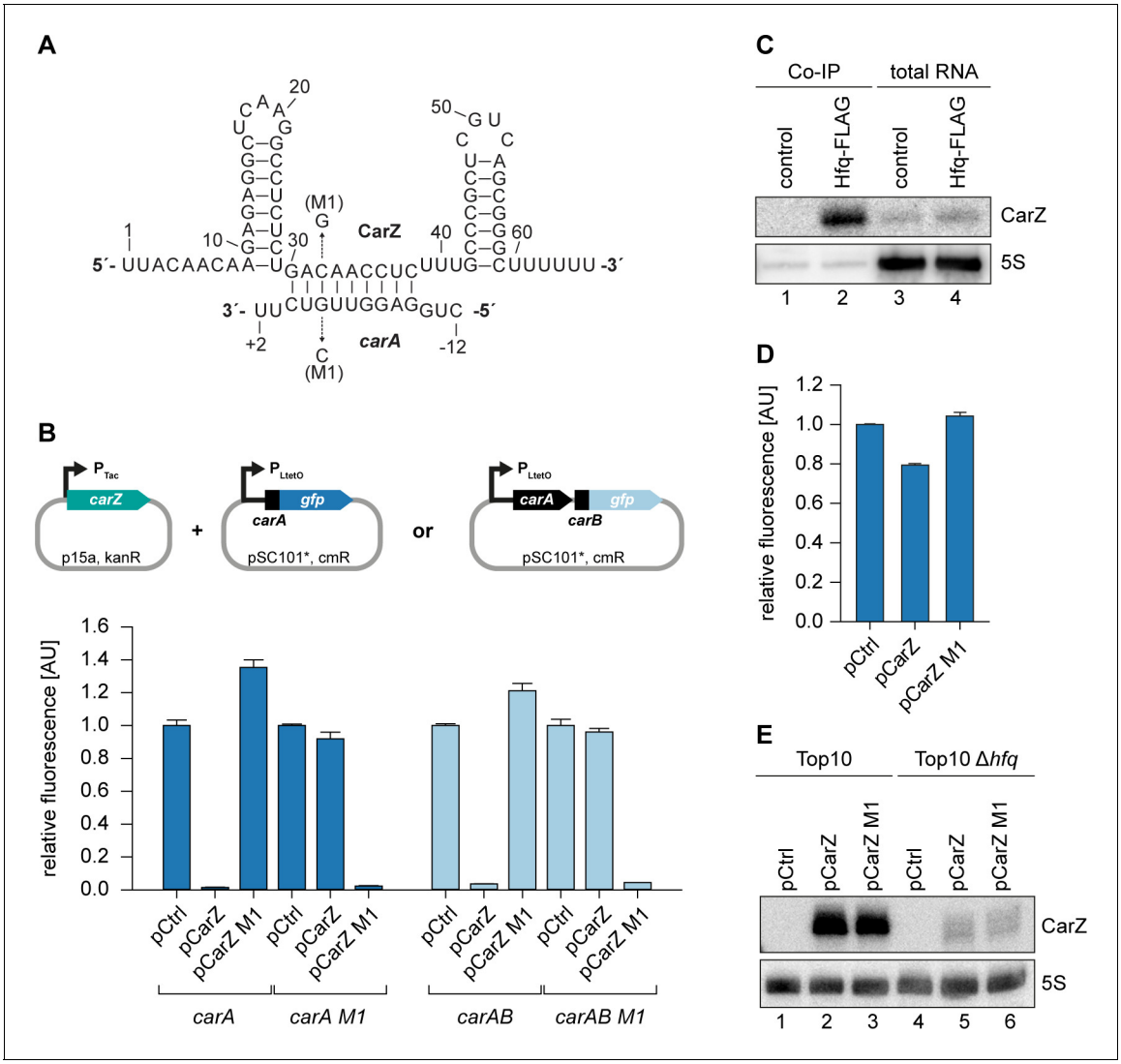

Figure 7—figure supplement 1. Hfq-dependent, post-transcriptional repression of CarA and CarB by CarZ. (**A**) Predicted CarZ secondary structure and base-pairing to *carA*. Arrows indicate the single nucleotide mutations tested in (**B**). (**B**) *E. coli* strains carrying translational reporter plasmids for *carA::gfp* or *carAB::gfp* were co-transformed with a control plasmid or the indicated CarZ expression plasmids. Transcription of the reporter and *carZ* were driven by constitutive promoters. Cells were grown to OD_{600} = 1.0 and fluorophore production was measured. GFP levels of strains carrying the control plasmid were set to 1. Error bars represent the SD of three biological replicates. (**C**) *V. cholerae* wild-type (control) and *hfq*::3XFLAG (Hfq-FLAG) strains were grown to stationary phase (OD_{600} of 2.0), lysed, and subjected to immunoprecipitation using the anti-FLAG antibody. RNA samples of lysate (total RNA) and co-immunoprecipitated fractions were analyzed on Northern blots. 5S rRNA served as loading control. (**D**) *E. coli* Δ*hfq* strains carrying the translational *carA::gfp* reporter plasmid and either a control plasmid or the indicated CarZ expression plasmids were grown to OD_{600} = 1.0 and fluorophore production was measured. GFP levels of the control strain were set to 1. Error bars represent the SD of three biological replicates. (**E**) *E. coli* wild-type or Δ*hfq* strains carrying the translational *carA::gfp* reporter plasmid and either a control plasmid or the indicated CarZ expression plasmids were grown to OD_{600} = 1.0. RNA samples were analyzed for CarZ levels by Northern blot; 5S rRNA served as loading control.

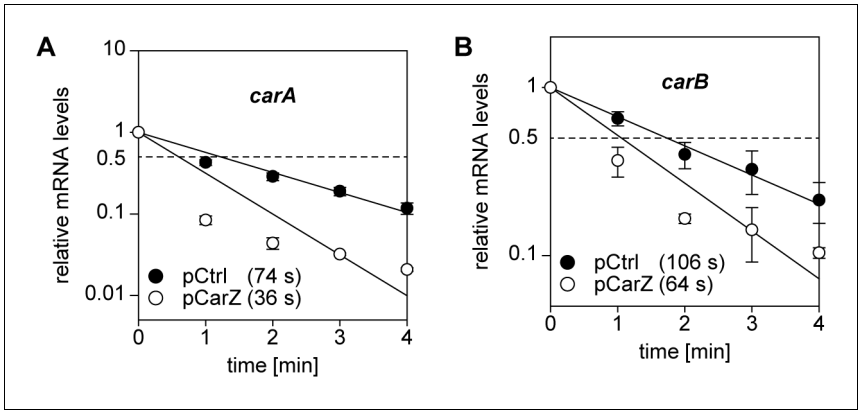

Figure 7—figure supplement 2. CarZ induces *carAB* degradation. (**A, B**) *V. cholerae* Δ*carZ* strains carrying either pBAD1K-ctrl (pCtrl) or pBAD1K-*carZ* (pCarZ) were grown to late exponential phase (OD_{600} of 1.0) and treated with L-arabinose (0.2% final conc.) to induce sRNA expression. After 15 min of induction, rifampicin was added to terminate transcription. RNA samples were obtained at the indicated time points and transcript levels of *carA* (**A**) and *carB* (**B**) were monitored by qRT-PCR. Error bars represent the SD of three biological replicates.

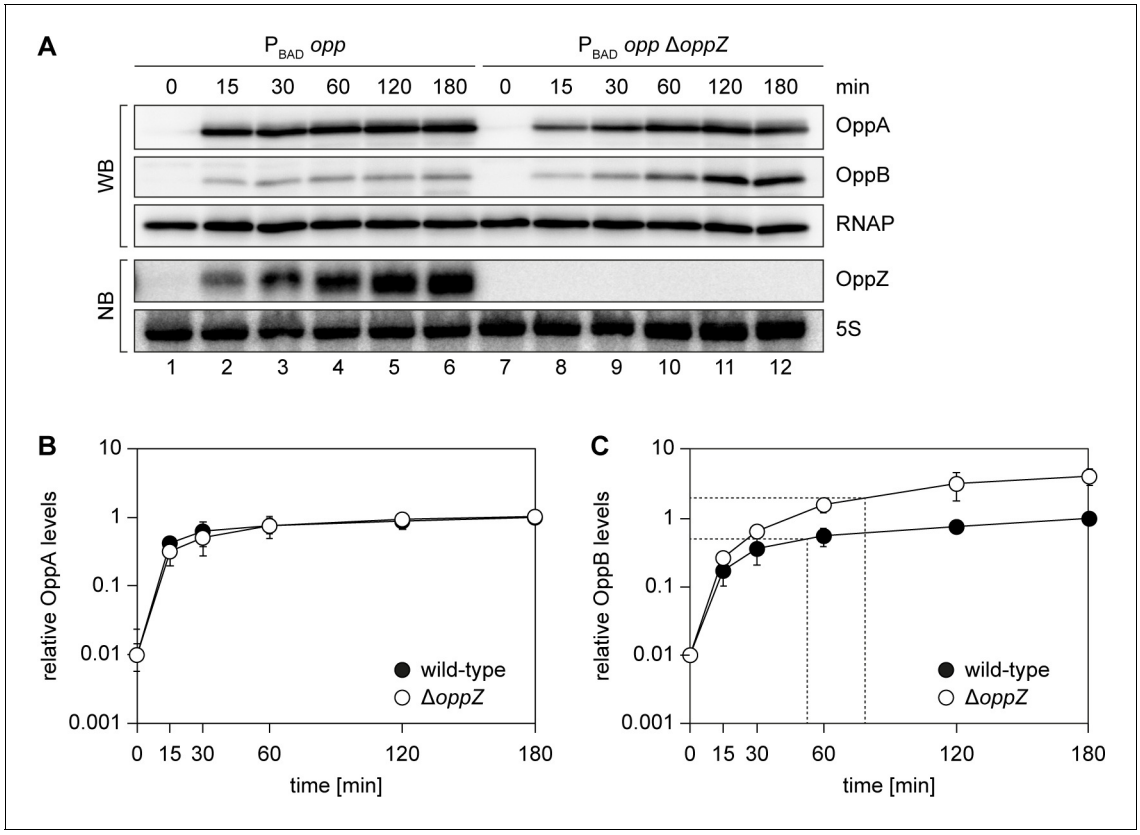

Figure 8. Modified kinetics of gene induction by autoregulatory OppZ. (**A**) Expression of the *opp* operon including the *oppA*::3XFLAG and *oppB*::3XFLAG genes and the native *oppZ* gene (lanes 1–6) or an *oppZ* deletion (lanes 7–12) was induced from the pBAD promoter at late exponential phase (OD_{600} of 1.0) by the addition of L-arabinose (0.2% final conc.). Protein and RNA samples were obtained at the indicated time points and tested for OppA and OppB production by Western blot and for OppZ expression by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively. (**B, C**) Quantification of OppA (**B**) or OppB (**C**) levels from the experiment in (**A**); error bars represent the SD of three biological replicates. Data are presented as fold regulation of OppA or OppB in Δ*oppZ* compared to the wild-type. Dashed lines in (**C**) indicate the time points of half-maximum OppB expression.

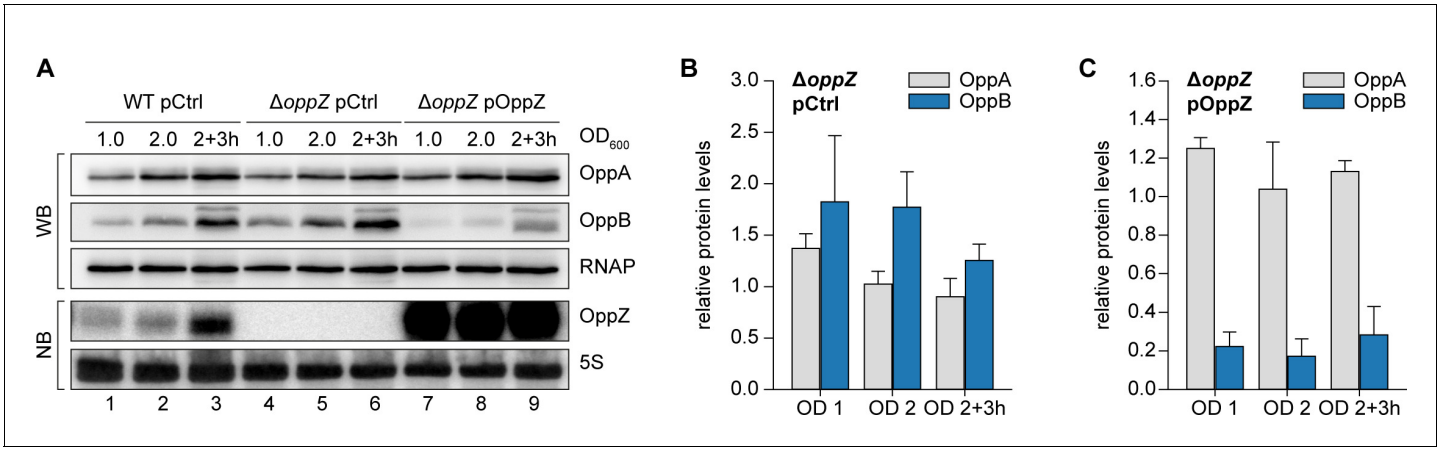

Figure 8—figure supplement 1. OppZ-dependent repression of OppA and OppB protein levels. (**A**) *V. cholerae* wild-type and Δ*oppZ* strains carrying the *oppA*::3XFLAG and *oppB*::3XFLAG genes and either a control plasmid or a constitutive OppZ expression plasmid were grown to obtain protein and RNA samples at the indicated OD_{600}. OppA and OppB production were analyzed by Western blot and OppZ expression was tested by Northern blot. RNAP and 5S rRNA served as loading controls for Western and Northern blots, respectively. (**B**) Quantification of (**A**), bars show fold regulation of OppA and OppB in Δ*oppZ* compared to the wild-type; error bars represent the SD of four biological replicates. (**C**) Quantification of (**A**), bars show fold regulation of OppA and OppB upon OppZ overexpression in the Δ*oppZ* background compared to the wild-type control; error bars represent the SD of four biological replicates.

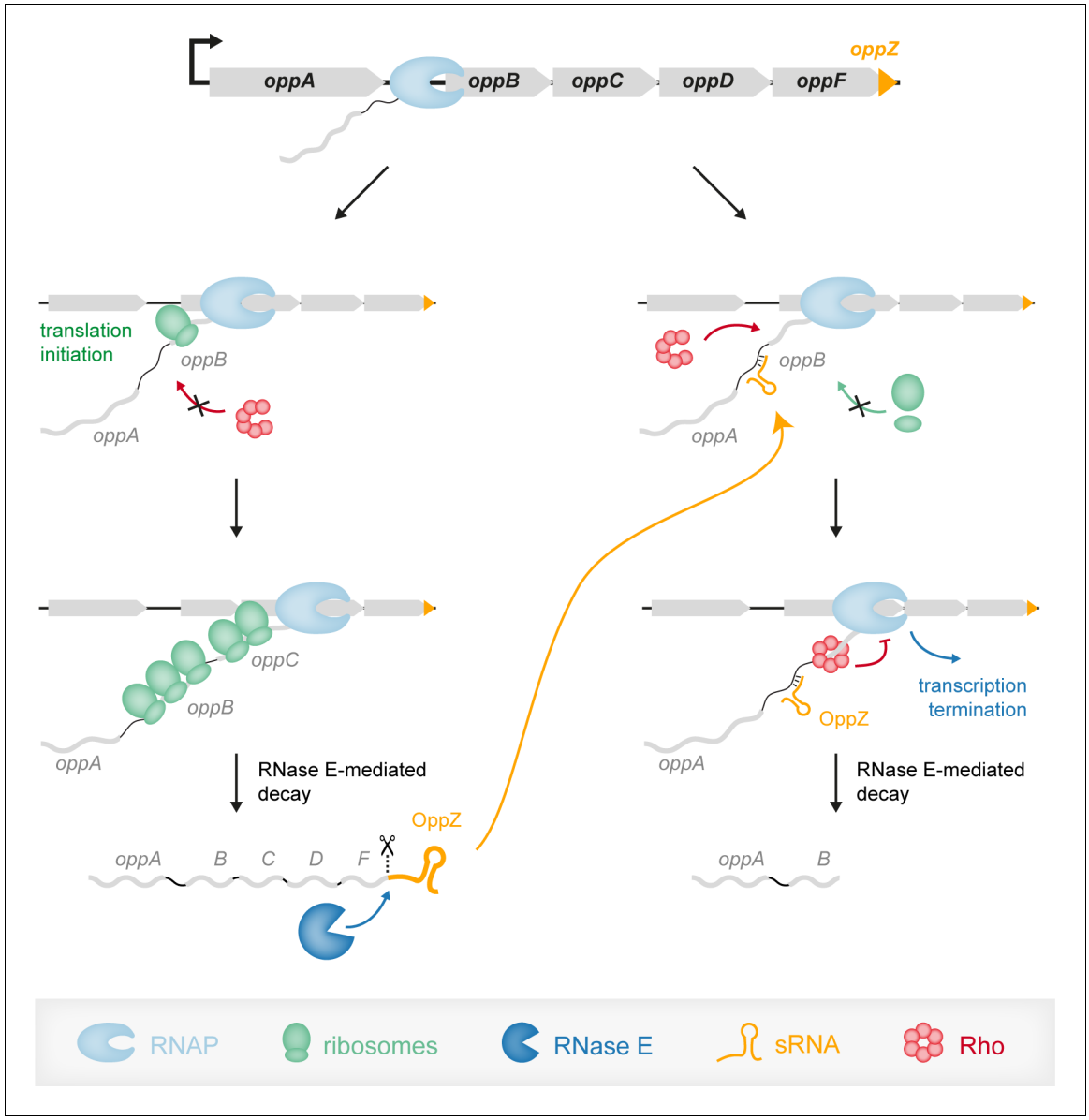

Figure 9. Model of the OppZ-dependent mechanism of *opp* regulation. Transcription of the *oppABCDF* operon initiates upstream of *oppA* and in the absence of OppZ (left) involves all genes of the operon as well as OppZ. In this scenario, all cistrons of the operon are translated. In the presence of OppZ (right), the sRNA blocks translation of *oppB* and the ribosome-free mRNA is recognized by termination factor Rho. Rho catches up with the transcribing RNAP and terminates transcription pre-maturely within *oppB*. Consequently, *oppBCDF* are not translated and OppZ is not produced.