Coordinate regulation of the expression of SdsR toxin and its downstream pphA gene by RyeA antitoxin in *Escherichia coli*

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In *Escherichia coli*, SdsR and RyeA, a unique pair of mutually cis-encoded small RNAs (sRNAs), act as toxin and antitoxin, respectively. SdsR and RyeA expression are reciprocally regulated; however, how each regulates the synthesis of the other remains unclear. Here, we characterized the biosynthesis of the two sRNAs during growth and investigated their coordinate regulation using *sdsR* and *ryeA* promoter mutant strains. We found that RyeA transcription occurred even upon entry of cells into the stationary phase, but its apparent expression was restricted to exponentially growing cells because of its degradation by SdsR. Likewise, the appearance of SdsR was delayed owing to its RyeA-mediated degradation. We also found that the *sdsR* promoter was primarily responsible for transcription of the downstream *pphA* gene encoding a phosphatase and that *pphA* mRNA was synthesized by transcriptional read-through over the *sdsR* terminator. Transcription from the σ70-dependent *ryeA* promoter inhibited transcription from the σ5-dependent *sdsR* promoter through transcriptional interference. This transcriptional inhibition also downregulated *pphA* expression, but RyeA itself did not downregulate *pphA* expression.

There are over 100 noncoding small RNAs (sRNAs) in *Escherichia coli*1–7; these can be divided into two categories: cis-acting and trans-acting. *Cis*-acting sRNAs, which are cis-encoded, act as antisense RNAs because they bind to sense mRNAs transcribed on the opposite DNA strand, thereby up- or downregulating their expression8–10. *Trans*-acting sRNAs have been widely studied and shown to regulate target mRNAs by base pairing with them through seed regions, usually with the help of the RNA-binding protein Hfq11–13. A single *trans*-acting sRNA can affect a variety of physiological events by interacting with multiple target mRNAs6,14–17. Therefore, sRNAs play a pivotal role in coordinating various aspects of cellular metabolism by fine-tuning the expression of their target genes.

Two sRNAs, SdsR and RyeA, have a number of characteristics that make them unique. They are cis-encoded sRNAs for each other4,18–20, while SdsR also function as a *trans*-acting sRNA18,21. SdsR acts as a regulator of multiple mRNAs by base-pairing with *tolC*, *mutS*, and *yhcB* in *E. coli*18,22,23, and with several mRNAs, including *ompD*, in *Salmonella*22,24. Unlike SdsR, RyeA has not yet been reported to function as a *trans*-acting sRNA. Recently, our group showed that SdsR and RyeA act as toxin and antitoxin, respectively18. The toxin function of SdsR is mediated by repression of *yhcB* encoding an inner membrane protein, which is involved in cell envelope biogenesis and cell shape maintenance16–27. Since both the toxin and antitoxin are sRNAs, the SdsR/RyeA pair represents a novel type of toxin-antitoxin system.

SdsR and RyeA show reciprocal expression patterns18. RyeA expression is dominant in the mid-exponential phase, whereas SdsR expression becomes higher starting from the late exponential phase to the stationary phase4. Therefore, SdsR, as an RpoS-dependent sRNA, is highly expressed during the stationary phase, but is barely expressed in the exponential phase.

The *pphA* gene, downstream of *sdsR*, encodes a phosphatase, also called PrpA, that is similar to *Salmonella* PrpA28 and λ-PP, a phosphoprotein phosphatase of bacteriophage lambda29. PphA plays a role in the protein misfolding response pathway by positively modulating the CpxR/CpxA two-component system, which activates *htrA* transcription30. It is likely that there are other target proteins of PphA because additional phosphoproteins accumulate in a *pphA* mutant30. It has been reported that *pphA* transcription is induced by heat shock and that its...
promoter, which is located far upstream of the sdsR promoter, has some homology to the promoter consensus sequences of RNA polymerase σ70-holoenzyme (Eσ70). However, a subsequent study suggested that pphA may not be heat-shock inducible. Expression of pphA is also induced during biofilm formation and upon urea stress. Therefore, it remains unclear how pphA expression is regulated.

In this study, we examined biosynthesis of the two sRNAs during growth and coordinate regulation of SdsR toxin and its downstream pphA gene by RyeA. We found that both SdsR and pphA expression are under control of the sdsR promoter and tightly down-regulated during exponential growth by expression of ryeA.

Results

Biosynthesis of SdsR and RyeA. As there has been some confusion in the literature regarding biosynthesis of SdsR and RyeA in E. coli, we set to clarify their biosynthesis. To determine the precise transcription initiation sites from each promoter, we subcloned the sdsR (−79 to +11 relative to its transcription start site) and ryeA (−80 to +10 relative to its most downstream transcription start site) promoter-containing DNA fragments to plasmid pKK232-8 to generate ryeA-CAT and sdsR-CAT transcriptional fusions, respectively. We then analyzed sdsR-CAT and ryeA-CAT fusion transcripts using primer extension (Fig. 1A,B). An analysis of primer extension products revealed that the sdsR promoter starts transcription at a site 1 nt downstream of the previously reported 5′ end of E. coli SdsR, which corresponds to the 5′ end of Salmonella SdsR. On the other hand, the ryeA promoter starts transcription at three sites, of which the most downstream site is the previously predicted 5′ end. In parallel, we performed 5′ RACE experiments using total cellular RNAs with or without the E. coli RNA pyrophosphatase (RppH) treatment and the RACE products were analyzed on an agarose gel (Fig. 1C). The predicted RyeA band (a) was detected at comparable amounts in both RppH-treated and untreated RNAs, whereas the predicted SdsR band (b) was observed only in RppH-treated RNA. These results suggest that RyeA and SdsR have different phosphorylation status at the 5′ end: SdsR retains 5′ triphosphate, but RyeA carries 5′ monophosphate. Each RACE band (the corresponding gel area of band b was used for SdsR in RppH-untracted RNA) was eluted from the gel and subjected to DNA sequencing analysis (Table 1). The 5′ end of primary transcripts and their 3′ termini are generated by RppH-treated RNA, not RppH-untreated, corresponds to its transcription start. On the other hand, RyeA showed only one 5′ end corresponding to its most downstream transcription start site regardless of being treated with RppH. These results suggest that while SdsR has a triphosphate at the 5′ end as a primary transcript, RyeA largely exists as a single processed transcript with a monophosphate at the 5′ end, which could be formed by removing 1 or 2 nucleotides, or pyrophosphate from three different primary RyeA transcripts.

The 3′ ends of SdsR and RyeA were analyzed by 3′ RACE (Fig. 1C and Table 1). A sequence analysis of RACE products suggested that SdsR has heterogeneous 3′ ends that terminate at base positions ranging from +100 to +102 relative to its own transcription start site, whereas RyeA has 3′ ends of +261 to +270 relative to the most downstream transcription start site.

In vitro transcription was carried out to determine whether the 3′ ends of SdsR and RyeA correspond to their transcription termini. Plasmid DNA, pSdsR (−379/+222), containing an sdsR/ryeA transcription unit consisting of −379 to +222 relative to the sdsR transcription start site was used as a template for in vitro transcription assays (Fig. 2A). Since the sdsR promoter is known to be σ70-dependent, we used both RNA polymerase σ70-holoenzyme (Eσ70) and Eσ70. As shown in the left half gel of Fig. 2B,C, Eσ70 in the absence of Eσ70 (Eσ70/Eσ70, 1/0) generated almost exclusively RyeA transcripts although it transcribed sdsR at about 10% levels as compared to the RyeA transcripts. On the other hand, Eσ70 alone (Eσ70/Eσ70, 0/1) produced only SdsR transcripts without generating RyeA transcripts. These results suggest that transcription of sdsR is mostly mediated by Eσ70, but that ryeA transcription are mediated solely by Eσ70. SdsR transcripts of ~100 nt and RyeA transcripts of ~270 nt were detected, indicating that primary and secondary transcripts observed in vivo are generated by Rho-independent termination. We also found a read-through transcript of ~310 nt that passes over the sdsR terminator and terminates at the following rnpB terminator, suggesting that genes downstream of sdsR could be regulated by sdsR transcription.

Regulation of SdsR and RyeA biosynthesis. To determine the effects of SdsR on the biosynthesis of RyeA, or vice versa, we constructed sdsR and ryeA promoter mutant strains in which their −10 elements were inactivated by changing each of them to CTCCGAG. We then examined levels of SdsR and RyeA in the promoter mutant cells during growth (Figs 3 and S1). As expected, SdsR and RyeA were not detected in the sdsR and ryeA promoter mutant strains, respectively (Fig. 3A). In wild-type cells, RyeA was expressed during the early exponential phase, and its expression sharply decreased in the mid-exponential phase. The sdsR promoter mutation increased RyeA synthesis during the exponential phase and allowed its continued synthesis in the stationary phase. On the other hand, SdsR expression started in the late-exponential phase in wild-type cells, but appeared at an earlier stage in ryeA promoter mutant cells (Fig. 3B). Since the larger ryeA covers the entire sdsR, the whole 104-nt SdsR sequence can base-pair with RyeA. We performed analysis of mutual degradation to determine whether interaction between two sRNAs leads to degradation of each other (Fig. S2). We ectopically co-expressed both SdsR and RyeA, but expression of SdsR was increased at a fixed expression level of RyeA, or vice versa. We found that when one is overexpressed, degradation of the other is facilitated, suggesting that this extensive base-pairing leads to degradation of both sRNAs. Because SdsR and RyeA are degraded each other, it is likely that the early appearance of SdsR in ryeA promoter mutant cells and the prolongation of RyeA expression to the stationary phase in sdsR promoter mutant cells are attributable to the absence of mutual degradation of the two sRNAs. The lack of RyeA degradation products of about 60 nt in sdsR promoter mutant cells further supports reciprocal regulation of the two sRNAs through degradation of each other. This mutual degradation could serve to further restrict RyeA expression to the exponential phase and SdsR expression to the stationary phase.
Figure 1. Identification of 5′ and 3′ ends of RyeA and SdsR. (A) Schematic representation of the region containing the sdsR/ryeA locus and its nucleotide sequence. Promoter regions (−35 and −10) of sdsR and ryeA are underlined, and their transcription start sites are indicated by arrows. (B) Primer extension analysis of ryeA-CAT and sdsR-CAT fusion transcripts. The regions +341 to +132 (containing the ryeA promoter) and −79 to −11 (containing the sdsR promoter) were cloned into pKK232-8 to generate ryeA-CAT and sdsR-CAT plasmids, respectively. Total cellular RNA extracts were prepared from either MG1655 cells containing the ryeA-CAT or sdsR-CAT plasmid grown for 2 h, 6 h, or 10 h at 37 °C. The 32P-labeled primer CAT_R was used to analyze ryeA-CAT and sdsR-CAT fusion transcripts. Primer extension products were analyzed on a 5% polyacrylamide sequencing gel containing 8 M urea. The DNA ladders (G, A, T and C) were prepared by dideoxy sequencing using the template plasmid DNA and the same primer. Loading amounts are indicated below the lanes. The transcription start nucleotides are indicated by arrows. (C) RACE analysis. The 5′ or 3′ RACE products (primary PCR products or nested PCR products) were analyzed on 2% agarose gels. Predicted RACE products were indicated by a, b, c, and d. For 5′ RACE, E. coli RNA pyrophosphatase (RppH)-treated RNA and untreated RNA were used. M, 100 bp size markers; R, RyeA; S, SdsR.
The tight growth phase-specific control of SdsR and RyeA may be needed for SdsR- or RyeA-mediated regulation of certain genes in specific growth phases. To identify such genes, we performed an RNA-seq analysis of ryeA and sdsR promoter mutant cells as well as wild-type cells. For this RNA-seq analysis, cultures were sampled in both the exponential phase (3h) and stationary phase (8h), and genes in sdsR or ryeA promoter mutant cells that showed a greater than 2-fold change and a p-value < 0.05 were selected for further characterization. We identified 17 genes (7 upregulated and 10 downregulated) in the exponential phase and 5 genes (2 upregulated and 3 downregulated) in the stationary phase for ryeA promoter mutant cells (Tables S1 and S2). In contrast, sdsR promoter mutant cells showed many changed genes: 171 genes (110 upregulated and 51 downregulated) in the exponential phase and 55 genes (33 upregulated and 22 downregulated) in the stationary phase (Tables S3–S5). Selected genes were confirmed by qRT-PCR analysis (Table S6), which showed that RNA-seq data were reliable. A Gene Ontology (GO) analysis showed that many of the genes that were altered by the sdsR promoter mutation, either downregulated or upregulated, were related to transport function and encoded membrane proteins (Fig. S3), an observation consistent with previous RNA-seq analysis of SdsR-overexpressing cells.

As expected, the RNA-seq data revealed upregulation of RyeA and SdsR in sdsR and ryeA promoter mutant cells, respectively (Fig. 4A,B). We also examined the RNA-seq data to see how expression of known SdsR target genes is affected by the sdsR or ryeA promoter mutation (Table S7). The sdsR promoter mutation increased tolC and yhcB expression by about 2-fold in the stationary phase and exponential phase, respectively. In contrast, the ryeA promoter mutation led to a slight decrease in tolC and yhcB expression, suggesting that RyeA can modulate SdsR-mediated gene regulation. However, mutS, another target for SdsR, was not shown expression that would be expected from sdsR or ryeA promoter mutant cells, implying that it may not be a primary target of SdsR.

Interestingly, the RNA-seq data showed that the pphA gene, downstream of sdsR, was downregulated in sdsR promoter mutant cells and upregulated in ryeA promoter mutant cells (Fig. 4C). An analysis of pphA expression by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and semi-quantitative RT-PCR confirmed the same downregulation in sdsR promoter mutant cells and upregulation in ryeA promoter mutant cells (Fig. 4D,E). These data indicate that pphA expression may be regulated by the sdsR/ryeA regulatory circuit.

pphA is co-transcribed with SdsR from the sdsR promoter. The previously reported pphA promoter, located about 180 bp upstream of the sdsR promoter, is known as a heat-shock promoter. Therefore, transcription from this promoter should pass through the sdsR/ryeA locus to transcribe the pphA gene; furthermore, the sdsR promoter can generate pphA transcripts. To determine which promoter makes the greater contribution to pphA expression, we constructed various transcriptional sdsR-lacZ fusions and analyzed their transcriptional activities using LacZ assays (Fig. 5). The promoter region containing a DNA fragment 200 bp upstream and 100 bp downstream (−379 to −170 relative to the 5′ end of SdsR) from the previously known pphA transcription site showed little LacZ activity at 37 °C and even at 42 °C. However, a promoter region extended to include the sdsR/ryeA locus (+222) exhibited high LacZ activity at 37 °C that was not significantly further increased at 42 °C. Therefore, it is unlikely that the previously reported heat inducible pphA promoter is responsible for the observed LacZ activity of the −379/+222 construct. Furthermore, the sdsR promoter mutation sharply decreased the LacZ activity of sdsR(−379/+222)-lacZ, indicating that the sdsR promoter is a major contributor to pphA transcription. The −329 to +222 promoter region was fused to the CAT gene in plasmid pKK232-8 and the 5′ ends of the fused mRNA, transcribed in vivo, were analyzed by primer extension analysis (Fig. S4). Most extension products were from sdsR transcripts, and no extension products from the reported pphA promoter were detected. These data, taken together with in vitro data showing read-through transcripts traversing the sdsR terminator (Fig. 2B), suggest that the sdsR promoter rather than the previously reported pphA promoter is responsible for pphA transcription.

Effects of ryeA transcription on expression of SdsR and sdsR-pphA dicistronic mRNA. Next, we examined whether ryeA transcription affected pphA expression using the ryeA promoter mutant. To avoid possible effects of chromosomally expressed SdsR and RyeA, we constructed the sdsR-lacZ fusions in an sdsR/ryeA-knockout background. The LacZ activity of the sdsR(−379/+222)-lacZ fusion was increased by about 4-fold by the ryeA promoter mutation (Fig. 5). This increase could be attributable to the absence of RyeA (acting in trans) or ryeA transcription itself (acting in cis). To discriminate between these two possibilities, we analyzed LacZ activity following ectopic expression of RyeA. To determine conditions for ectopically expressing RyeA at levels comparable to those generated by the endogenous ryeA promoter, we varied the concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG) used to induce expression (Fig. 6A). We found that RyeA levels induced by

| 5′ ends of RNAa | SdsRb | RyeAc |
|----------------|-------|-------|
| −RppH | +3 (1), +31 (2), +32 (1), +34 (1), NDa (5) | +1 (11), +33 (1), +36 (3), NDa (5) |
| +RppH | −106 (1), +31 (1), NDa (2) | +1 (17), +25 (1), +36 (1), NDa (1) |
| 3′ ends of RNAa | +100 (3), +101 (10), +102 (2), NDa (5) | +261 (1), +263 (7), +269 (3), +270 (9) |

Table 1. Determination of 5′ and 3′ ends of SdsR and RyeA. The RACE products a and b shown in Fig. 1C and c and d in Fig. 1D were cloned by T- blunt vector and analyzed by DNA sequencing. In case of SdsR in RppH-untreated RNA, the corresponding gel area of band b was used for analysis. The positions of both ends at which each RNA terminator terminates are represented relative to the most downstream transcription start for RyeA. The numbers in parentheses indicate the frequency of occurrence. Untreated and treated with RppH are indicated by −RppH and +RppH, respectively. ND, non-detected.
0.005 mM IPTG were comparable to those produced by the ryeA promoter. Induction of RyeA caused no decrease in LacZ activity (Fig. 6B), suggesting that the absence of RyeA is not responsible for the increase in LacZ activity. Therefore, it is likely that ryeA transcription, not RyeA, inhibits pphA expression by reducing read-through transcription from the sdsR promoter. Then we examined how ryeA transcription affects the read-through transcription during the growth using the sdsR(−379/+222)-lacZ fusion. We found that the ryeA transcription represses LacZ expression at all growth phases and delays its expression 3 h to the stationary phase (Fig. S5). To further confirm that read-through transcripts are increased by the ryeA mutation, we inserted a Brevibacterium albidum tRNAArg sequence between the sdsR sequences and the CAT coding sequence in the sdsR-CAT fusion constructs and examined exogenous tRNA expression as well as SdsR in an sdsR/ryeA-knockout background (Fig. 7). The reason that we used heterologously expressed B. albidum tRNAArg was because the tRNA was previously shown to be metabolically stable in E. coli and detectable by Northern blot analysis without cross-hybridization with E. coli tRNAs. The ryeA promoter mutation caused an increase in tRNAArg expression, confirming that the ryeA mutation increases read-through transcription. Taken together, these data show that pphA expression is inhibited by ryeA transcription itself, and not by RyeA.
To examine whether the increase in read-through transcripts was caused by reduced termination efficiency at the sdsR terminator, we introduced a terminator mutation into the sdsR(−79/+222)-lacZ fusion (Fig. 8). This terminator mutation did not affect the ryeA promoter mutation-induced increase in LacZ activity, suggesting that the increase in read-through transcripts caused by the ryeA promoter mutation does not result from reduced termination efficiency. Therefore, it seems likely that transcription from the ryeA promoter interferes with transcription of sdsR.

Because it is known that transcriptional interference can occur in DNA constructs with convergent promoters, we tested whether positive supercoiling induced by transcription from the ryeA promoter could affect transcription from the sdsR promoter. For this purpose, we treated cells with novobiocin, a gyrase subunit B inhibitor. Growth of E. coli cells was inhibited by novobiocin with a 50% inhibitory concentration (IC50) of 100 μg/ml (Fig. S6A). We examined effects of ryeA transcription on sdsR transcriptional interference can be due to promoter occlusion, colliding RNA polymerases, or transcription-induced changes in DNA supercoiling that affect initiation of transcription. Promoter occlusion is not likely because the two promoters are far apart. We tested whether positive supercoiling induced by transcription from the ryeA promoter could affect transcription from the sdsR promoter. For this purpose, we treated cells with novobiocin, a gyrase subunit B inhibitor. Growth of E. coli cells was inhibited by novobiocin with a 50% inhibitory concentration (IC50) of 100 μg/ml (Fig. S6A). We examined effects of ryeA transcription on sdsR transcriptional interference can be due to promoter occlusion, colliding RNA polymerases, or transcription-induced changes in DNA supercoiling that affect initiation of transcription. Promoter occlusion is not likely because the two promoters are far apart. We tested whether positive supercoiling induced by transcription from the ryeA promoter could affect transcription from the sdsR promoter. For this purpose, we treated cells with novobiocin, a gyrase subunit B inhibitor. Growth of E. coli cells was inhibited by novobiocin with a 50% inhibitory concentration (IC50) of 100 μg/ml (Fig. S6A). We examined effects of ryeA transcription on sdsR transcriptional interference can be due to promoter occlusion, colliding RNA polymerases, or transcription-induced changes in DNA supercoiling that affect initiation of transcription. Promoter occlusion is not likely because the two promoters are far apart. We tested whether positive supercoiling induced by transcription from the ryeA promoter could affect transcription from the sdsR promoter. For this purpose, we treated cells with novobiocin, a gyrase subunit B inhibitor. Growth of E. coli cells was inhibited by novobiocin with a 50% inhibitory concentration (IC50) of 100 μg/ml (Fig. S6A).
Figure 4. Analysis of RyeA, SdsR, and pphA RNA expression. Expression dynamics of SdsR and pphA mRNA in ryeA promoter mutant cells. (A, B) Read counts for RyeA and SdsR from RNA-seq data are plotted against ryeA and sdsR chromosomal positions. WT, MG1655 cells; ryeAPm, ryeA promoter mutant cells; sdsRPm, sdsR –10 promoter mutant cells. E, exponential phase cells (3 h post-inoculation); S, stationary phase cells (8 h post-inoculation). (C) RNA read counts for pphA mRNA are plotted against pphA chromosomal positions. Since a specific region of higher RNA-seq reads is AU-rich, the higher efficiency of random reverse transcription priming on the AU-rich sequence may cause that transcript depth. (D) qRT-PCR analysis of changes in pphA mRNA levels by the ryeA or sdsR –10 promoter mutation. Total cellular RNAs were isolated from WT and the promoter mutant cells at 8 h post-inoculation, and subjected to qRT-PCR. Fold changes relative to WT cells are shown. (E) The same total RNAs were subjected to RT-PCR. RT-PCR products were analyzed in a 2% agarose gel. In (D), mean ± SD; n = 5–6; *P ≤ 0.05, **P ≤ 0.001 by Student’s t-test.
transcription in the presence of novobiocin at 100μg/ml (Fig. S6B). Since SdsR levels could be affected by RyeA in trans, we analyzed the novobiocin effects in LacZ activity from sdsR(−379/+222)-lacZ fusion rather than those in SdsR levels. Novobiocin had little effect on the increase of LacZ activity by the ryeA mutation, suggesting that transcription-induced changes in DNA supercoiling contribute little to the observed transcriptional interference. On the other hand, when we examined the ryeA and sdsR promoter activities during growth using lacZ transcriptional fusions, the ryeA and sdsR promoters showed transcription activities in all the growth phases, although ryeA promoter is more active in the exponential phase than the sdsR promoter (Fig. S7). These results altogether suggest the possibility that two oppositely transcribing RNA polymerases collide in the cell. Therefore, it seems likely that ryeA transcription inhibits transcription from the sdsR promoter through transcriptional interference, which may occur by RNA polymerase collision sometime between RNA polymerase binding to the

Figure 5. LacZ analysis for sdsR and pphA transcription. (A) Schematic diagrams of various sdsR-lacZ transcriptional fusion constructs are shown along with their LacZ activities for DJ480ΔryeA/sdsR cells grown for 2 h (E) and 10 h (S) post-incubation at 37 °C or 42 °C. sdsRm, sdsR −10 promoter mutation; ryeAmp, ryeA −10 promoter mutation. (B,C) The LacZ activities are presented in bar graph (mean ± SD; n = 3).
Figure 6. Effects of RyeA on sdsR transcription. (A) Ectopically expressed RyeA in DJ480ΔryeA/sdsR cells carrying the sdsR(−379/+222)-lacZ or sdsR(−379/+222ryeAPm)-lacZ fusion were analyzed. Total cellular RNAs from pRyeA-containing cells induced with different IPTG concentration were analyzed by Northern blotting. pRyeA, RyeA-expressing plasmid derived from vector pHM4T. (B) LacZ activities were measured in cells ectopically expressing RyeA by induction with 0.005 mM IPTG. Exponential (E) and stationary (S) phase cells (grown for 2 h and 10 h, respectively) were used for LacZ assays (mean ± SD; n = 3).

Figure 7. Analysis of read-through transcripts traversing the sdsR terminator. (A) Schematic representation of sdsR-tRNAArg-CAT fusion plasmids. The B. albidum tRNAArg gene was inserted in the region before the CAT gene of pKK232-8 plasmid. (B) Northern blot analysis of Arg-tRNA, RyeA, and SdsR. Total cellular RNA from cells carrying the sdsR(−379/+222)-tRNAArg-CAT or sdsR(−379/+222ryeAPm)-tRNAArg-CAT fusion plasmid were isolated and analyzed by Northern blotting. In each panel the spliced images from the same Northern membrane are shown with the insertion of a dividing lines between the spliced lanes.
**Discussion**

In this study, we first defined the transcription units and biogenesis of *sdsR* and *ryeA* in more detail, showing that 3′ ends of both SdsR and RyeA correspond to their own transcription termini. We identified three transcription initiation start sites from the *ryeA* promoter. The primary *ryeA* transcripts are trimmed at the 5′ end to form processed RNAs with 5′ monophosphate whose 5′ end corresponds to the most downstream transcription start, which can generate RyeA of 272 nt with the longest 3′ end. On the other hand, the 5′ end of SdsR, which is the same as the *sdsR* transcription initiation site, retains triphosphate. This may explain that RyeA has a much shorter half-life than SdsR in the cell because RNA with 5′ monophosphate is more vulnerable to degradation than RNA with 5′ triphosphate. The transcription of *sdsR* terminates at base positions ranging from +100 to +102 relative to its own transcription start site, generating the longest SdsR (102 nt) in the cell.

RyeA is expressed in the exponential phase, whereas SdsR expression starts in late-stationary phase. Since we showed here that RyeA and SdsR are almost exclusively transcribed in vitro by Eσ70 and EσS, respectively, acting at their respective promoters, the growth-dependent regulation of RyeA and SdsR should be mediated by the sigma factor selectivity of the two promoters. Promoter mutation analyses revealed that mutual degradation of SdsR and RyeA also contributes to discrete growth phase-dependent regulation of the expression of *sdsR* promoter and the transcription elongation step up until the RNA polymerase reaches the *ryeA* promoter by collision between oppositely transcribing RNA polymerases.

**Figure 8.** *sdsR* terminator-independency of transcriptional interference between the *sdsR* and *ryeA* promoter.
(A) Schematic diagrams of *sdsR*(-79/+222)-*lacZ* transcriptional fusion constructs are shown along with their LacZ activities for DJ480Δ*ryeA/sdsR* cells grown for 2 h (E) and 10 h (S) post-inoculation at 37°. (B) The LacZ activities are presented in bar graph. *sdsRTm*, *sdsR* terminator mutation; *ryeAPm*, *ryeA* −10 promoter mutation (mean ± SD; n = 3).
Each sRNA. Furthermore, RyeA transcription can interfere with SdsR transcription, generating less SdsR when RyeA transcription occurs. As a result of this biosynthetic pathway, more restricted stationary phase expression of SdsR can be achieved through the coupled action of degradation of SdsR by RyeA expressed in the exponential phase and inhibition of SdsR transcription through transcriptional interference by RyeA transcription from the $\sigma^{70}$-dependent ryeA promoter.

RNA-seq analysis showed that expression of pphA, encoding a phosphatase, downstream of sdsR was decreased by the sdsR promoter mutation and increased by the ryeA promoter mutation, suggesting that pphA expression is controlled by the sdsR/ryeA regulatory circuit. We showed that pphA mRNA is transcribed as an sdsR-pphA dicistronic RNA from the $\sigma^{70}$-dependent sdsR promoter rather than from the previously reported heat-inducible pphA promoter. Thus, pphA mRNA may not be transcribed from the heat-inducible promoter, as suggested previously. However, pphA expression differs from SdsR expression in that it is not affected in trans by RyeA although the ryeA transcription represses pphA expression. Therefore, pphA could be expressed at an earlier growth phase than SdsR.

Trans-acting sRNAs usually downregulate expression of multiple target genes by inhibiting translation initiation of mRNA or by inducing degradation. Our RNA-seq analysis showed that the sdsR promoter mutation upregulated more than 100 genes, but the ryeA promoter mutation led to only a few upregulated genes, suggesting that a major function of RyeA is to downregulate SdsR as a cis-encoded sRNA. The finding that the well-known SdsR target gene tolC was repressed by the ryeA promoter mutation and activated by the sdsR mutation supports this possibility.

Our study showed that pphA expression begins with transcription from the sdsR promoter, and requires read-through transcription over the sdsR terminator and continues transcription into the pphA coding region. RNA-seq data showed that there was a considerable amount of read-through RNA over the sdsR terminator, but the amount of RNA continued to decrease with progression into the pphA open reading frame. Therefore, it is likely that other regulatory systems, such as premature termination or translational control, in addition to the sdsR/ryeA regulatory circuit are involved in pphA regulation. This remains to be determined in the future.

Antisense RNAs are expressed through convergent transcription, and their expression promotes transcriptional interference. Transcriptional interference can come into play through various mechanisms, depending on the promoter strength and transcription velocity. In this context, the sdsR/ryeA transcription unit is interesting because transcription levels from the ryeA and sdsR promoter vary during growth, leading to different transcriptional interference levels depending on growth conditions. It is likely that the sdsR/ryeA region at which transcriptional interference between two RNA polymerases occurs depends on the growth phase. SdsR transcription can be affected by convergent ryeA transcription at both initiation and elongation steps because the entire...
sdsR transcription unit is encompassed by the ryeA gene. Like SdsR expression, pphA expression is also affected by transcriptional interference by ryeA transcription.

To summarize, we herein characterized regulation of the expression of the toxin SdsR, including its downstream pphA gene, and the antitoxin RyeA (Fig. 9). SdsR expression is tightly regulated during growth through transcription from the σ70-dependent sdsR promoter, RyeA-mediated degradation of SdsR, and transcriptional interference from the σ70-dependent ryeA promoter. On the other hand, downstream pphA expression, like that of SdsR, is also under control of both sdsR and ryeA promoters, but is unaffected by RyeA. Therefore, the sdsR/ ryeA regulatory circuit plays a critical role in tightly controlling growth-dependent expression of SdsR toxin and pphA to ensure that they are not expressed during the exponential phase.

### Experimental Procedures

#### Strains, plasmids, and oligonucleotides.

All strains and plasmids used in this study are listed in Table 2. All primers and oligonucleotides used in this study are shown in Table 3. To generate sdsR and ryeB promoter mutant strains, each −10 element was changed to ‘CTCGAG’ using scarless mutagenesis, as described previously. A series of lysogen-containing lacZ transcriptional fusion constructs was prepared. Briefly, various promoter regions around ryeA/sdsR were amplified, and the resulting fragment was cloned between the EcoRI sites of the pRS1553 vector to generate lacZ transcriptional fusion plasmids. Lysogens were constructed by transforming E. coli strain DJ480 with the various fusion constructs and transfecting with λRS468 to construct the corresponding lacZ fusion lysogens. Single-copy integration was confirmed by PCR. For point mutations in sdsR-lacZ fusions, site-directed mutagenesis was performed, as described previously. DJ480ΔsdsR/ryeA strain was generated from MG1655ΔsdsR/ryeA by P1-mediated transduction and confirmed by sequence analysis of the amplified, knocked-out region. pRyeA and pSdsR carrying a pBR322 origin ectopically express IPTG-inducible RyeA and SdsR, respectively. To generate sdsR- or ryeA-CAT fusion plasmids, sdsR or ryeA promoter-containing DNA fragments were obtained via PCR amplification of genomic DNA. The resulting PCR products were digested with BamHI/HindIII and ligated into pKK232–8. To generate sdsR-tRNAArg-CAT fusion plasmids, a B. albidum tRNAArg sequence was amplified by PCR and inserted immediately upstream of the CAT gene in the sdsR-CAT fusion plasmids. Template DNA plasmids for in vitro transcription were prepared by cloning sdsR or ryeA promoter-containing DNA fragments into the HindIII/EcoRI site of plasmid pLS16. The oligonucleotides employed are listed in Table 3.

#### Primer extension.

An SdsR +59R primer (5′-GCT TCT GGG AGA AGA CCG-3′) was 5′ end-labeled with [γ−32P]ATP using T4 polynucleotide kinase. The primer was then used to analyze sdsR(−379/+131)-CAT fusion transcripts. Total cellular RNA was isolated from cells carrying the sdsR(−379/+131)-CAT fusion plasmid. The labeled primer was used for primer extension analysis, as previously described.

| Name | Description | Source |
|------|-------------|--------|
| MG1655 | E. coli MG1655 wild type | Laboratory stock |
| ryeAPm | MG1655 chromosomal ryeA promoter mutant (mutation on -10 promoter substituted to XhoI site) | This study |
| sdsRPm | MG1655 chromosomal sdsR promoter mutant (mutation on -10 promoter substituted to XhoI site) | This study |
| MG1655ΔsdsR/ryeA | MG1655ΔryeA/sdsR-kan | This study |
| DJ480 | E. coli DJ480 wild type used for lacZ fusion | This study |
| DJ480ΔsdsR/ryeA | P1 transduction from MG1655ΔsdsR/ryeA | This study |

| Name | Description | Source |
|------|-------------|--------|
| pWRG99 | Plasmid carrying temperature-sensitive λ-red recombinase and I-SceI endonuclease | This study |
| pWRG100 | Template DNA plasmid for constructing scarless mutants | This study |
| pKK232-8 | A plasmid encoding cat gene encoding cam acetyltransferase (CAT) for chloramphenicol resistance | This study |
| pL2A | A derivative of pGEM3 carrying B. albidum tRNAArg | This study |
| pRS1553 | A cloning vector for construction of lacZ transcriptional fusion | This study |
| pHM4T | A derivative of pHM1, AmpR, IPTG-inducible RNA expression vector | This study |
| pRyeA-ara | A derivative of pHM4T carrying ryeA full sequence, with a mutation on -10 element of sdsR | This study |
| pSdsR-ara | A derivative of pHM4T carrying sdsR full sequence | This study |
| pLS16 | In vitro transcription template vector | This study |
| pAKA-ara | A derivative of pAKA, TcR, arabinose-inducible RNA expression vector | This study |
| pRyeA-ara | A derivative of pAKA-ara carrying ryeA full sequence | This study |
| pSdsR-ara | A derivative of pAKA-ara carrying sdsR full sequence | This study |

Table 2. Strains and plasmids used in this study.
| Name            | Sequence 5’-3’                  | Use                                                                 |
|-----------------|--------------------------------|----------------------------------------------------------------------|
| RyeA-p1         | GCGGCCGCAACTGCTCAAGACAACC      | Chromosomal ryeA -10 promoter mutant cloning                          |
| RyeA-p2         | TCACTCGAGACACACTCTCTACTG       | Chromosomal ryeA -10 promoter mutant cloning                          |
| RyeA-p3         | TGTTGCTTCGAGGTGACTATAAAG       | Chromosomal ryeA -10 promoter mutant cloning                          |
| RyeA-p4         | CAAGCTTGGAAACCTTGGGCTCTGC      | Chromosomal ryeA -10 promoter mutant cloning                          |
| SdsR-p1         | GCGGCCGCAACTGCTCAAGACAACC      | Chromosomal sdsR -10 promoter mutant cloning                          |
| SdsR-p2         | TCACTCGAGACACACTCTCTACTG       | Chromosomal sdsR -10 promoter mutant cloning                          |
| SdsR-p3         | TGTTGCTTCGAGGTGACTATAAAG       | Chromosomal sdsR -10 promoter mutant cloning                          |
| SdsR-p4         | CAAGCTTGGAAACCTTGGGCTCTGC      | Chromosomal sdsR -10 promoter mutant cloning                          |
| SdsR-pRcom1     | ATAGCCTTTTGATTTCCAAT           | Chromosomal sdsR -10 promoter mutant cloning                          |
| SdsR-pRcom2     | CTGTATTCGGTCCAGGGAAA           | Chromosomal sdsR -10 promoter mutant cloning                          |
| 5RACE_F         | GATATGCGCGAATTCCTGTAGAAC       | 5’ RACE primer                                                       |
| 5RACE_RyeAR     | CCGCCTCAGTTCTTTCAACC           | 5’ RACE GSP1 primer                                                  |
| 5RACE_SdsRR     | GAGCATTGGCCTGCTCTGGAACACAGT    | 5’ RACE GSP2 primer                                                  |
| 3RACE_RyeAF     | AAATGCGCCGGAACCGAAATGC         | 3’ RACE primer                                                       |
| 3RACE_SdsRF     | GCAAGGCAACTAAGCCTGCA           | 3’ RACE primer                                                       |
| 5RACE_Nested    | CCTGTAGAAGGAAACGACTAGAAG       | 5’ RACE primer                                                       |
| 5RACE_Nested_RyeAR | GCAAAGGGCACAATGCTGCA         | 5’ RACE primer                                                       |
| 5RACE_Nested_SdsRR | TCGGTCAGGGGAAGGTTGGCTC       | 5’ RACE GSP2 primer                                                  |
| 5RACE_R         | GAGCATTGGCCTGCTCTGGAACACAGT    | 3’ RACE primer                                                       |
| 5RACE_RyeAF     | AAATGCGCCGGAACCGAAATGC         | 3’ RACE primer                                                       |
| 3RACE_SdsRF     | GCAAGGCAACTAAGCCTGCA           | 3’ RACE primer                                                       |
| 5RACE_RyeAF     | AAATGCGCCGGAACCGAAATGC         | 3’ RACE primer                                                       |
| 3RACE_SdsRF     | GCAAGGCAACTAAGCCTGCA           | 3’ RACE primer                                                       |
| 5RACE_Nested    | CGGCCGCTAAAGGACAGTGAAG         | 3’ RACE primer                                                       |
| 3RACE_Nested_RyeAR | GCAAAGGGCACAATGCTGCA         | 3’ RACE primer                                                       |
| 3RACE_Nested_SdsRR | TCGGTCAGGGGAAGGTTGGCTC       | 3’ RACE primer                                                       |
| AvrArCF         | CCGTTCGCTCTGCTGGGCCCCC        | pBAD-AraC cloning into pAKA                                            |
| AvrCREcoRI      | CGGGATCCAGCTGATGACCACC         | pBAD-AraC cloning into pAKA                                            |
| HD3pphAPF       | CCCAAGCTTGGGAAGGGTGTGCTG      | −379 +222 plating into pLS16                                           |
| ERIphhAR        | CGGAATTCGGCTCTGCTGACTACTAGCCTA | −379 +222 plating into pLS16                                           |
| BamHIHisRPF     | CGGGATCCGCTGCTCTGCTGCTG       | sdsR-CAT fusion                                                       |
| HinDIIIHisRPR   | CGGAATTCGCTTGGCTCTTGCC        | sdsR-CAT fusion                                                       |
| HINDIIIryeAPF   | CGGAATTCGCTTGGCTCTTGCTG       | ryeA-CAT fusion                                                       |
| BAMYrhyeAPR     | CGGGATCTTGGCGCTCTGCTG         | ryeA-CAT fusion                                                       |
| BAMYHI-379PF    | CGGGATCTTGGCGCTCTGCTG         | sdsR(-379 +311)-CAT fusion                                            |
| HinddI +131R    | CCCAAGCTTGGGAAGGGTGTGCTG      | sdsR(-379 +311)-CAT fusion                                            |
| tRNA_F          | TACAAGCTCTGGCTGTAGGCTG         | sdsR-trNA-3’-CAT fusion                                               |
| tRNA_R          | TATAAGCTTTGGCTGTCGGGCGGGG     | sdsR-trNA-3’-CAT fusion                                               |
| ERI-379F        | CGGAATTCGGCTCTGCTGCTG         | sdsR-lacZ fusion                                                      |
| ERI-79F         | CGGAATTCAGCTTGGCTGACACC       | sdsR-lacZ fusion                                                      |
| ERI +104F       | CGGAATTCATGCTTAAAGAAGCCAGAACG | sdsR-lacZ fusion                                                      |
| ERI +142F       | CGGAATTCATGCTTAAAGAAGCCAGAACG | sdsR-lacZ fusion                                                      |
| ERIA-80F        | CGGAATTCGGCTCTGCTGCTGACTAATCC | ryeA-lacZ fusion                                                      |
| BHI-170R        | CGGGATCCGCTCTGCTGCTGGAATAGT   | sdsR-lacZ fusion                                                      |
| BHI +111R       | CGGAATTCGCTTGGCGGCCCCC        | sdsR-lacZ fusion                                                      |
| BHI +222R       | CGGAATTCGCTTGGCGGCCCCC        | sdsR-lacZ fusion                                                      |
| BHI +237R       | CGGGATCCGCTTGGCGGCCCCC        | sdsR-lacZ fusion                                                      |
| BHI +140R       | CGGGATCCGCTTGGCGGCCCCC        | ryeA-lacZ fusion                                                      |
| CAT_R           | GGTGTTATACCGCTGTTTTTTC        | Primer extension primer for RyeA and SdsR                             |
| SdsR + 59R      | GCTCTTGGGAGAGCTGGAAG          | Primer extension primer for pphA                                     |
| RyeA_NP         | CGGGATCCGCTTGGCTTCTACTTCTG    | RyeA Northern probe                                                  |
| SdsR_NP         | CGGGATCCGCTTGGCTTCTACTTCTG    | SdsR Northern probe                                                  |
| 5SRNA + 90R     | GAGACCCACATCCCACATCTGG        | 5S RNA Northern probe                                                 |
| tRNA_NP         | GGAACCTTGACTCTTCCCTGAGCTA     | tRNA Northern probe                                                   |
| pphA_F          | TATCAGGAGAATTGCGCCTATC        | pphA mRNA qRT-PCR                                                    |
| pphA_R          | AGCTCTCAGGTCTGCTCTGCTGAGTA    | pphA mRNA qRT-PCR                                                    |
| asmA_F          | GATGGCCGCGGGAGATAACT          | asmA mRNA qRT-PCR                                                    |
| asmA_R          | CTTGCTGCTGCTGCTGCTGCTTAA     | asmA mRNA qRT-PCR                                                    |
| aphA_F          | GAGCAAGCAGCAGGGAATAGC         | aphA mRNA qRT-PCR                                                    |
| aphA_R          | CGGGATCCGCTGGGAATAGC          | aphA mRNA qRT-PCR                                                    |
| stpA_F          | CGGCGTCAGGGCGAATATAAAA       | stpA mRNA qRT-PCR                                                    |
| stpA_R          | AGATCGAGGAAATCGTGGAGAGA       | stpA mRNA qRT-PCR                                                    |

Continued
The table below shows the oligonucleotide sequences used in the study:

| Name         | Sequence 5′-3′                                      | Use                          |
|--------------|-----------------------------------------------------|------------------------------|
| ytfE_F       | GTGAACGCGCGCTCTTATT                                 | ytfE mRNA qRT-PCR            |
| ytfE_R       | GGGTGTACGCGGAGTTTG                                 | ytfE mRNA qRT-PCR            |
| ynfF_R       | TCAAGCTGCAAGGGTTTAC                                 | ynfF mRNA qRT-PCR            |
| ytfE_R       | GGGCTTCGTCCCAACTTATC                                 | ytfE mRNA qRT-PCR            |
| tolC_F       | TAG TAA CCC GGA ATT GCG TAA G                       | tolC mRNA qRT-PCR,18         |
| tolC_R       | AGC CGT TGC TAT AGG TGA T AAT C                    | tolC mRNA qRT-PCR,18         |
| putA_F       | GGCCAATGAGCCAGATGAA                                 | putA mRNA qRT-PCR            |
| putA_R       | GCTGTTCAGCCAGCATAGA                                 | putA mRNA qRT-PCR            |
| gapA_F       | GCACACCAACTGGCTGGCT                                 | gapA mRNA qRT-PCR,30         |
| gapA_R       | GCCGCCGCGCATCTTGTGA                                 | gapA mRNA qRT-PCR,30         |
| PM_RyeAR     | GCCTGACTTTTATAGCTACGAGACACAACACTCTACTGTCC          | ryeA promoter mutation       |
| SdsRtMR      | TCCAGGGAAATGGCTCTTGGGAGAGGCCGTCG                  | sdsR terminator mutation     |
| SdsRtMF      | CATTTCCCTGGAGGCAATACAG GAACTCCTGT                  | sdsR terminator mutation     |
| PM_SdsRF     | CCTTACTCGAGGATGCGACGCCAG                         | sdsR promoter mutation       |
| PM_SdsRR     | GTCATCCTCAGATGAAAGGCAGACGCCAG                       | sdsR promoter mutation       |

**Table 3. Oligonucleotide sequences.**

**RACE assays.** 5′ and 3′ RACE analysis for RyeA and SdsR were performed as described previously47. Total cellular RNAs from mid-exponential phase (4h) and stationary phase (8h) cells were used for RyeA and SdsR, respectively. For 5′ RACE total cellular RNA was treated with 5 unit of *E. coli* RppH (New England Biolabs) in a 50 μl reaction before RNA ligation.

**In vitro transcription by *E. coli* RNA polymerase.** In vitro transcription using *E. coli* RNA polymerase was performed as described previously46. *E. coli* RNA polymerase holoenzyme *E. coli* RNA polymerase was purchased from New England Biolabs. σ7 (RpoS) was purified using the plasmid described previously46. σ7 was reconstructed by combining the core enzyme (New England Biolabs) and σ7 in a molar ratio of 1:2. After 1 h reaction at 37 °C, the reaction was terminated by adding phenol/chloroform.

**Northern blotting.** *E. coli* cells were grown overnight in LB broth in the presence of appropriate antibiotics. Overnight cultures were diluted 1:100 in fresh LB medium and further cultured at 37 °C. Total cellular RNA was extracted at the desired time points using the acidic hot-phenol method, as described previously25. RNA was generated in vitro using the T7 RibomAX Express Large Scale RNA Production System (Promega). Northern blot analysis was carried out as described previously35. Briefly, 5–10 μg of total RNA was fractionated on a 5% polyacrylamide gel containing 7 M urea and electropheramotransferred to a Hybond-XL membrane (Amersham Biosciences). Membranes were hybridized with 32P-labeled DNA probes in PerfectHyb Plus Hybridization Buffer (Sigma Aldrich) and analyzed using an FLA 7000 Image Analyzer (Fuji).

**Mutual degradation assays of SdsR and RyeA.** MG1655ΔsdsR/ryeA cells were co-transformed with a plasmid pair of pSdsR-ara/pRyeA or pRyeA-ara/pSdsR. Overnight cultures of the transformed cells were diluted (1:100) into LB medium containing ampicillin (100 μg/ml) and tetracycline (10 μg/ml), and grown at 37 °C for 2 h. Arabinose of 0.2% was added into the culture and cells were grown for 5 min or 25 min. Then 0.1 mM IPTG was added into each culture and cells were further grown further. Aliquots of the cell culture were taken in intervals and their RyeA and SdsR contents were analyzed by Northern blotting.

**RNA-seq sample preparation and analysis.** *E. coli* MG1655 wild-type, ryeA promoter mutant, and sdsR promoter mutant cells were grown at 37 °C overnight. After diluting 1:100, overnight cultures were grown for 3 h (exponential phase) and 8 h (stationary phase); total RNA was extracted at each point. All preparation procedures were as previously described18. All RNA sequencing and alignment procedures were conducted by ChunLab. Ribosomal RNA was depleted using a Ribot-one rRNA removal kit (Epicentre) according to the manufacturer’s instructions. Libraries for Illumina sequencing were generated using a TruSeq Stranded mRNA Sample Prep kit (Illumina) according to the manufacturer’s protocol. RNA sequencing was performed on the Illumina HiSeq 2500 platform using single-end 50-bp sequencing. Sequence data for the reference genome were retrieved from the NCBI database. Quality-filtered reads were aligned to the reference genome sequence using Bowtie2.

Transcript abundance was measured as Relative Log Expression (RLE). To screen for mRNAs whose levels differed by more than 2-fold versus wild-type cells, we filtered for mRNAs with EdgeR p-values < 0.05. For GO analyses, the 171 genes and 55 genes found to be up- or downregulated in the exponential phase and stationary phase, respectively, were compared. The sdsR promoter mutation was sorted according to their GO categories (http://amigo.geneontology.org)38.

**LacZ assay.** Three colonies for each strain were cultured overnight in LB medium with/without ampicillin (100 μg/ml), after which overnight cultures were diluted 1:100 and cultured in fresh medium containing 0.02% arabinose and 1 mM IPTG. Cultures were incubated for 2 h for exponential phase and 10 h for stationary phase. LacZ activity was assayed as described previously48.
Semi-quantitative RT-PCR and qRT-PCR. RNA extraction and DNase treatment were performed as described above for RNA-seq. cDNA was synthesized using M-MLV reverse transcriptase (Enzymomics) with specific primers for semi-quantitative RT-PCR. qRT-PCR was performed on an Exicycler 96 system (Bioneer) using Prime Q-master mix (Genet Bio). Expression levels of each mRNA of interest were normalized to that of the gapA gene. All experiments were performed according to the manufacturer’s instructions.

Data Availability
RNA-seq raw data for this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and are accessible through the GEO Series accession number GSE122921.

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**Author Contributions**

Y.L., J.S.C. and H.P. designed the research. J.S.C., H.P. and W.K. performed the experiments and analyzed the data. Y.L. and J.S.C. co-wrote the manuscript.

**Additional Information**

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