

1 **Determinants of target prioritization and regulatory hierarchy for the bacterial**  
2 **small RNA SgrS**

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35 **ABSTRACT**

36           The mechanisms by which small RNA (sRNA) regulators select and prioritize  
37 target mRNAs remain poorly understood, but serve to promote efficient responses to  
38 environmental cues and stresses. We sought to uncover mechanisms establishing  
39 regulatory hierarchy for a model sRNA, SgrS, found in enteric bacteria and produced  
40 under conditions of metabolic stress when sugar transport and metabolism are  
41 unbalanced. SgrS post-transcriptionally controls a nine-gene regulon to restore growth  
42 and homeostasis under stress conditions. An in vivo reporter system was used to  
43 quantify SgrS-dependent regulation of target genes and established that SgrS exhibits a  
44 clear preference for certain targets, and regulates those targets efficiently even at low  
45 SgrS levels. Higher SgrS concentrations are required to regulate other targets. The  
46 position of particular targets in the regulatory hierarchy is not well-correlated with the  
47 predicted thermodynamic stability of SgrS-mRNA interactions or the SgrS-mRNA  
48 binding affinity as measured in vitro. Detailed analyses of SgrS interaction with *asd*  
49 mRNA demonstrate that SgrS binds cooperatively to two sites and remodels *asd* mRNA  
50 secondary structure. SgrS binding at both sites increases the efficiency of *asd* mRNA  
51 regulation compared to mutants that have only a single SgrS binding site. Our results  
52 suggest that sRNA selection of target mRNAs and regulatory hierarchy are influenced  
53 by several molecular features. The sRNA-mRNA interaction, including the number and  
54 position of sRNA binding sites on the mRNA and cofactors like the RNA chaperone Hfq  
55 seem to tune the efficiency of regulation of specific mRNA targets.

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57

58 **IMPORTANCE**

59           To survive, bacteria must respond rapidly to stress and simultaneously maintain  
60 metabolic homeostasis. The small RNA (sRNA) SgrS mediates the response to stress  
61 arising from imbalanced sugar transport and metabolism. To coordinate the stress  
62 response, SgrS regulates genes involved in sugar uptake and metabolism. Intrinsic  
63 properties of sRNAs such as SgrS allow them to regulate extensive networks of genes.  
64 To date, sRNA regulation of targets has largely been studied in the context of “one  
65 sRNA-one target”, and little is known about coordination of multi-gene regulons and  
66 sRNA regulatory network structure. Here, we explore the molecular basis for regulatory  
67 hierarchy in sRNA regulons. Our results reveal a complex interplay of factors that  
68 influence the outcome of sRNA regulation. The number and location of sRNA binding  
69 sites on mRNA targets and the participation of an RNA chaperone dictate prioritized  
70 regulation of targets to promote an efficient response to stress.

71

## 72 INTRODUCTION

73 Bacteria live in diverse niches, often encountering rapidly changing and stressful  
74 environments. Bacterial stress responses can mitigate the negative effects of stress on  
75 cell structure and function. Usually stress responses are coordinated by molecules—  
76 either RNAs or proteins, that alter expression of a regulon comprised of multiple genes.  
77 Coordinated control of the regulon prepares the cell to survive or adapt to the stress (1,  
78 2). Proteins control expression of target regulons by binding to DNA sequences and  
79 modulating the frequency of transcription initiation, but RNAs often modulate gene  
80 expression post-transcriptionally. A prevalent type of RNA regulator in bacteria is  
81 referred to simply as small RNA (sRNA). These sRNAs are often produced in response  
82 to a particular stress, and regulate target mRNAs through base pairing interactions that  
83 modify mRNA translation or stability (3, 4). In diverse bacteria, hundreds of sRNAs have  
84 been identified (5-7). While the majority of sRNAs have not been characterized, many  
85 studies suggest that sRNA regulatory networks are as extensive and complex as those  
86 controlled by proteins (8, 9).

87 A large body of work has illuminated base pairing-dependent molecular  
88 mechanisms of post-transcriptional regulation by sRNAs (10, 11). The sRNA SgrS  
89 (sugar-phosphate stress sRNA) has been an important model for discovery of both  
90 negative and positive mechanisms of target mRNA regulation. SgrS is induced in  
91 response to metabolic stress associated with disruption of glycolytic flux and  
92 intracellular accumulation of sugar phosphates (also referred to as glucose-phosphate  
93 stress) (12, 13). SgrS regulates at least 9 genes and promotes recovery from glucose-  
94 phosphate stress. SgrS-dependent repression of mRNAs encoding sugar transporters

95 (*ptsG*, *manXYZ*) reduces uptake of sugars to prevent further sugar-phosphate  
96 accumulation (Fig. 1) (12, 14, 15). Activation of a sugar phosphatase (*yigL*) mRNA  
97 promotes dephosphorylation and efflux of accumulated sugars (16), and repression of  
98 other mRNAs is hypothesized to reroute metabolism to promote recovery from stress  
99 (Fig. 1) (17). Each target of SgrS is regulated by a distinct molecular mechanism. How  
100 different mechanisms of regulation yield effects of variable magnitude with respect to  
101 mRNA stability and translation is an open question.

102       Temporally-ordered and hierarchical patterns of gene regulation carried out by  
103 protein transcription factors have been characterized in many systems (18-21). These  
104 regulatory patterns allow cells to efficiently respond to environmental signals by  
105 prioritizing induction or repression of products needed to respond to those signals.  
106 Protein regulators establish a hierarchy of regulation based on their affinities for binding  
107 sites in the operator regions of different target genes. As the concentration of active  
108 regulator increases, genes are sequentially regulated based on binding site affinity (22).  
109 There is growing evidence that sRNAs also regulate their target genes hierarchically  
110 (23, 24). However, the mechanisms involved in establishing and maintaining prioritized  
111 regulation of sRNA targets are not known.

112       We hypothesize that the temporal progression of target regulation by SgrS is  
113 specifically optimized to promote efficient recovery from glucose-phosphate stress (Fig.  
114 1). To test this hypothesis, first defined the efficiency of SgrS regulation of each target  
115 and found that SgrS indeed prioritizes regulation of some targets over others. We  
116 examined the factors that determine regulatory efficiency, including the the arrangement  
117 and strength of SgrS target binding sites and the roles of other factors like RNase E and

118 Hfq. Detailed characterization of a specific SgrS-mRNA target interaction revealed  
119 cooperative binding of SgrS to two binding sites and a requirement for both binding sites  
120 for maximal SgrS-dependent regulation. Collectively, our results upheld the hypothesis  
121 that sRNAs regulate expression of genes in their target regulons hierarchically.  
122 Features of each sRNA-mRNA pair and molecular mechanisms of regulation precisely  
123 determine the regulatory priority for each target.

## 124 **RESULTS**

125 **SgrS differentially regulates targets at the level of translation.** Previous studies  
126 suggested the possibility of a hierarchy of regulatory effects carried out by the small  
127 RNA SgrS, which controls translation of a diverse set of mRNA targets (11, 12, 15, 16,  
128 25). To study this, we used a two-plasmid system to control expression of SgrS and  
129 target translational fusions (Fig. 2A). All target transcript fragments fused to *gfp* contain  
130 experimentally confirmed SgrS binding sites. Regulation of target translation by SgrS  
131 was measured as described previously (24).

132 To quantify translational regulation by SgrS and facilitate comparisons of  
133 regulatory efficiency among targets, we analyzed the data as described by Levine, et al.  
134 (24). Activity of reporter fusions was measured by monitoring GFP fluorescence over  
135 time. By plotting the GFP fluorescence (RFU) as a function of growth ( $OD_{600}$ ) for target-  
136 *gfp* fusions in the absence of SgrS, we defined “basal activity” at different inducer  
137 concentrations (example in Fig. S1A). This method of quantifying translational fusion  
138 activity accounts for the fact that fluorescence levels are not directly proportional to  
139 inducer concentrations ((24) and Fig. S1A). While the absolute values for basal activity  
140 differ among different target fusions, all fusions responded to induction in a dose-

141 dependent manner (Fig. S2A). Similar plots (RFU/OD<sub>600</sub>) were generated for each  
142 fusion induced in the presence of SgrS (examples Fig. S1B-F). We define “regulated  
143 activity” as the slope of the curve (RFU/OD<sub>600</sub>) under conditions where both the fusion  
144 and SgrS are induced (example in Fig. S1B). As levels of SgrS increase, clear patterns  
145 of repression or induction are seen for all target fusions (Figs S1B-F and S2B-F).

146 To define the efficiency of regulation of each target we plotted regulated activity  
147 as a function of basal activity for *ptsG*, *manX*, *asdl*, *purR*, and *yigL*. When there is no  
148 SgrS-mediated regulation, a line with a slope of 1 is seen for all targets (Fig. 2B-F).  
149 Slopes less than 1 indicate that the fusion is repressed by SgrS. This is true for *ptsG*,  
150 *manX*, *asdl* and *purR* reporter fusions (Figure 2B-E). Slopes greater than 1 are  
151 indicative of activation by SgrS, which is true only for *yigL* (Fig. 2F). Importantly, the  
152 magnitude of regulation was responsive to SgrS levels. As concentrations of SgrS  
153 inducer (aTc) increased, slopes of lines for repressed targets were correspondingly  
154 reduced (Fig. 2B-E). This was not the case for *yigL*, the only positively regulated target  
155 of SgrS (Fig. 2F). The magnitude of activation did not increase beyond a maximal level  
156 obtained at 20 ng/mL of inducer. While the basis of this difference is unclear, it likely  
157 reflects the inherently different molecular mechanisms of regulation: mRNA stabilization  
158 for *yigL* and translational repression for all other targets.

159 We then compared regulatory efficiency among different targets at different  
160 levels of SgrS induction. At the two lowest levels of SgrS induction (10ng/mL and 20  
161 ng/mL aTc), only *ptsG* and *yigL* showed substantial repression and activation,  
162 respectively (Fig. 3A, B). In contrast, *manX*, *asdl* and *purR* fusions yielded curves  
163 whose slopes remained at ~1, indicating no regulation at these lower levels of SgrS.

164 Our interpretation of these results is that *ptsG* and *yigL* are the high-priority or  
165 “strongest” targets of SgrS, since they are regulated preferentially when SgrS levels are  
166 low. With increasing SgrS levels (20-50 ng/ml aTc), regulation of “weaker” targets  
167 *manX*, *asdl* and *purR* became apparent (Fig. 3C, D, E). As SgrS levels increased, *ptsG*  
168 repression became more efficient up to a maximal repression at 40 ng/mL of aTc, and it  
169 remained the most strongly repressed target at all levels of SgrS. Collectively, these  
170 data suggest that SgrS targets are preferentially regulated in the following order: 1/2)  
171 *ptsG* and *yigL*, 3) *manX*, 4) *asdl*, and 5) *purR* (Fig. 3A-E). We hypothesize that the  
172 position of each target within the regulatory hierarchy is determined by characteristics of  
173 SgrS-target mRNA interactions and the mechanism of SgrS-dependent regulation.

174 **Differences in in vitro binding affinity are not correlated with regulation efficiency.**

175 One of the initial steps in sRNA-mediated regulation is formation of base-pairing  
176 interactions with the target mRNA. Binding of the sRNA with its target mRNA is  
177 dependent on sequence complementarity and RNA secondary structure. We examined  
178 the characteristics of SgrS-target mRNA binding in vitro to determine whether the  
179 strength of binding is correlated with the target hierarchy observed at the level of  
180 translation.

181 Electrophoretic mobility shift assays (EMSAs) were performed to measuring  
182 binding of SgrS to its target mRNAs *ptsG*, *manX*, *purR*, *yigL* and *asd*. Binding of SgrS to  
183 *ptsG* had a  $K_D$  of  $0.11 \pm 0.01 \mu\text{M}$  (Fig. 4A, B), which was lower than  $K_D$ s for SgrS  
184 binding to most of the other targets (Fig. 4A-E). SgrS-*manX* mRNA binding had a  $K_D$  of  
185  $19.7 \pm 2.78 \mu\text{M}$  (Fig. 4A, C) which is weaker than the interaction with *ptsG* (Fig. 4B), but  
186 stronger compared to *purR* and *yigL* (Fig. 4A). Three different fragments of *asd* mRNA



187 were tested, because previous work demonstrated that SgrS pairs at two distinct sites  
188 on *asd* mRNA (17). The first site, *asdl*, is adjacent to the ribosome binding site and is  
189 sufficient for modest SgrS-dependent translational repression. The second site, *asdll*, is  
190 in the coding sequence of *asd*, 60-nt downstream of the start codon. When both sites  
191 are present, *i.e.*, on *asdl-II*, stronger SgrS-dependent translational repression is  
192 observed (17). Surprisingly, while *asdl* (containing only the upstream SgrS binding site)  
193 regulation is less efficient compared to *manX* (Fig. 3A-E), *in vitro* it binds SgrS more  
194 strongly with a  $K_D$  of  $0.15 \pm 0.04 \mu\text{M}$  (Fig. 4A, D), which is comparable to SgrS-*ptsG*  
195 binding (Fig. 4A, B). SgrS interaction with *asdll* was very weak (Fig. 4A). We could not  
196 determine  $K_D$  values for SgrS interaction with *asdll*, *purR* and *yigL*, due to limitations in  
197 obtaining high enough concentrations of RNA, but it is apparent that SgrS binding to  
198 these targets is much weaker compared to *ptsG*, *manX* and *asdl* (Fig. 4A).

199 Results of EMSAs with SgrS and *asdl-II* (containing both SgrS binding sites)  
200 revealed apparent binding cooperativity. SgrS binding to *asdl-II* has a  $K_D$  of  $0.07 \pm 0.01$   
201  $\mu\text{M}$  (Fig. 4E, F), even slightly lower than that of SgrS-*ptsG* mRNA binding. Moreover, we  
202 observed two shifted species that correspond to one or two SgrS sRNAs pairing with a  
203 single *asdl-II* transcript (Fig. 4E).

204 **Structural analyses of SgrS-*asd* mRNA interactions.** Our data thus far indicate that  
205 SgrS regulates mRNA targets in a hierarchical fashion (Figs. 2, 3). SgrS-mRNA binding  
206 affinities alone do not explain the target hierarchy, as SgrS-*ptsG* mRNA and SgrS-*asd*  
207 mRNA interactions have very similar  $K_D$ s, but *ptsG* is much more efficiently regulated  
208 than *asd* at all concentrations of SgrS (Fig. 3). To further understand the features that

209 influence the efficiency of target regulation, we performed more detailed analyses of  
210 SgrS-*asd* mRNA interactions.

211 Previous work demonstrated that SgrS binding site I encompasses nt +31 to +49  
212 and site II from nt +110 to +127 ((17), Fig. 5A) We used IntaRNA (26, 27) to predict the  
213 free energy ( $\Delta G$ ) for SgrS interactions with *asd* mRNA segments containing both sites,  
214 or each site individually (Fig. 5B). IntaRNA accounts for the energy necessary to open  
215 double-stranded regions of RNA secondary structure, to make them accessible for  
216 pairing. We first analyzed SgrS interactions with *asdI-II* mRNA (encompassing nt +1 to  
217 +240), which we denote as “structured” (Fig. 5B). Interaction of SgrS with *asd* site I has  
218 a predicted  $\Delta G$  of -10.5 kcal/mol, while SgrS pairing with site II has a  $\Delta G$  of -1.1  
219 kcal/mol (Fig. 5B, structured). The  $\Delta G$  for interactions between SgrS and the isolated  
220 binding sites, are -18 kcal/mol for site I and -7.4 kcal/mol for site II (Fig. 5B, isolated).  
221 These predictions suggest that SgrS interaction with site II is less favorable, particularly  
222 in the context of the longer structured *asd* mRNA.

223 We investigated the structure of *asdI-II* with selective 2'-hydroxyl acylation  
224 analyzed by primer extension (SHAPE), where flexible nts are modified by N-  
225 methylisotoic anhydride (NMIA), while nts constrained in helices are not reactive. In the  
226 absence of SgrS, the sequence encompassing the *asd* ribosome binding site (+44 to  
227 +50) is located within a structured loop (+36 to +69) on top of a short stem (+31 to +35  
228 pairing with +70 to +74) (Fig. 5C, Fig. S3). The nts in site I (+31 to +49, Fig. 5A) are  
229 located on the 5' side of the stem-loop structure. Most of the nts in this structure are  
230 reactive, which is indicative of a flexible conformation that is accessible for ribosome  
231 binding or base pairing with the seed sequence of SgrS (Fig. 5C). The seed interaction

232 of SgrS likely promotes opening of the structure. Downstream of the site I stem-loop  
233 structure is a highly structured second stem (+83 to +155) that contains site II in the  
234 apical region (+110 to +129) (Fig. 5C, Fig. S3). Site II is sequestered in a helix and  
235 would not be accessible to base pair with SgrS (Fig. 5C). In light of binding cooperativity  
236 observed in Fig. 4E, we hypothesize that SgrS pairing with site I induces rearrangement  
237 of *asd* mRNA secondary structure to facilitate interaction with site II.

238 We next used SHAPE to probe changes to the *asdI-II* structure in the presence  
239 of SgrS. The reactivity of site I nt +31 to +49 decreased as the concentration of SgrS  
240 increased (Fig. 5D), with the exception of nt +41 which is not predicted to base pair with  
241 SgrS (Fig. 5A). The SHAPE reactivity plateaued between 5 and 10-fold excess SgrS  
242 (Fig. S3E,F), which suggests that binding to site I was saturated. This is consistent with  
243 a strong base-pairing interaction between SgrS and site I. In contrast, the reactivity of  
244 the site II nts +110 to +129 decreased more slowly and to a lesser extent (Fig. 5D),  
245 consistent with a weaker and cooperative interaction. Fewer site II nts showed changes  
246 in SHAPE reactivity upon addition of SgrS; this is likely due to the highly structured  
247 nature of site II in the absence of SgrS.

248 The reactivity of nts outside of the SgrS binding sites also changed in the  
249 presence of SgrS (Fig. 5E). When a mutant SgrS that is not predicted to bind to *asdI-II*  
250 was used, minimal changes in SHAPE reactivity were observed, which suggests that  
251 the changes in the presence of wild-type SgrS are due to the interactions between SgrS  
252 and *asdI-II* mRNA and not due to the presence of additional RNA in the system (Fig.  
253 5E). This indicates that SgrS binding changes the overall structure of the *asd* RNA. A  
254 secondary structure predicted using the SHAPE data suggests that these changes are

255 limited to opening the SgrS binding sites and extending the site II stem (Fig. 5C). It is  
256 worth noting an important caveat to these analyses. The structure prediction algorithms  
257 were not designed to account for intermolecular interactions, so this analysis may not  
258 be able to capture the *in vivo* relevant structure of *asd* mRNA in complex with SgrS.  
259 Nonetheless, SHAPE data are consistent with other analyses in demonstrating binding  
260 of SgrS to *asd* mRNA, prominently at site I and to a lesser extent at site II.

261 **Optimal repression by SgrS involves both pairing sites within *asd* mRNA.** To  
262 further investigate the role of the two SgrS pairing sites on *asd*, we performed stochastic  
263 optical reconstruction microscopy (STORM) coupled with single-molecule *in situ*  
264 hybridization (smFISH) to monitor SgrS regulation of *asd-lacZ* variants *asdl*, *asdII*, and  
265 *asdl-II* (Fig. 6A) at single molecule resolution. In these experiments bacteria were grown  
266 in the presence of L-arabinose to induce expression of chromosomal *asd-lacZ* variants.  
267 Glucose-phosphate stress was induced for 10 min by the addition of 1%  $\alpha$ -methyl D-  
268 glucopyranoside ( $\alpha$ MG). 3D super-resolution images show *asd-lacZ* mRNAs (Fig. 6B-D,  
269 green) and SgrS (Fig. 6B-D, red), as projected on 2D planes, with cells outlined.

270 Numbers of *asd-lacZ* mRNAs and SgrS sRNAs were counted and represented as “copy  
271 number per cell” in histograms, with average copy number per cell indicated above the  
272 histogram (Fig. 6B-D). SgrS induction reduced the copy number of *asdl-lacZ* mRNA by  
273 3-fold (Fig. 6B, green) when SgrS was induced to high levels after 10 min treatment with  
274  $\alpha$ MG (Fig. 6B, red and S4A, B). On the contrary, the copy number of *asdII-lacZ* mRNAs  
275 (Fig. 6C, green) was not strongly affected in the presence of high SgrS levels after  $\alpha$ MG  
276 treatment (Fig. 6C, red and Fig. S4C, D). Copy numbers of *asdl-II-lacZ* mRNA (Fig. 6D,  
277 green) were reduced by ~8-fold after 10 min of  $\alpha$ MG induction (Fig. 6D, red, Fig.

278 S6E,F). These data demonstrate that both binding sites on *asd* mRNA are important for  
279 efficient SgrS-dependent regulation of mRNA stability.

280 We next examined the roles of the two SgrS binding sites in the efficiency of  
281 translational regulation. SgrS regulation of an *asdI-II* translational fusion was compared  
282 to regulation of an *asdI* fusion (Fig. 7A). By plotting regulated activity as a function of  
283 basal activity as described above, we determined that SgrS repression of *asdI-II* was  
284 more efficient than repression of *asdI* across the entire range of SgrS expression levels  
285 (Fig. 7B), a result in line with our previous study (17). Comparison to other targets  
286 indicated that *asdI-II* is regulated more efficiently than *manX*, *asdI* and *purR*, at all  
287 concentrations of SgrS (Fig. 7C).

288 We then compared SgrS regulation of *asdI* and *asdI-II* in the *rne701* mutant  
289 strain deficient in degradosome assembly (28). We determined basal activity (Fig. S5A)  
290 and regulated activity (Fig. S5B-F) of *asdI* and *asdI-II* translational *gfp* fusions at  
291 different levels of SgrS induction. Reminiscent of our data in the wild-type strain (Fig.  
292 7A), SgrS regulated *asdI-II* more efficiently compared to *asdI* in the *rne701* mutant (Fig.  
293 7D). Moreover, when compared to SgrS regulation of other targets, *asdI-II* was  
294 repressed most efficiently (Fig. 7E) in the *rne701* mutant. Taken together the data  
295 indicate that the second binding site on *asd* mRNA enhances the stringency of SgrS-  
296 mediated regulation. Moreover, addition of the second binding site on *asd* changes its  
297 regulatory priority relative to other targets in the SgrS regulon.

298 **Transcriptional regulation of *asd* by SgrS.** We observed that the *asdI-II* transcript is  
299 more abundant than *asdI* (Fig. 6B, D). Since the constructs used in that experiment  
300 were expressed from a heterologous promoter, we postulate that increased levels of

301 *asdI-II* mRNA compared to *asdI* mRNA must be due to increased mRNA stability or  
302 transcription elongation of the *asdI-II* construct compared to *asdI*. We constructed *asdI*  
303 and *asdI-II* transcriptional fusions to *lacZ* expressed from an inducible promoter (Fig.  
304 8A) to test whether SgrS can regulate *asd* transcripts independent of translational  
305 regulation. Consistent with observations from smFISH, the *asdI-II-lacZ* transcriptional  
306 fusion had substantially higher activity compared to *asdI-lacZ* (Fig. 8B). While SgrS  
307 regulated both reporter fusions, *asdI-II* repression was more efficient (3.3-fold  
308 repression) than *asdI* (2.1-fold repression) (Fig. 8B). SgrS still regulated both fusions in  
309 the *rne701* mutant strain (Fig. 8B). Importantly, SgrS-dependent degradation of other  
310 SgrS targets *ptsG* mRNA (29), and *manXYZ* mRNA (15, 25) was abolished in  
311 degradosome mutants. Together with our previous work, these observations suggest  
312 that SgrS regulates *asd* by two independent mechanisms: translational repression by  
313 pairing at site I (directly occluding the ribosome binding site) and reducing mRNA levels  
314 by promoting mRNA turnover and/or inhibiting transcription elongation.

## 315 **DISCUSSION**

316 In this study, we set out to define the hierarchy of regulation by a model bacterial  
317 sRNA. SgrS is a good model for this study because it has a modestly-sized regulon,  
318 and the mechanisms of regulation of several targets have been characterized in detail  
319 (16, 17, 25, 30). Our results demonstrate a clear pattern of prioritized regulation of  
320 mRNA targets (Fig. 2B-F, Fig. 3A-E). Two targets in particular, *ptsG* and *yigL*, were  
321 “high-priority” targets that were efficiently regulated even at low levels of SgrS  
322 production. Other targets, *manX*, *purR*, and *asd*, were less impacted by SgrS and were  
323 only regulated when SgrS was produced at higher levels.

324 We investigated features of sRNA-mRNA interactions that could impact the  
325 overall efficiency of SgrS-mediated regulation. In vitro SgrS-mRNA interactions as  
326 measured by EMSA defined  $K_D$ s that were not well-correlated with in vivo regulatory  
327 efficiency (Fig. 4A-F, Fig. 3A-E). Two targets stood out in the comparison of in vivo  
328 regulation and in vitro SgrS-mRNA interactions. The *yigL* mRNA interaction with SgrS  
329 was barely detectable in vitro (Fig. 4A), but in vivo, *yigL* translation was maximally  
330 activated at low SgrS levels (Fig. 2F). Conversely, the translation of *asdI* was modestly  
331 regulated by SgrS in vivo (Fig. 2E), but the in vitro SgrS-*asdI* interaction was  
332 comparable to that of SgrS-*ptsG*, the strongest in vivo regulatory effect. These apparent  
333 contradictions between in vitro interactions and in vivo regulatory efficiency led us to  
334 further explore SgrS regulation of *asd*.

335 Previous work demonstrated that SgrS has two binding sites on *asd* mRNA: site I  
336 overlaps that *asd* ribosome binding site and site II is ~60 nt downstream in the *asd*  
337 coding sequence ((17) and Fig. 5A). EMSAs demonstrated SgrS pairing at site I alone,  
338 but pairing at site II alone was undetectable. Binding of SgrS to an *asd* mRNA  
339 containing both sites I and II was cooperative (Fig. 4E,F). Structural analyses of *asd*  
340 mRNA in the absence and presence of SgrS demonstrated that SgrS indeed pairs  
341 preferentially at site I over site II and induces substantial structural rearrangement in the  
342 mRNA (Fig. 5C-E, Fig. S3). Quantification of SgrS-dependent degradation of *asd* mRNA  
343 showed that site I is important, but sites I and II together promote the most efficient  
344 regulation (Fig. 6B-D, Fig. S4). Similar to binding and regulation of mRNA degradation,  
345 SgrS interactions at both sites I and II on *asd* mRNA improve the efficiency of  
346 translational regulation (Fig. 7B,C, Fig. S2). These results suggest that the number and

347 position of sRNA binding sites on mRNA targets control regulation in vivo in ways that  
348 could not be predicted based on in vitro characterization of sRNA-mRNA binding.

349 In many cases, sRNA-mediated regulation of translation is thought to indirectly  
350 affect mRNA stability by making untranslated mRNA more susceptible to degradation by  
351 RNase E. There are also examples of sRNA regulation, including SgrS regulation of  
352 *yigL* (16), where modulation of mRNA stability is translation-independent. Truncation of  
353 RNase E (encoded by *rne*), removing the C-terminal scaffold for degradosome  
354 assembly, often prevents sRNA-mediated degradation of mRNA targets (15, 31, 32). If  
355 translational regulation is the primary function of an sRNA on a given mRNA target, the  
356 regulation should be preserved in *rne* mutant backgrounds. For SgrS targets, the  
357 regulatory hierarchy is mostly preserved in an *rne701* degradosome mutant background  
358 (Fig. 7, compare C and E), suggesting that for most targets, regulation of RNA stability  
359 is not the primary mechanism of control by SgrS. Interestingly, the high-priority target  
360 *ptsG* was a notable exception. In the wild-type background, *ptsG* is the most efficiently-  
361 regulated target (Fig. 7C), whereas in the *rne701* host, it is weakly regulated. This  
362 defect could be overcome by increasing SgrS expression levels (Fig. S6B). This result  
363 suggests that RNase E-dependent degradation of *ptsG* mRNA is more important for its  
364 efficient regulation by SgrS compared to other targets, where efficient regulation does  
365 not depend on subsequent target degradation. This is consistent with the fact that *ptsG*  
366 mRNA levels decrease at least 10-fold whereas other targets exhibit a modest 2-fold  
367 decrease in mRNA levels upon SgrS expression (17). Our recent study quantifying  
368 SgrS-dependent mRNA degradation at single molecule resolution indicated that *ptsG*  
369 mRNA exhibits faster degradation kinetics than *manXYZ* mRNA (31), which could



370 enhance the efficiency of regulation in a wild-type but not *rne701* mutant background  
371 where translational regulation and mRNA degradation are uncoupled.

372 One of our ultimate goals is to define at a molecular level the mechanisms by  
373 which sRNAs select and prioritize regulation of their targets. The current study  
374 implicates features of sRNA-mRNA interactions such as number and strength of sRNA  
375 binding sites on each mRNA target and accessory factors such as RNase E in dictating  
376 regulatory hierarchy. Another factor that is likely to play an important role in setting  
377 regulatory priority is the RNA chaperone Hfq. EMSAs demonstrated Hfq binding to  
378 *ptsG*, *manX*, *purR*, *yigL*, *asdl*, *asdlI* and *asdl-II* mRNAs (Fig. S7A) with similar  $K_D$  values  
379 for all targets (Fig. S7B). Previous work has shown that sRNAs compete for binding to  
380 Hfq, and this competition affects their regulatory ability (33, 34). Different sRNAs can  
381 bind to distinct sites on Hfq and this impacts their regulation of particular targets (34,  
382 35). Additional work will be required to determine what role Hfq plays in establishing the  
383 hierarchy of regulatory effects in sRNA regulons.

384 Most sRNA-mRNA interactions are conceived of as single binding site  
385 interactions, but we have already identified two SgrS targets that deviate from this  
386 model and have shown that additional binding sites can play important roles in  
387 regulation and change regulation efficiency (17, 25). We have not yet discovered the  
388 specific mechanism of regulation of *asd* mRNA by SgrS, but have shown definitively  
389 that both binding sites are required for strong regulation. SgrS-dependent control of  
390 both transcriptional and translational *asd* reporter fusions is not impacted in RNase E  
391 degradosome deficient strains (Fig. 7B,D, Fig. 8B), suggesting that the regulation is not  
392 strictly dependent on translation or mRNA turnover. Future work will test the hypothesis

393 that SgrS acts on *asd* mRNA at the level of transcription elongation, perhaps by an  
394 attenuation mechanism.

395 In *Vibrio*, quorum sensing-regulated Qrr sRNAs regulate multiple targets by  
396 distinct mechanisms and differences in those mechanisms influence the dynamics and  
397 strength of regulation (23). Strong and rapid regulation is achieved by sRNAs acting  
398 catalytically where the sRNA pairs with and promotes mRNA degradation but is then  
399 recycled for use on another mRNA target. A sequestration mechanism, where formation  
400 of the sRNA-mRNA complex is the terminal outcome of regulation, results in slower and  
401 weaker sRNA-dependent regulation of the target mRNA. For Qrr sRNAs, these  
402 regulatory mechanisms seem to depend on which region of the sRNA is pairing with a  
403 given target and whether the sRNA-mRNA interaction is strong or weak (23). While  
404 some of the same features of SgrS-mRNA interactions may be relevant in determining  
405 regulatory efficiency, we note that the SgrS seed sequence responsible for pairing with  
406 all mRNA targets characterized thus far is encompassed by a short (~20 nt) mostly  
407 single-stranded region of SgrS (12, 15-17). Moreover, we did not see a good correlation  
408 between strong versus weak binding in vitro and in vivo regulatory efficiency. It may be  
409 true that the “rules” governing regulatory efficiency and specific outcomes are different  
410 for each individual sRNA. Work on more model sRNAs will be needed to illuminate  
411 broad general principles.

412 Beyond defining interesting molecular features of sRNA-mRNA interactions,  
413 defining regulatory hierarchy for sRNA regulons is important for understanding bacterial  
414 physiology. The vast majority of sRNA regulons remain undefined, and thus sRNA  
415 functions unknown. For novel sRNAs, distinguishing high-priority from weaker targets

416 may provide crucial clues to the predominant role of the sRNA in cell physiology. For  
417 SgrS, the regulatory hierarchy we have defined here is perfectly consistent with growth  
418 studies demonstrating the primary importance of SgrS regulation of sugar transport and  
419 efflux under glucose-phosphate stress conditions (36). The hierarchy of regulation by  
420 sRNAs likely evolved to promote rapid and efficient responses to environmental signals  
421 that would provide cells with a competitive growth advantage in their specific niche. It  
422 will be critical to develop tools to more rapidly characterize sRNA regulatory hierarchy to  
423 better understand functions of the hundreds of uncharacterized sRNAs in diverse  
424 bacteria.

## 425 **MATERIALS AND METHODS**

426 **Strain and plasmid construction.** List of strains and plasmids used in this study are  
427 listed in Table S1. All strains used in this study are derivatives of *E. coli* K-12 strain  
428 MG1655. Oligonucleotide primers and 5' biotinylated probes used in this study are listed  
429 in Table S2 and were acquired from Integrated DNA Technologies. Chromosomal  
430 alleles were moved between strains by P1 *vir* transduction (37) and inserted using  $\lambda$   
431 Red recombination (38, 39).

432 Translational reporter fusion alleles  $P_{BAD}$ -*asdI-II-lacZ* (MBP151F/MBP193R  
433 primers),  $P_{BAD}$ -*asdI-lacZ* (MBP151F/MBP151R primers) and  $P_{BAD}$ -*asdII-lacZ*  
434 (MBP193F/MBP193R primers) were constructed by PCR amplifying desired fragments  
435 using primers containing homologies to  $P_{BAD}$  and *lacZ*. Similarly, transcriptional fusions  
436  $P_{BAD}$ -*asdI-II-lacZ* (MBP151F/MBP206R3 primers) and  $P_{BAD}$ -*asdI-lacZ*  
437 (MBP151F/MBP206R1 primers) were generated by PCR amplification using forward  
438 primer with homology to  $P_{BAD}$  and reverse primers containing *lacZ* RBS and *lacZ*

439 homology. PCR products were then recombined into PM1205 strain using  $\lambda$  Red  
440 homologous recombination.

441 Plasmid harboring SgrS under the control of  $P_{tet0-1}$  promoter was constructed by  
442 PCR amplifying *sgrS* from *E. coli* MG1655 chromosomal DNA using oligos containing  
443 NdeI and BamHI restriction sites. Resulting PCR product and vector pZA31R (24) were  
444 digested by NdeI and BamHI (New England Biolabs) restriction endonucleases.  
445 Digestion products were ligated using DNA Ligase (New England Biolabs) to produce  
446 pZAMB1 plasmid containing  $P_{tet0-1}$ -*sgrS* allele.

447 Plasmid pZEMB8 containing  $P_{lac0-1}$ -*ptsG-gfp* was constructed by PCR amplifying  
448 *ptsG* from MG1655 chromosomal DNA using oligos containing KpnI and EcoRI  
449 restriction sites. Resulting PCR products and vector pZE12S (24) were digested by KpnI  
450 and EcoRI restriction endonucleases. Digestion products were ligated using DNA  
451 Ligase to produce pZEMB2. Superfolder *gfp* (from now on just *gfp*) was amplified from  
452 pXG10-SF (40) using oligos containing KpnI and XbaI restriction sites. pZEMB2 and the  
453 resulting PCR product were digested with KpnI and XbaI, and ligated with DNA Ligase  
454 to produce pZEMB8. Plasmids with translational reporter fusions  $P_{lac0-1}$ -*manX-gfp*  
455 (pZEMB10),  $P_{lac0-1}$ -*yigL-gfp* (pZEMB15),  $P_{lac0-1}$ -*purR-gfp* (pZEMB25),  $P_{lac0-1}$ -*asdl-gfp*  
456 (pZEMB26) and  $P_{lac0-1}$ -*asdl-II-gfp* (pZEMB27) were constructed by restriction cloning  
457 into pZEMB8 using KpnI and EcoRI restriction endonucleases.

458 **Media and reagents.** Bacteria were cultured in Luria-Bertani (LB) broth medium or LB  
459 agar plates (37) at 37°C, unless stated otherwise. Bacteria were grown in MOPS  
460 (morpholine-propanesulfonic acid) rich defined medium (Teknova) with 0.2% fructose  
461 for reporter fluorescence assays. Where necessary, media was supplemented with

462 antibiotics at following concentrations: 100 µg/ml ampicillin (Amp), 25 µg/ml  
463 chloramphenicol (Cm), 25 µg/ml kanamycin (Kan) and 50 µg/ml spectinomycin (Spec).  
464 Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used at concentrations of 0.1-1.5  
465 mM for induction of P<sub>lacO-1</sub> promoter, anhydrotetracycline (aTc) 0-50 ng/ml for induction  
466 of P<sub>tetO-1</sub> promoter and 0.000002%-0.2% L-arabinose for induction of P<sub>BAD</sub> promoter,  
467 unless otherwise noted. To induce glucose-phosphate stress, 0.5% α-methylglucoside  
468 (αMG) was added to the growth medium.

469 **Reporter fluorescence assay.** Bacterial strains were cultured overnight in MOPS rich  
470 medium supplemented with 0.2% fructose, Amp, Cm and subcultured 1:100 to fresh  
471 medium with appropriate inducers (IPTG, aTc) in 48 well plates. Relative fluorescence  
472 units (RFU) and optical density (OD<sub>600</sub>) were measured over time. “GFP expression”  
473 was calculated by plotting RFU over OD<sub>600</sub> and determining the slopes of linear  
474 regression plots for each IPTG concentration in exponentially growing cells in the  
475 presence of aTc to induce SgrS expression. “Promoter activity” was calculated by  
476 plotting RFU over OD<sub>600</sub> and determining the slopes of linear regression plots for each  
477 IPTG concentration in exponentially growing cells in the absence of aTc.

478 ***In vitro* transcription and radiolabeling.** Template DNA for *in vitro* transcription was  
479 generated by PCR using gene-specific oligonucleotides containing the T7 promoter  
480 sequence. Following oligonucleotides were used to generate specific template DNA:  
481 MBP84F/MBP213R–*ptsG* (+1 to +240), O-JH218/MBP214R–*manX* (+1 to +240),  
482 MBP56F/MBP215R–*asdl-II* (+1 to +240), MBP56F/MBP222R–*asdl* (+1 to +110),  
483 MBP226F/MBP226R–*asdlII* (+71 to +310), MBP65F/MBP174R–*purR* (+1 to +230),  
484 MBP216F/MBP216R–*yigL* (-191 to +50 relative to ATG translation start of *yigL*)

485 MBP234F/MBP234R–*gfp* (+1 to +240) and O-JH219/O-JH119 were used to generate  
486 full-length *sgrS* template DNA. *In vitro* transcription of DNA templates was performed  
487 according to specifications of MEGAscript T7 Kit (Ambion). *In vitro* transcribed RNA was  
488 5'-end labeled with radioisotope <sup>32</sup>P using the KinaseMax Kit (Ambion). Samples were  
489 cleaned by passing through Illustra ProbeQuant G-50 Micro Columns (GE Healthcare).  
490 Than samples were cleaned once more with phenol-chloroform: isoamyl alcohol  
491 (Ambion) and labeled RNA precipitated with Ethanol:3M NaAc (30:1).

492 **RNA-RNA gel electrophoretic mobility shift assay.** Different concentrations of  
493 unlabeled mRNA were mixed with 0.02 pmol of 5'-end labeled SgrS. Samples were  
494 denatured at 95°C for 1 min, placed on ice for 5 min, and incubated at 37°C for 30 min  
495 in 1x binding buffer (20mM Tris-HCL (pH 8.0), 1mM DTT, 1mM MgCl<sub>2</sub>, 20 mM KCl,  
496 10mM Na<sub>2</sub>HPO<sub>4</sub> (pH 8.0)) (41). Non-denaturing loading buffer was added and samples  
497 resolved for 6 h at 40 V on native 5.6% PAGE.

498 **Protein-RNA gel electrophoretic mobility shift assay.** 0.02 pmol of 5'-end labeled  
499 mRNA was denatured at 95°C for 1 min., placed on ice for 5 min. Different  
500 concentrations of purified Hfq protein (His-tagged) were added. Samples were  
501 incubated at 37°C for 30 min in 1x binding buffer (20mM Tris-HCL (pH 8.0), 1mM DTT,  
502 1mM MgCl<sub>2</sub>, 20 mM KCl, 10mM Na<sub>2</sub>HPO<sub>4</sub> (pH 8.0)). Non-denaturing loading buffer was  
503 added and samples resolved for 1h 30 min at 20 mA on native 4.0% PAGE (41).

504 **SHAPE.** The *asdI-II* RNA (0.15 μM) and SgrS RNA (0.075 μM, 0.15 μM, 0.30 μM, 0.75  
505 μM, 1.5 μM, or 3.0 μM) were folded separately as in (42) using a modified SHAPE  
506 buffer (100 mM HEPES [pH 8.0], 2 mM MgCl<sub>2</sub>, 40 mM NaCl). For each SgrS  
507 concentration, the SgrS RNA or the equivalent volume of 0.5X TE was added to the

508 *asdI-II* RNA and the samples were incubated at 37°C for 30 min. The RNAs were  
509 modified with *N*-methylisatoic anhydride (NMIA, 6.5 mM; Sigma-Aldrich) and collected  
510 by ethanol precipitation as in (42). Parallel primer extension inhibition and sequencing  
511 reactions were performed using fluorescently labeled primers complementary to the 3'  
512 end of the *asdI-II* RNA (5'-AGATCAAAGGCATCCTGAAG, 22.5 nM; Applied  
513 Biosystems, ThermoFisher Scientific) as in (43) with minor modifications. Prior to primer  
514 binding the RNAs were denatured and snap cooled and the reactions were carried out  
515 for 20 min at 52°C, followed by 5 min at 65°C. The cDNAs were analyzed on a 3730  
516 DNA Analyzer (Applied Biosystems, Inc.). The data were processed and SHAPE  
517 reactivity (difference between the frequency of primer extension products at each  
518 nucleotide in +NMIA vs. -NMIA samples) was derived using the QuShape software (44).  
519 Data for each nucleotide were averaged with statistical outliers removed and normalized  
520 using the 2-8% rule (45). Relative reactivity was calculated by subtracting normalized  
521 SHAPE reactivity in the absence of the SgrS RNA from reactivity in the presence of the  
522 WT or MT SgrS RNA.

523 **Single-molecule fluorescence *in situ* hybridization (smFISH).** The *asdI-lacZ*  
524 (MB170), *asdII-lacZ* (MB183) and *asdI-II-lacZ* (MB171) strains were grown overnight at  
525 37 °C, 250 rpm in LB Broth Miller (EMD) with 25 µg/ml kanamycin (Kan) and 50 µg/ml  
526 spectinomycin (Spec). The next day, the overnight culture was diluted 100-fold into  
527 MOPS EZ rich defined medium (Teknova) with 0.2% (w/w) sodium succinate, 0.02%  
528 glycerol and 0.01% L-arabinose, for *asdI-lacZ* and *asdII-lacZ* strains, and was allowed  
529 to grow at 37 °C till the OD<sub>600</sub> reached 0.15-0.25. The concentration of L-arabinose used  
530 for *asdI-II-lacZ* was 0.002%. α-methyl D-glucopyranoside (αMG) (Sigma-Aldrich) was

531 added to the culture to a desired concentration to introduce sugar phosphate stress and  
532 induce SgrS sRNA expression. After 10 minutes of induction, the culture was taken out  
533 and fixation was performed by mixing with formaldehyde (Fisher Scientific) at a final  
534 concentration of 4%.

535  $\Delta sgrS$  and  $\Delta lacZ$  strains were grown in LB Broth Miller (EMD) at 37 °C, 250 rpm  
536 overnight. Then the cultures were diluted 100-fold into MOPS EZ rich defined medium  
537 (Teknova) with 0.2% glucose and allowed to grow at 37 °C till the OD<sub>600</sub> reached 0.2.  
538 The cells were then fixed by mixing with formaldehyde (Fisher Scientific) at a final  
539 concentration of 4%. TK310 cells were grown overnight, similar to the knockout strains.  
540 The overnight culture was then diluted 100-fold into MOPS EZ rich defined medium  
541 (Teknova) with 0.2% glucose and 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG,  
542 Sigma-Aldrich) and allowed to grow at 37 °C for 30 minutes. The cells were then fixed in  
543 the same procedure as mentioned before.

544 The fixation and permeabilization of the cells were done using the methods  
545 published previously (46). After fixing with 4% formaldehyde, the cells were incubated at  
546 room temperature for 30 minutes. The cells were then centrifuged at 600 g for 7 minutes  
547 and the pellets were washed with 1X PBS 3 times. The cells were then permeabilized  
548 with 70% ethanol for 1 hour at room temperature and stored at 4 °C before fluorescence  
549 *in situ* hybridization.

550 The smFISH probes were designed using Stellaris Probe Designer and the  
551 orders were placed from Biosearch Technologies (<https://www.biosearchtech.com/>).  
552 The labeling of the probes was performed using equal volumes of each probe. The final  
553 volume of sodium bicarbonate was adjusted to 0.1 M by adding 1/9 reaction volume of 1



554 M sodium bicarbonate (pH = 8.5). The probe solution was mixed with 0.05-0.25 mg of  
555 Alexa Fluor 647 or Alexa Fluor 568 succinimidyl ester (Life Technologies) dissolved in 5  
556  $\mu$ L DMSO. The dye was kept about 20-25 fold in molar excess relative to the probes.  
557 After incubation with gentle vortexing in the dark at 37 °C overnight, the reaction was  
558 quenched by adding 1/9 reaction volume of 3 M sodium acetate (pH = 5). Unconjugated  
559 dyes were removed by ethanol precipitation first and then by P-6 Micro Bio-Spin  
560 Column (Bio-Rad).

561 A previously published protocol (46) was used for the hybridization procedure. 60  
562  $\mu$ l of permeabilized cells were washed with FISH wash solution (10% formamide in 2X  
563 SSC (Saline Sodium Citrate) buffer) and resuspended in 15  $\mu$ l hybridization buffer (10%  
564 dextran sulfate, 1 mg/ml *E. Coli* tRNA, 0.2 mg/ml BSA, 2 mM vanadyl ribonucleoside  
565 complexes, 10% formamide in 2X SSC) with probes. The number of probes used for  
566 sRNA SgrS was 9 and they were labeled with Alexa Fluor 647. The number of probes  
567 for mRNA *lacZ* was 24 and they were labeled with Alexa Fluor 568. The concentration  
568 of the labeled probes for SgrS and *lacZ* mRNA were 50 nM and 15 nM each. The  
569 reactions were incubated in the dark at 30 °C overnight. The cells were then  
570 resuspended in 20X volume FISH wash solution and centrifuged. They were then  
571 resuspended in FISH wash solution, incubated for 30 minutes at 30 °C and centrifuged  
572 and this was repeated 3 times. The cells were pelleted after the final washing step and  
573 resuspended in 20  $\mu$ l 4X SSC and stored at 4 °C for imaging. The labeled cells were  
574 immobilized in poly-L-lysine (Sigma-Aldrich) treated 1.0 borosilicate chambered  
575 coverglass (Thermo Scientific™ Nunc™ Lab-Tek™). They were then imaged with  
576 imaging buffer (50 mM Tris-HCl (pH = 8.0), 10% glucose, 1%  $\beta$ -mercaptoethanol

577 (Sigma-Aldrich), 0.5 mg/ml glucose oxidase (Sigma-Aldrich) and 0.2% catalase  
578 (Calbiochem) in 2X SSC).

579 **Single-molecule localization-based super-resolution imaging.** An Olympus IX-71  
580 inverted microscope with a 100X NA 1.4 SaPo oil immersion objective was used for the  
581 3D super-resolution imaging. The lasers used for two-color imaging were Sapphire 568-  
582 100 CW CDRH, Coherent (568nm) and DL640-100-AL-O, Crystalaser (647nm) and  
583 DL405-025, Crystalaser (405nm) was used for the reactivation of Alexa 647 and Alexa  
584 568 fluorophores. The laser excitation was controlled using mechanical shutters  
585 (LS6T2, Uniblitz). A dichroic mirror (Di01-R405/488/561/635, Semrock) was used to  
586 reflect the laser lines to the objective. The objective collected the emission signals and  
587 then they made their way through an emission filter (FF01-594/730-25, Semrock for  
588 Alexa 647 or HQ585/70M 63061, Chroma for Alexa 568) and excitation laser was  
589 cleaned up using notch filters (ZET647NF, Chroma, NF01-568/647-25x5.0 and NF01-  
590 568U-25, Semrock). They were then imaged on a 512x512 Andor EMCCD camera  
591 (DV887ECS-BV, Andor Tech). Astigmatism was introduced by placing a cylindrical lens  
592 with a focal length of 2 m (SCX-50.8-1000.0-UV-SLMF-520-820, CVI Melles Griot) in  
593 the emission path between two relay lenses with focal lengths of 100 mm and 150 mm  
594 each and this helped us to do 3D imaging. In this setup, each pixel corresponded to 100  
595 nm. We used the CRISP (Continuous Reflective Interface Sample Placement) system  
596 (ASI) to keep the z-drift of the setup to a minimum. The image acquisition was  
597 controlled using the storm-control software written in Python by Zhuang's group and  
598 available at GitHub.

599           The imaging of the sample began with a DIC image of the sample area.  
600           Subsequently two-color super-resolution imaging was performed. 647nm excitation was  
601           used first and after image acquisition was completed for Alexa Fluor 647, 568nm  
602           excitation was used to image Alexa Fluor 568. 405nm laser power was increased slowly  
603           to compensate for fluorophore bleaching and also to maintain moderate signal density.  
604           We stopped imaging when most of the fluorophores had photobleached and the highest  
605           reactivation laser power was reached.

606           The raw data acquired using the acquisition software was analyzed using the  
607           same method as described in previously published work (31), which was a modification  
608           of the algorithm published by Zhuang's group (47, 48). The clustering analysis on the  
609           localization data was performed using MATLAB codes in the same method as described  
610           previously (31). Background signal was estimated using  $\Delta sgrS$  and  $\Delta lacZ$  strains and  
611           they were prepared, imaged and analyzed as described before. TK310 cells were  
612           prepared, imaged and analyzed in the same way as a low copy *lacZ* mRNA sample for  
613           copy number calculation. The copy number calculation was also performed using  
614           MATLAB codes as described previously (31).

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627

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- 755

## 756 **FIGURE LEGENDS**

### 757 **Figure 1. Model for SgrS target prioritization during glucose-phosphate stress.**

758 Glucose or the analogs  $\alpha$ MG and 2-deoxyglucose are phosphorylated during transport  
759 through the phosphotransferase system proteins EIICB<sup>Glc</sup> (PtsG) or EIICD<sup>Man</sup> (ManYZ).  
760 If sugar-phosphates are not metabolized, the glucose-phosphate stress response is  
761 triggered, and the transcription factor SgrR becomes active and promotes *sgrS*  
762 transcription. The RNA chaperone Hfq promotes SgrS-mediated translational repression  
763 of *ptsG* and *manXYZ* mRNAs, reducing synthesis of sugar transporters. SgrS stabilizes  
764 *yigL* mRNA, promoting sugar phosphatase (YigL) synthesis. SgrS-mediated repression  
765 of *asd*, *purR*, *foIE* and *adiY* likely reroutes metabolism to restore homeostasis during

766 stress recovery. The hypothetical sequence of regulatory events following stress  
767 induction is represented from left to right as SgrS levels increase over time. When SgrS  
768 concentrations are low, only the highest priority targets are regulated. When stress  
769 persists and concentrations of SgrS increase, lower priority targets are regulated.

770 **Figure 2. Efficiency of target regulation by SgrS.** A) Representation of genetic  
771 constructs in two compatible plasmids used to study target regulation by SgrS. One  
772 plasmid contains full-length SgrS under the control of the aTc-inducible  $P_{tet}$  promoter. A  
773 second plasmid contains a  $P_{lac}$  promoter and the relevant region encoding each SgrS  
774 target (including the SgrS binding site) translationally fused to a superfolder *gfp* (*sfgfp*)  
775 reporter gene. B-F) Regulated activity was plotted as a function of basal activity (see  
776 text for description) for (B) *ptsG*, (C) *manX*, (D) *purR*, (E) *asdl*, and (F) *yigL* fused to  
777 *sfgfp* reporter gene. Without SgrS-mediated regulation we obtained a line with a slope  
778 =1. The plots with slopes <1, indicate repression of (B) *ptsG*, (C) *manX*, (D) *purR* and  
779 (E) *asdl* by SgrS. The plot with a slope of >1 are indicative of activation of (F) *yigL*.

780 **Figure 3. Regulatory hierarchy established by SgrS.** Regulated activity was plotted  
781 as a function of basal activity for *ptsG*, *manX*, *purR*, *asdl*, and *yigL* fusions. Lack of  
782 SgrS regulation is indicated by a line with a slope =1. The plots with slopes <1, indicate  
783 repression (*ptsG*, *manX*, *purR* and *asdl*) by SgrS. The plot with slope >1 indicates  
784 activation (*yigL*). Target fusion activity was monitored at different levels of SgrS  
785 induction by aTc: (A) 10 ng/ml, (B) 20 ng/ml, (C) 30 ng/ml, (D) 40 ng/ml, (E) 50 ng/ml.

786 **Figure 4. SgrS binding with target mRNAs *in vitro*.** A) SgrS was labeled with  $^{32}P$  and  
787 incubated with unlabeled target transcripts at final concentrations of 0 $\mu$ M - 16  $\mu$ M .  
788 Electrophoretic mobility shift assays (EMSAs) were performed after incubating full-



789 length SgrS (+1 to +227) with its target transcripts (A) *ptsG* (+1 to +240), *manX* (+1 to  
790 +240), *purR* (+1 to +230), *yigL* (-191 to +50 relative to ATG translation start of *yigL*),  
791 *asdl* (+1 to +110), and *asdlI* (+71 to +310). B-D) Targets transcripts (B) *ptsG* (+1 to  
792 +240), (C) *manX* (+1 to +240), (D) *asdl* (+1 to +110) were labeled with  $^{32}\text{P}$  and  
793 incubated with unlabeled SgrS. EMSAs were performed to resolve complex formation.  
794 Band densities were measured for biological replicates (n, top left) and plotted to  
795 determine dissociation constant ( $K_D$ , bottom right) values for (B) *ptsG*, (C) *manX*, and  
796 (D) *asdl*. E) EMSA of radiolabeled SgrS in the presence of increasing concentrations of  
797 *asdl-II* transcript. Shift in mobility corresponding to one or two SgrS bound to *asdl-II* is  
798 denoted as Site I-SgrS\* and Sites I-II-SgrS\* respectively. F) Quantification of SgrS  
799 binding with radiolabeled *asdl-II* (+1 to +240), as described above.

800 **Figure 5. Secondary structure of 5' end of *asd*.** A) Diagram showing base-pairing  
801 interactions of SgrS with binding sites I and II of *asd* mRNA. B) Energy of interaction  
802 predicted by IntaRNA (26). "Structured" indicates pairing between full length SgrS (+1 to  
803 +227) and *asdl-II* (+1 to +180). Plotted is the energy of interactions at either site I (*asdl*)  
804 or site II (*asdlI*). "Isolated" indicates interactions between isolated binding sites: SgrS  
805 (+158 to +176) with *asdl* (+31 to +49) and SgrS (+158 to +178) pairing with *asdlI* (+110  
806 to +129). C) The structure of the *asdl-II* RNA alone or in complex with SgrS was probed  
807 with NMIA and the modified RNA was analyzed by primer extension inhibition. SHAPE  
808 reactivity (difference between the frequency of primer extension products at each  
809 nucleotide in +NMIA vs. -NMIA samples) was then used as a parameter in the Vienna  
810 RNAprobing WebServer (49) to predict the secondary structure of the *asdl-II* RNA.  
811 Colors indicate SHAPE reactivity as following: red, highly reactive ( $\geq 0.8$ ); gold, reactive

812 (0.4-0.79); green, moderately reactive (0.2-0.39); blue, minimally reactive (0.1-0.19);  
813 grey, unreactive ( $< 0.01$ ). Distinct structures were observed in the absence of SgrS and  
814 in the presence of saturating concentrations of SgrS. The SHAPE reactivity of *asdI-II*  
815 RNA alone (left) or in the presence of 5-fold excess SgrS (right) is mapped to the  
816 predicted secondary structures. (D) SHAPE reactivity as a function of SgrS  
817 concentration for each binding site (top, site I; bottom, site II). Only nucleotides with a  
818 significant ( $\geq 0.1$ ) change in reactivity are shown. Error bars denote SEM,  $n = 9$ . (E)  
819 Relative SHAPE reactivity (difference in the SHAPE reactivity in the presence of SgrS  
820 vs. the absence of SgrS) of the *asdI-II* RNA in the presence of wild-type (top) or mutant  
821 (bottom) SgrS. Error bars denote SEM,  $n = 9$  (WT), 6 (MT). The *asdI-II* RNA  
822 nucleotides are numbered below the X-axis and the SgrS binding sites are indicated.

823 **Figure 6. STORM imaging of SgrS regulation of *asd* variants.** A) Illustration of *asdI-*  
824 *II*, *asdI* and *asdII* translationally fused to *lacZ* reporter with SgrS binding sites I and II  
825 marked. B-D) 2D projection of 3D super-resolution images of SgrS and *lacZ* mRNA for  
826 the different *asd-lacZ* variants, labeled by smFISH, before and after 10 min induction  
827 with 1%  $\alpha$ MG. (B), (C) and (D) correspond to *asdI-lacZ*, *asdII-lacZ*, and *asdI-II-*  
828 *lacZ* shown in (A). Probability distributions of RNA copy numbers in individual cells for  
829 30-250 cells are plotted next to the representative images.

830 **Figure 7. SgrS binding cooperativity allows for improved repression of *asd***  
831 **translation.** A) Illustration of *asdI* and *asdI-II* constructs with SgrS binding sites marked.  
832 Graphs show comparison of SgrS regulation of *asdI* and *asdI-II* variants in (B) wild-type  
833 and (D) *rne701* mutant by plotting regulated activity over basal activity at various SgrS  
834 expression levels (20-100 ng/ml aTc). Regulatory hierarchy of SgrS targets in (C) wild-

835 type and (E) *rne701* mutant strains. Regulation of target genes at one SgrS expression  
836 level (40 ng/ml aTc) are compared by plotting regulated activity over basal activity of the  
837 *gfp* reporter.

838 **Figure 8. SgrS regulation of transcriptional *asd-lacZ* fusions.**  $\beta$ -galactosidase  
839 activity of (A) *asdl-lacZ* (+1 to +64) and *asdl-II-lacZ* (+1 to +277) was (B) assayed in  
840 response to SgrS expression from a plasmid (and vector control) in WT and *rne701*  
841 background strains.

842

843 **Figure S1. Regulation of *ptsG* fusion by SgrS.** Example plots of *ptsG-gfp*  
844 translational fusion activity (RFU) over growth ( $OD_{600}$ ) at various IPTG inducer  
845 concentrations at (A) basal (uninduced) or (B-F) increasing SgrS expression levels.  
846 Slopes of the linear regression plots for each IPTG concentration were calculated to  
847 obtain (A) “basal expression” and (B-F) “regulated activity” values.

848 **Figure S2. Inducer concentration-dependent activity of target fusions.** (A) Basal  
849 activity (0 ng/ml aTc) or (B-F) regulated activity (10-50 ng/ml aTc) of *ptsG*, *manX*, *purR*,  
850 *asdl*, *asdl-II* and *yigL* fusions at varying IPTG concentrations (0-1.5 mM IPTG).

851 **Figure S3: SHAPE analysis of *asdl-II* RNA.** (A-G) SHAPE reactivity of the *asdl-II* RNA  
852 alone and in complex with increasing concentrations of SgrS (0.5, 1, 2, 5, 10, or 20X).  
853 The structure of the *asdl-II* RNA in the absence and presence of SgrS was probed with  
854 NMIA and the modified RNA was analyzed by primer extension inhibition. SHAPE  
855 reactivity is the difference between the frequency of primer extension products at each  
856 nucleotide in +NMIA vs. -NMIA samples. Colors indicate SHAPE reactivity as following:  
857 red, highly reactive ( $\geq 0.8$ ); gold, reactive (0.4-0.79); green, moderately reactive (0.2-

858 0.39); blue, minimally reactive (0.1-0.19); grey, unreactive ( $< 0.01$ ). (H) Relative SHAPE  
859 reactivity of the *asdI-II* RNA in the presence of wild-type vs mutant SgrS. Relative  
860 reactivity is the difference in the SHAPE reactivity in the presence of wild-type and  
861 mutant SgrS. Error bars denote SEM,  $n = 9$ . The *asdI-II* RNA nucleotides are numbered  
862 below the X-axis and the SgrS binding sites are indicated. (I-J) SHAPE reactivity as a  
863 function of mutant SgrS concentration for each binding site (I, site I; J, site II). The same  
864 nucleotides as in Fig. 5D are shown. Error bars denote SEM,  $n = 6$ .

865 **Figure S4. Quantification of SgrS and *asd* mRNA variants using STORM.** Copy  
866 number of *lacZ* mRNA vs. SgrS in 30-250 individual cells for the different *asd*-  
867 *lacZ* variants, (A-B) *asdI-lacZ*, (C-D) *asdII-lacZ*, and (E-F) *asdI-II-lacZ*, before (A,C,E)  
868 and after (B,D,F) 10 min 1%  $\alpha$ MG induction.

869 **Figure S5. Inducer concentration-dependent activity of target-*gfp* fusions.** (A)  
870 Basal activity (0 ng/ml aTc) or (B-F) regulated activity (20-100 ng/ml aTc) of *ptsG*,  
871 *manX*, *purR*, *asdI*, *asdI-II* and *yigL* fusions at varying IPTG concentrations (0-1.5 mM  
872 IPTG).

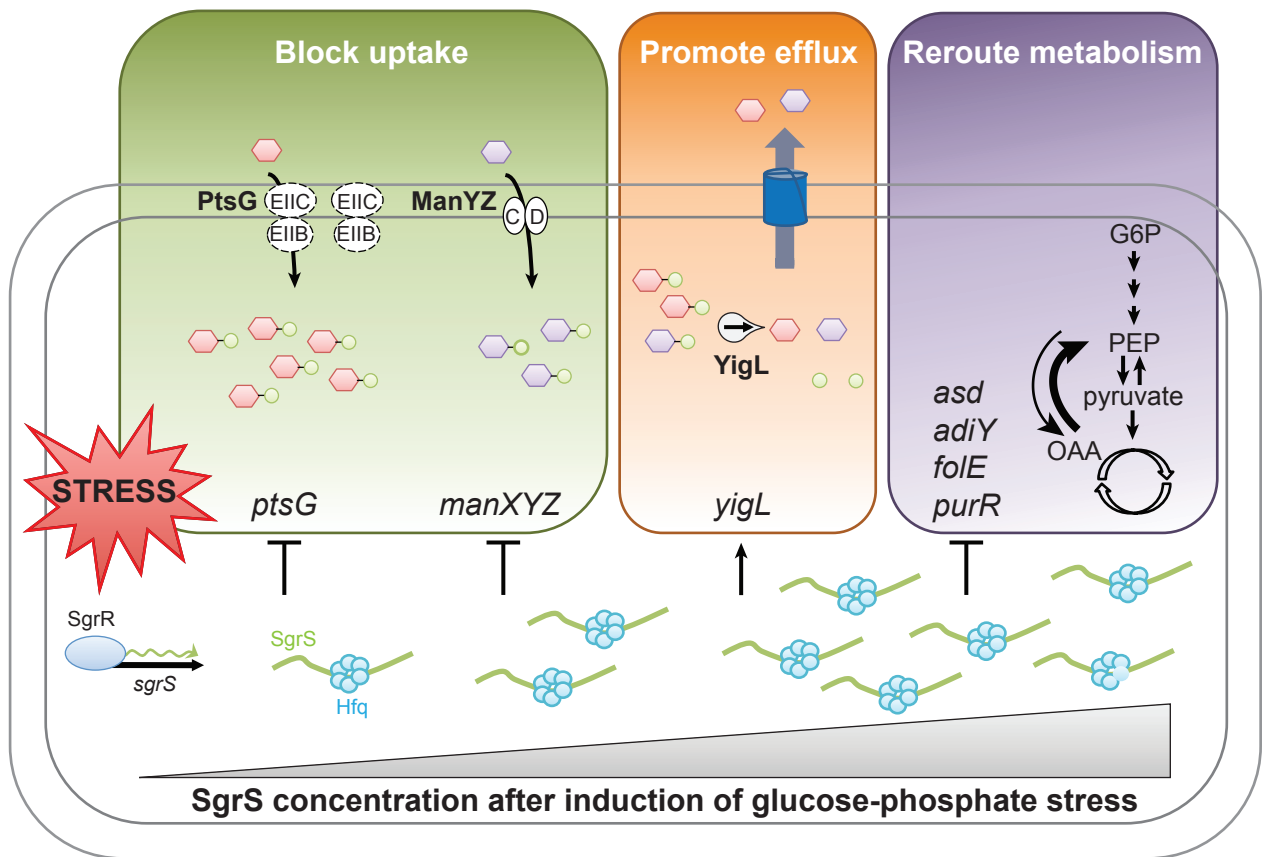
873 **Figure S6. Regulatory hierarchy established by SgrS in the RNase E mutant**  
874 **strain.** A) Regulated activity was plotted as a function of basal activity (see text for  
875 description) for *ptsG*, *manX*, *purR*, *yigL*, *asdI* and *asdI-II* fused to *sfgfp* reporter gene in  
876 the *rne701* mutant strain. Target fusion activity was monitored at different levels of SgrS  
877 induction by aTc: 20 ng/ml, 40 ng/ml, 60 ng/ml, 80 ng/ml, 100 ng/ml. B) Target  
878 regulation at different SgrS expression levels in the *rne701* mutant strain was compared  
879 to determine regulatory hierarchy. Without SgrS-mediated regulation we obtained a line  
880 with a slope =1. The plots with slopes  $< 1$ , indicate repression of *ptsG*, *manX*, *purR*, *asdI*

881 and *asdl-II* by SgrS. The plot with the slope of >1 is indicative of activation of *yigL*. B)  
882 Regulated activity was plotted as a function of basal activity for *ptsG*, *manX*, *purR*, *asdl*,  
883 and *yigL* fusions.

884 **Figure S7. Hfq binding with target mRNAs *in vitro*.** A) Target transcripts *ptsG* (+1 to  
885 +240), *manX* (+1 to +240), *purR* (+1 to +230), *yigL* (-191 to +50 relative to ATG  
886 translation start of *yigL*), *asdl* (+1 to +110), *asdlI* (+71 to +310) and *asdl-II* (+1 to +240)  
887 were labeled with <sup>32</sup>P and incubated with appropriate concentrations of Hfq protein.  
888 EMSAs were performed to resolve complex formation. Complexes of increasing size are  
889 marked I-IV, as was previously noted for Hfq (50). B) Band densities were measured for  
890 biological replicates (n, top left) and plotted to determine dissociation constant (K<sub>D</sub>,  
891 bottom right) values.

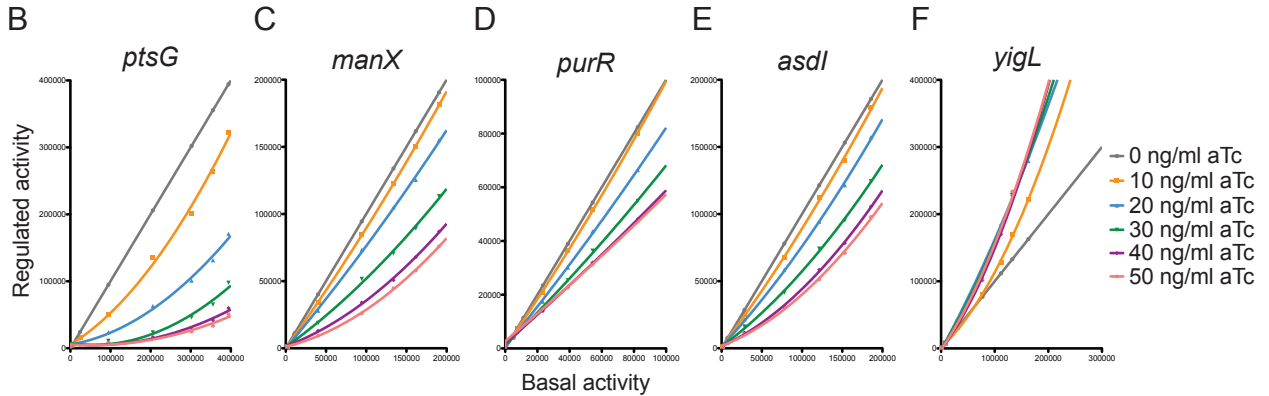
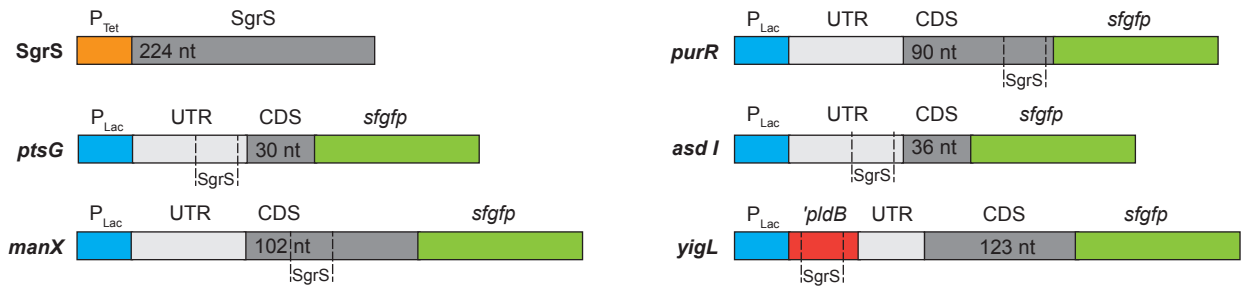
892 **Table S1. Strains and plasmids used in this study.**

893 **Table S2. Oligonucleotides used in this study.**

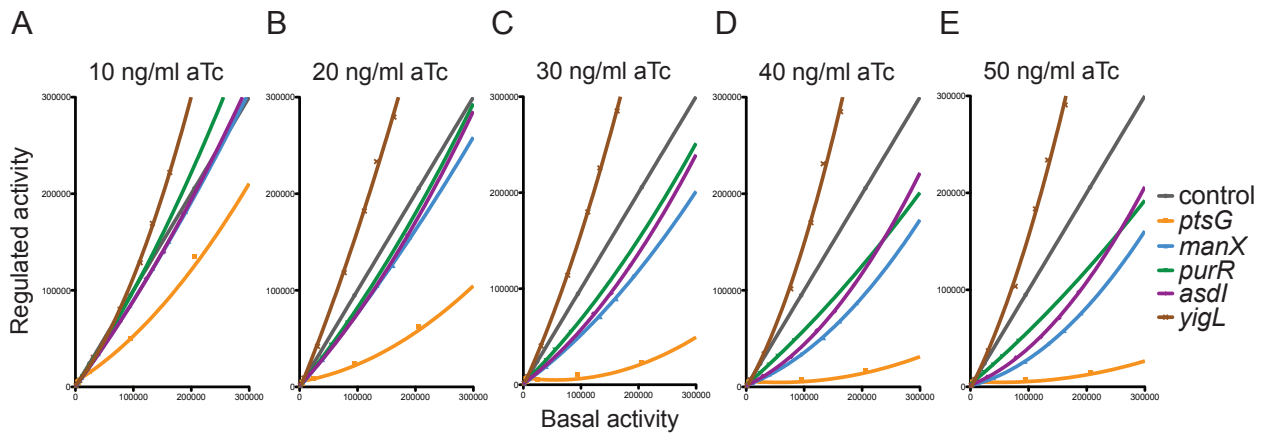


**Figure 1. Model for SgrS target prioritization during glucose-phosphate stress.** Glucose or the analogs  $\alpha$ MG and 2-deoxyglucose are phosphorylated during transport through the phosphotransferase system proteins EII<sup>C</sup>EII<sup>B</sup> (PtsG) or EII<sup>C</sup>EII<sup>D</sup> (ManYZ). If sugar-phosphates are not metabolized, the glucose-phosphate stress response is triggered, and the transcription factor SgrR becomes active and promotes *sgrS* transcription. The RNA chaperone Hfq promotes SgrS-mediated translational repression of *ptsG* and *manXYZ* mRNAs, reducing synthesis of sugar transporters. SgrS stabilizes *yigL* mRNA, promoting sugar phosphatase (YigL) synthesis. SgrS-mediated repression of *asd*, *purR*, *folE* and *adiY* likely reroutes metabolism to restore homeostasis during stress recovery. The hypothetical sequence of regulatory events following stress induction is represented from left to right as SgrS levels increase over time. When SgrS concentrations are low, only the highest priority targets are regulated. When stress persists and concentrations of SgrS increase, lower priority targets are regulated.

A



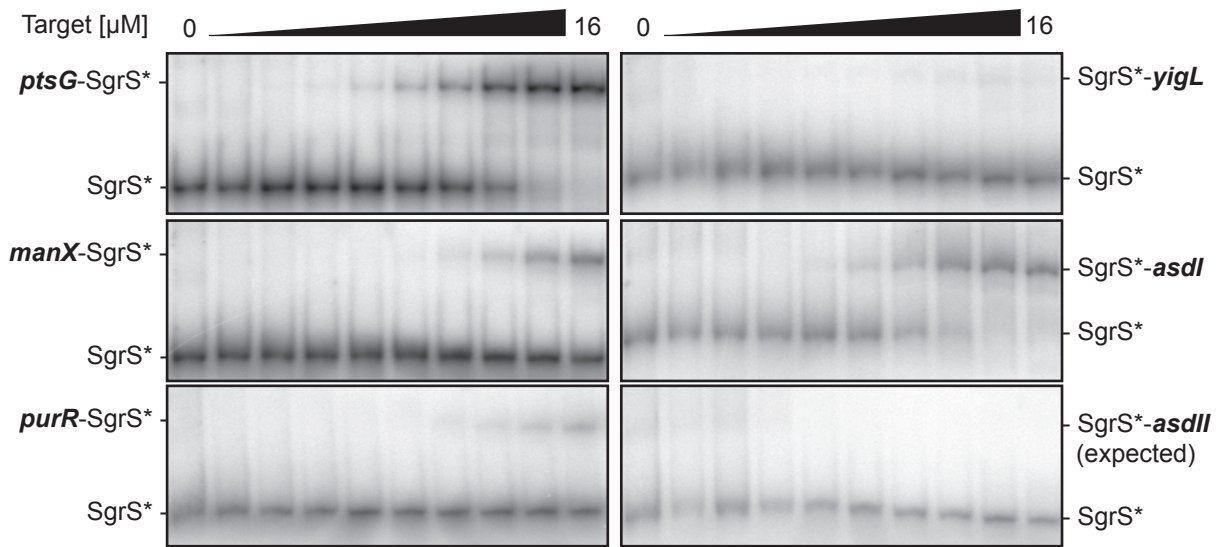
**Figure 2. Efficiency of target regulation by SgrS.** A) Representation of genetic constructs in two compatible plasmids used to study target regulation by SgrS. One plasmid contains full-length SgrS under the control of the aTc-inducible  $P_{tet}$  promoter. A second plasmid contains a  $P_{lac}$  promoter and the relevant region encoding each SgrS target (including the SgrS binding site) translationally fused to a superfolder *gfp* (*sfgfp*) reporter gene. B-F) Regulated activity was plotted as a function of basal activity (see text for description) for (B) *ptsG*, (C) *manX*, (D) *purR*, (E) *asdI*, and (F) *yigL* fused to *sfgfp* reporter gene. Without SgrS-mediated regulation we obtained a line with a slope =1. The plots with slopes <1, indicate repression of (B) *ptsG*, (C) *manX*, (D) *purR* and (E) *asdI* by SgrS. The plot with a slope of >1 are indicative of activation of (F) *yigL*.



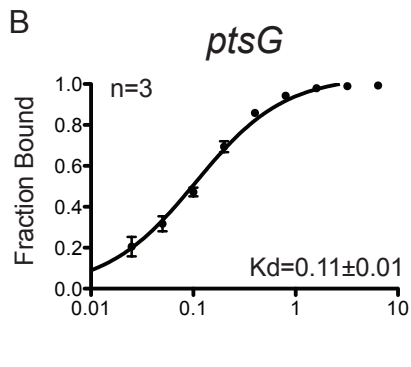
**Figure 3. Regulatory hierarchy established by SgrS.** Regulated activity was plotted as a function of basal activity for *ptsG*, *manX*, *purR*, *asdI*, and *yigL* fusions. Lack of SgrS regulation is indicated by a line with a slope =1. The plots with slopes <1, indicate repression (*ptsG*, *manX*, *purR* and *asdI*) by SgrS. The plot with slope >1 indicates activation (*yigL*). Target fusion activity was monitored at different levels of SgrS induction by aTc: (A) 10 ng/ml, (B) 20 ng/ml, (C) 30 ng/ml, (D) 40 ng/ml, (E) 50 ng/ml.



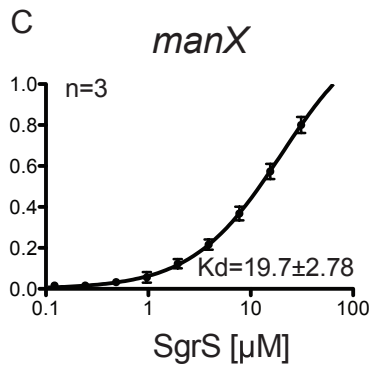
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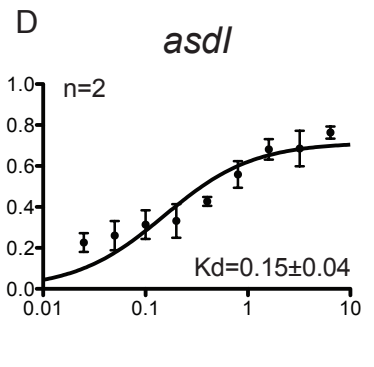
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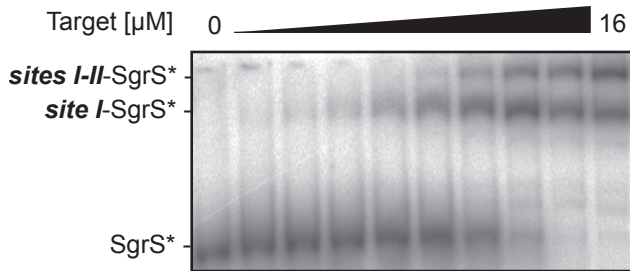
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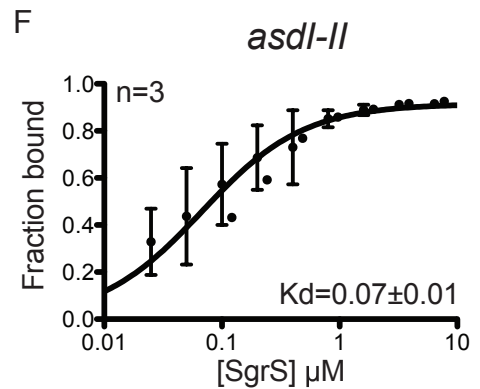


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**Figure 4. SgrS binding with target mRNAs *in vitro*.** A) SgrS was labeled with  $^{32}\text{P}$  and incubated with unlabeled target transcripts at final concentrations of  $0\mu\text{M}$  -  $16\mu\text{M}$ . Electrophoretic mobility shift assays (EMSAs) were performed after incubating full-length SgrS (+1 to +227) with its target transcripts (A) *ptsG* (+1 to +240), *manX* (+1 to +240), *purR* (+1 to +230), *yigL* (-191 to +50 relative to ATG translation start of *yigL*), *asdI* (+1 to +110), and *asdII* (+71 to +310). B-D) Targets transcripts (B) *ptsG* (+1 to +240), (C) *manX* (+1 to +240), (D) *asdI* (+1 to +110) were labeled with  $^{32}\text{P}$  and incubated with unlabeled SgrS. EMSAs were performed to resolve complex formation. Band densities were measured for biological replicates (n, top left) and plotted to determine dissociation constant ( $K_D$ , bottom right) values for (B) *ptsG*, (C) *manX*, and (D) *asdI*. E) EMSA of radiolabeled SgrS in the presence of increasing concentrations of *asdI-II* transcript. Shift in mobility corresponding to one or two SgrS bound to *asdI-II* is denoted as Site I-SgrS\* and Sites I-II-SgrS\* respectively. F) Quantification of SgrS binding with radiolabeled *asdI-II* (+1 to +240), as described above.

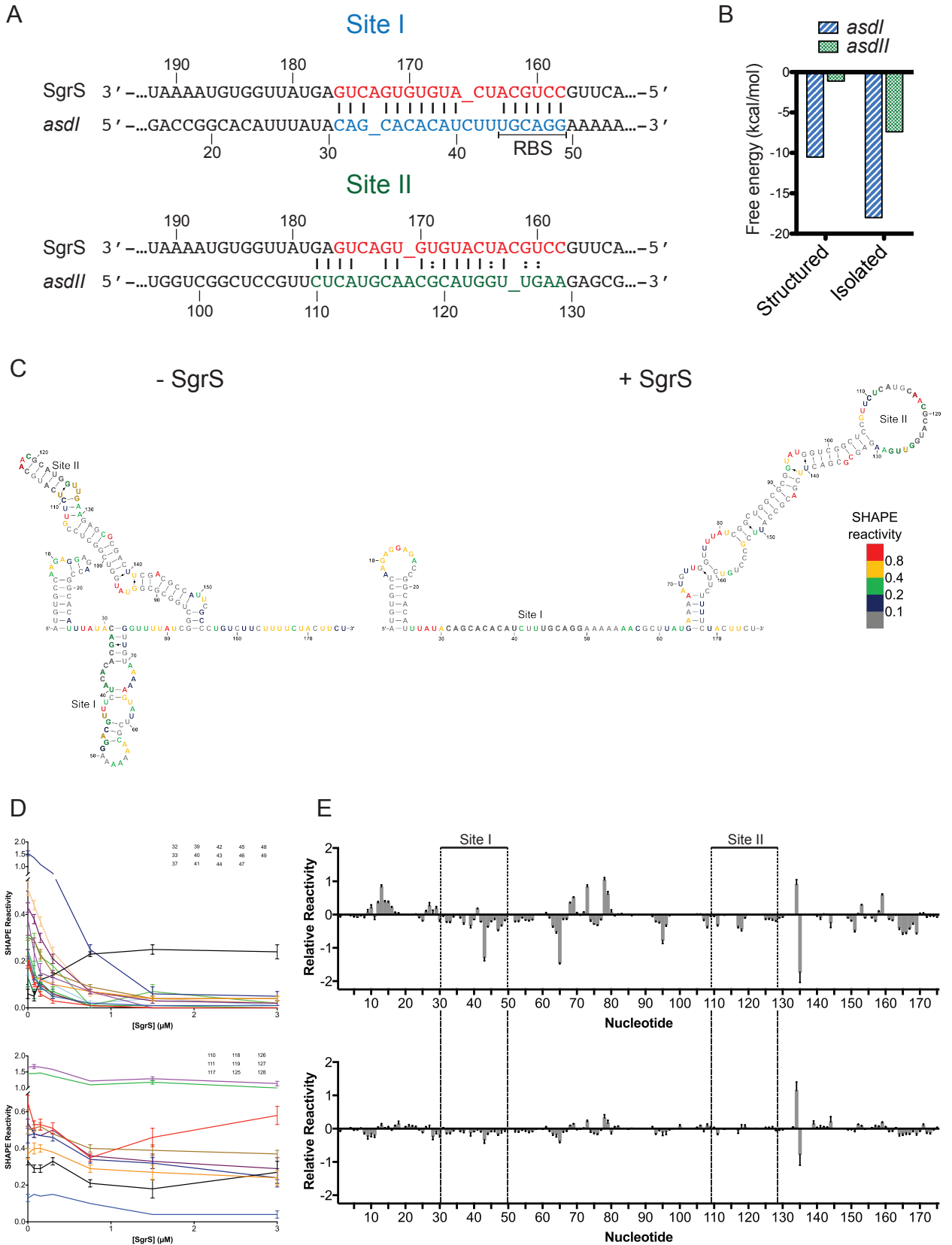
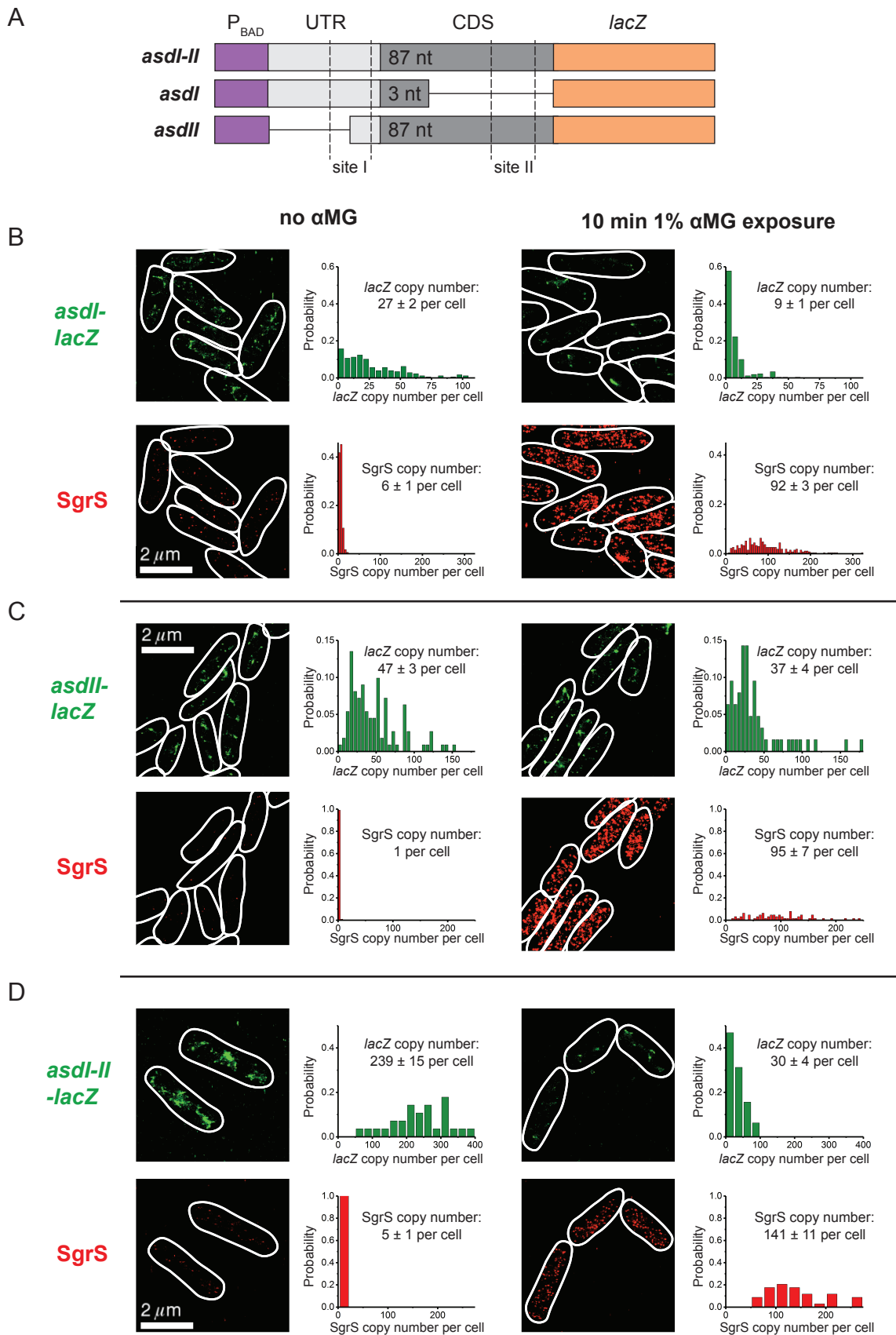
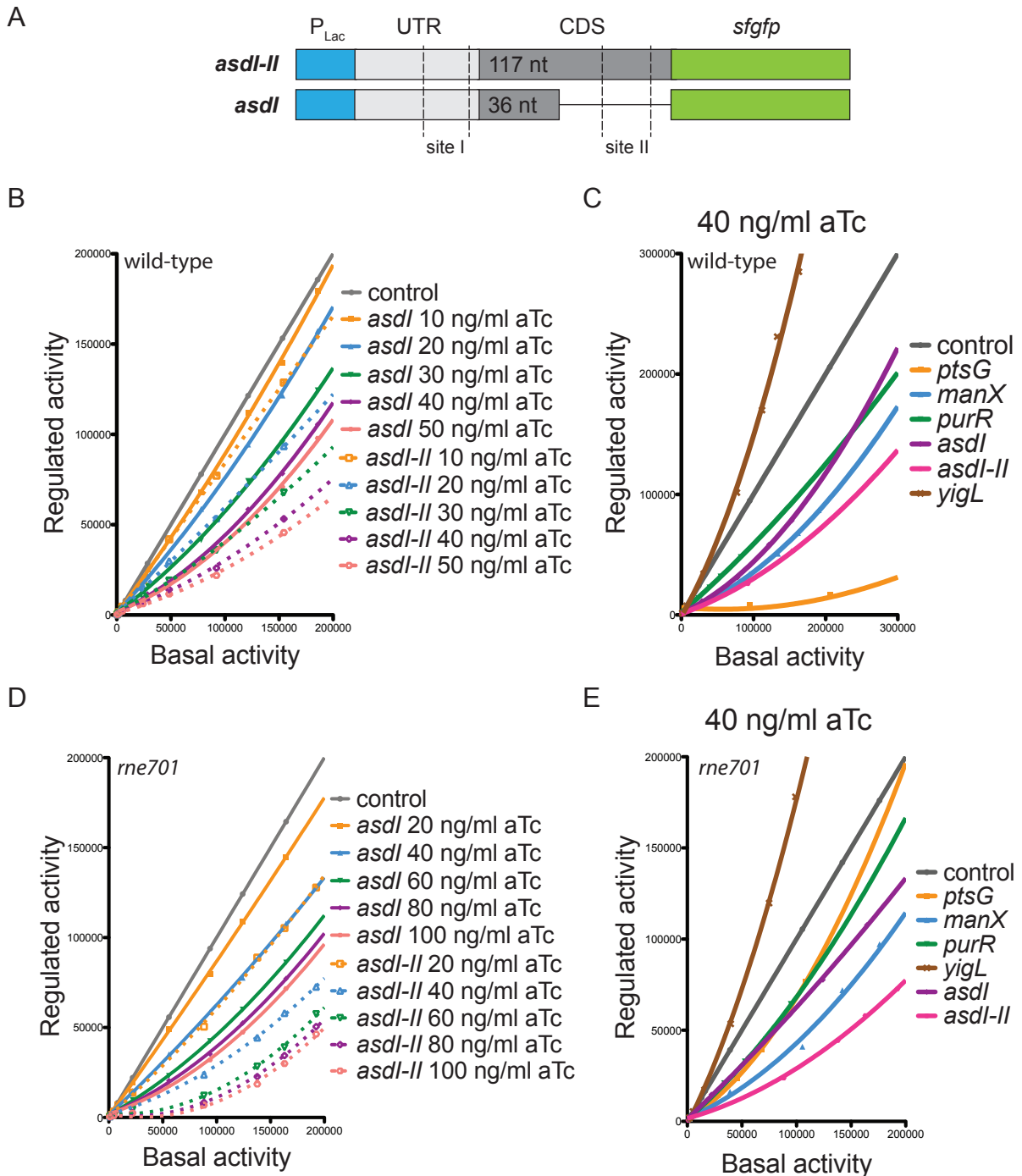


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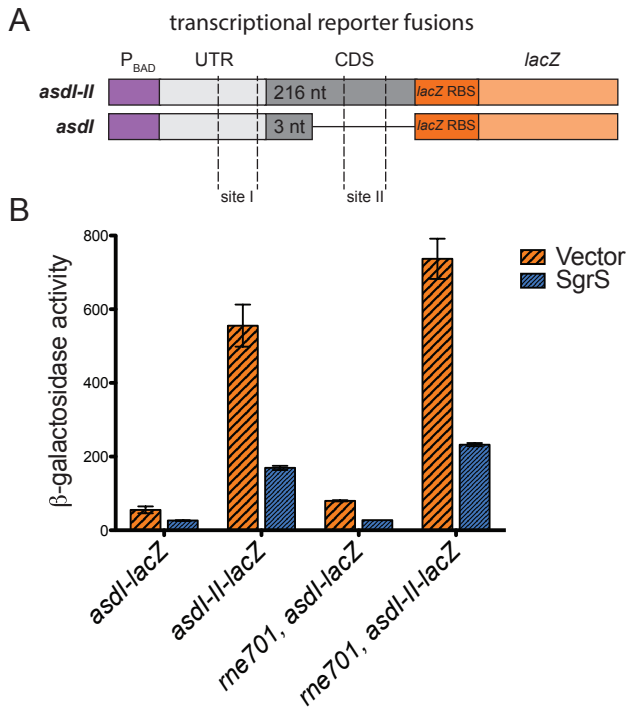
**Figure 5. Secondary structure of 5' end of *asd*.** A) Diagram showing base-pairing interactions of SgrS with binding sites I and II of *asd* mRNA. B) Energy of interaction predicted by IntaRNA (26). "Structured" indicates pairing between full length SgrS (+1 to +227) and *asdI-II* (+1 to +180). Plotted is the energy of interactions at either site I (*asdI*) or site II (*asdII*). "Isolated" indicates interactions between isolated binding sites: SgrS (+158 to +176) with *asdI* (+31 to +49) and SgrS (+158 to +178) pairing with *asdII* (+110 to +129). C) The structure of the *asdI-II* RNA alone or in complex with SgrS was probed with NMIA and the modified RNA was analyzed by primer extension inhibition. SHAPE reactivity (difference between the frequency of primer extension products at each nucleotide in +NMIA vs. -NMIA samples) was then used as a parameter in the Vienna RNAProbing WebServer (55) to predict the secondary structure of the *asdI-II* RNA. Colors indicate SHAPE reactivity as following: red, highly reactive ( $\geq 0.8$ ); gold, reactive (0.4-0.79); green, moderately reactive (0.2-0.39); blue, minimally reactive (0.1-0.19); grey, unreactive ( $< 0.01$ ). Distinct structures were observed in the absence of SgrS and in the presence of saturating concentrations of SgrS. The SHAPE reactivity of *asdI-II* RNA alone (left) or in the presence of 5-fold excess SgrS (right) is mapped to the predicted secondary structures. (D) SHAPE reactivity as a function of SgrS concentration for each binding site (top, site I; bottom, site II). Only nucleotides with a significant ( $\geq 0.1$ ) change in reactivity are shown. Error bars denote SEM,  $n = 9$ . (E) Relative SHAPE reactivity (difference in the SHAPE reactivity in the presence of SgrS vs. the absence of SgrS) of the *asdI-II* RNA in the presence of wild-type (top) or mutant (bottom) SgrS. Error bars denote SEM,  $n = 9$  (WT), 6 (MT). The *asdI-II* RNA nucleotides are numbered below the X-axis and the SgrS binding sites are indicated.



**Figure 6. STORM imaging of SgrS regulation of *asd* variants.** A) Illustration of *asdII*, *asdI* and *asdII* translationally fused to *lacZ* reporter with SgrS binding sites I and II marked. B-D) 2D projection of 3D super-resolution images of SgrS and *lacZ* mRNA for the different *asd-lacZ* variants, labeled by smFISH, before and after 10 min induction with 1%  $\alpha$ MG. (B), (C) and (D) correspond to *asdI-lacZ*, *asdII-lacZ*, and *asdI-II-lacZ* shown in (A). Probability distributions of RNA copy numbers in individual cells for 30-250 cells are plotted next to the representative images.



**Figure 7. SgrS binding cooperativity allows for improved repression of *asd* translation.** A) Illustration of *asdI* and *asdI-II* constructs with SgrS binding sites marked. Graphs show comparison of SgrS regulation of *asdI* and *asdI-II* variants in (B) wild-type and (D) *rne701* mutant by plotting regulated activity over basal activity at various SgrS expression levels (20-100 ng/ml aTc). Regulatory hierarchy of SgrS targets in (C) wild-type and (E) *rne701* mutant strains. Regulation of target genes at one SgrS expression level (40 ng/ml aTc) are compared by plotting regulated activity over basal activity of the *gfp* reporter.



**Figure 8. SgrS regulation of transcriptional *asd-lacZ* fusions.**  $\beta$ -galactosidase activity of (A) *asdI-lacZ* (+1 to +64) and *asdI-II-lacZ* (+1 to +277) was (B) assayed in response to SgrS expression from a plasmid (and vector control) in WT and *rne701* background strains.