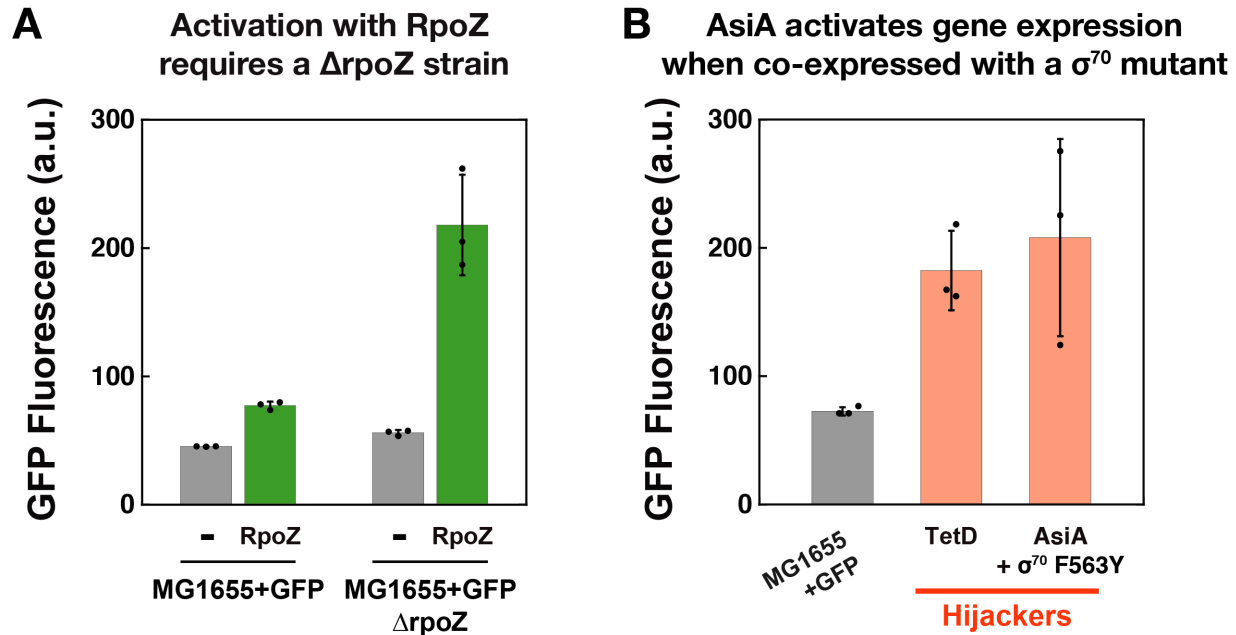


SUPPLEMENTARY INFORMATION

Synthetic CRISPR-Cas Gene Activators for Transcriptional Reprogramming in Bacteria

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Supplementary Figures



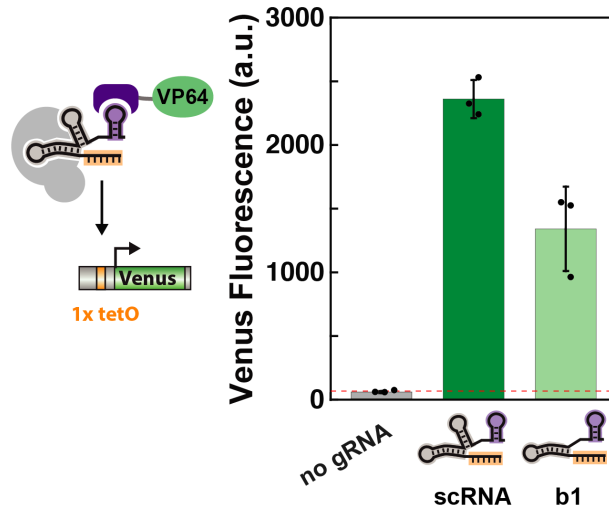
Supplementary Figure 1. Some candidate transcriptional activation domains require additional strain modifications for effective reporter gene activation.

A) CRISPRa with the MCP-RpoZ activator recruited via an scRNA is significantly increased in a $\Delta rpoZ$ host strain, consistent with that observed previously for other RpoZ fusion proteins including dCas9-RpoZ^{1,2}. GFP reporter strains were transformed with dCas9 and a 1x MS2 scRNA, and either with or without the MCP-RpoZ fusion protein.

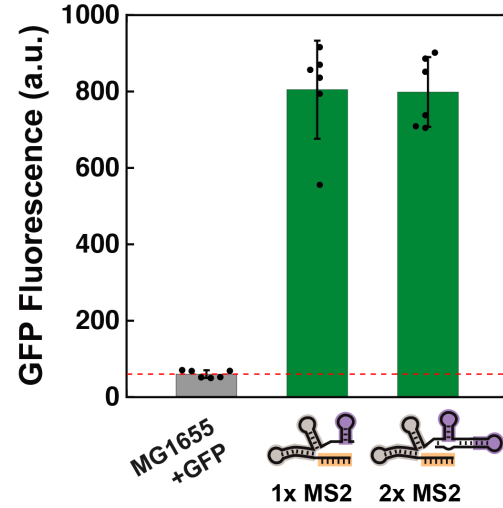
B) CRISPRa with an MCP-AsiA transcriptional activator, co-transformed with a σ^{70} F563Y mutant, produces GFP expression levels comparable to that obtained with MCP-TetD. The σ^{70} F563Y mutant prevents toxicity that occurs when AsiA is expressed alone and inhibits the activity of the endogenous σ^{70} subunit³. Attempts to transform MCP-AsiA into *E. coli* without co-transforming σ^{70} F563Y were unsuccessful unless a substantially weaker promoter (BBa_J23112) was used for MCP-AsiA, and no detectable GFP expression was observed with this construct (data not shown).

Values reported are GFP fluorescence levels measured by flow cytometry. Values are median $\pm$ s.d. for at least three biological replicates (specific values are indicated by black dots).

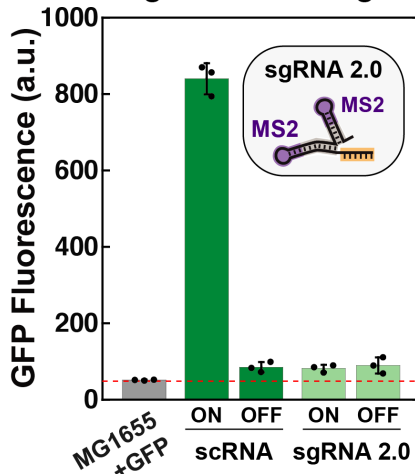
A Modified gRNA designs reduce activity in yeast



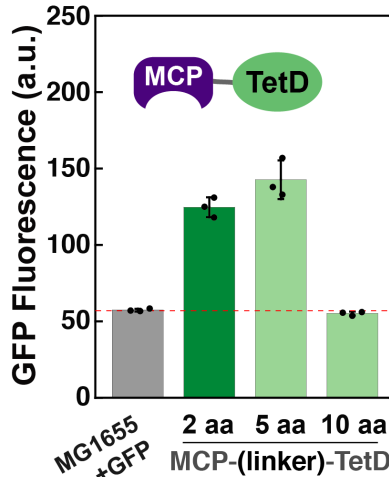
B Additional MS2 recruitment sites do not improve activity with SoxS_{R93A}



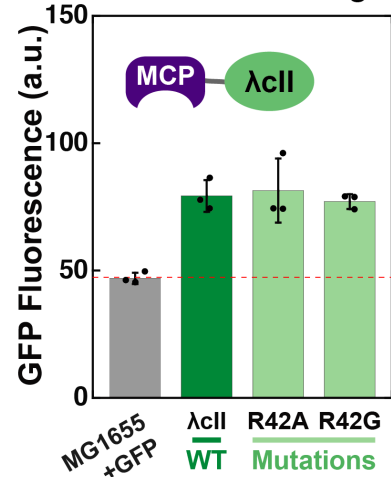
C sgRNA 2.0 design



D MCP-TetD linker



E λcII DNA binding



Supplementary Figure 2. Optimization of gene activation.

A) Removing the 3' terminator hairpin from a 1x MS2 scRNA decreases CRISPRa in a yeast reporter system. See Supplementary Methods for complete sequences of 1x MS2 scRNA and 1x MS2 scRNA.b1. Experiments were performed in yeast as previously described using a single TetO target site to activate a Venus fluorescent reporter gene by recruiting MCP-VP64, an activator of eukaryotic transcription⁴.

B) A 2x MS2 scRNA, which increases CRISPRa in yeast and human cells relative to a 1x MS2 scRNA⁴, does not improve GFP expression with CRISPRa in *E. coli*. The activator in this experiment is MCP-(5aa)-SoxS_{R93A} (Fig. 3).

C) sgRNA 2.0 does not activate GFP expression in *E. coli*. The sgRNA 2.0 design has two MS2 hairpins embedded within internal hairpins of the sgRNA and is very effective for CRISPRa in eukaryotic cells⁵. We expressed either 1x MS2 scRNA or sgRNA 2.0 with a W108 target site (ON)

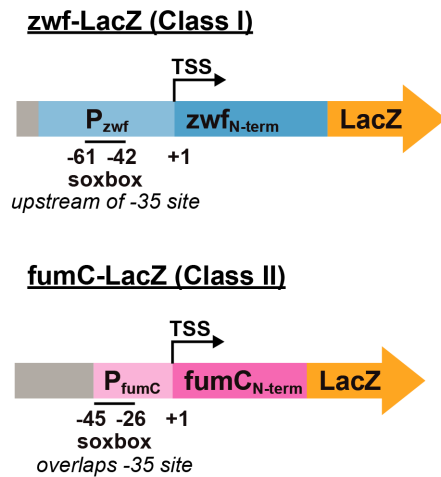
or an off-target RR2 sequence (OFF). The activator in this experiment is MCP-(5aa)-SoxS_{R93A} (Fig. 3).

D) Increasing the linker length between MCP and TetD from 2 to 5 amino acids modestly increases GFP expression, while increasing the linker further to 10 amino acids decreases GFP expression to background levels. The optimized 1x MS2.b1 scRNA was used in this experiment.

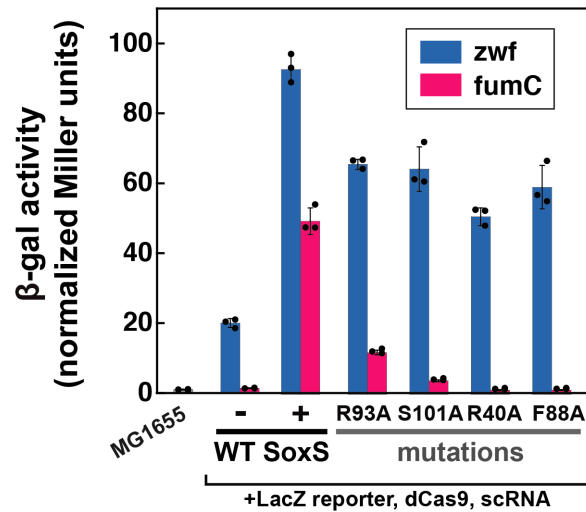
E) Mutations in the MCP- λ cII activator at R42, which is essential for DNA binding, retain activity when recruited via CRISPRa in *E. coli*, although the absolute level of GFP expression remains low compared to CRISPRa with MCP-SoxS. The unmodified 1x MS2 scRNA was used in this experiment. λ cII R42G was previously described⁶.

Values reported are Venus or GFP fluorescence levels measured by flow cytometry. Values are median $\pm$ s.d. for at least three measurements (specific values are indicated by black dots).

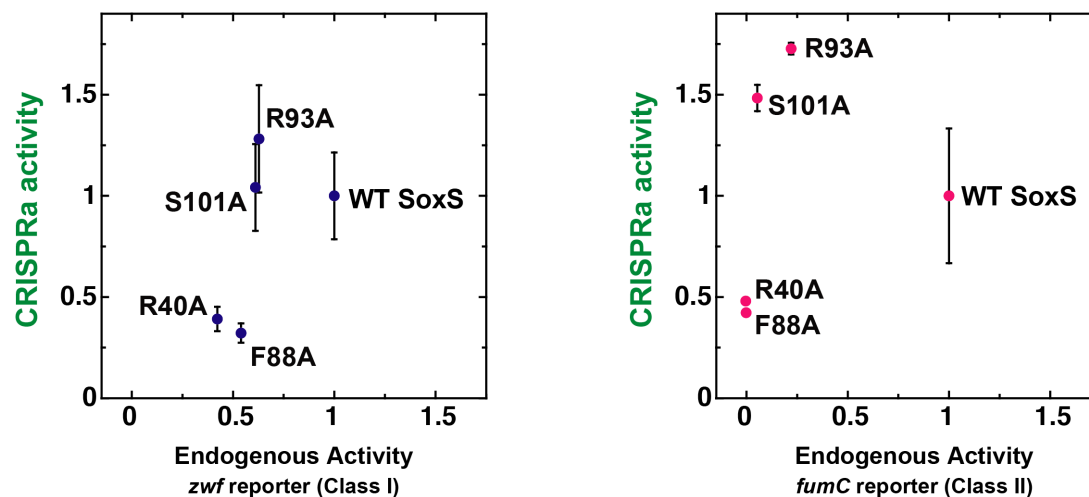
A SoxS Reporter Genes



B SoxS Reporter Activity



C Decoupling CRISPRa from Endogenous Activity with SoxS Mutants



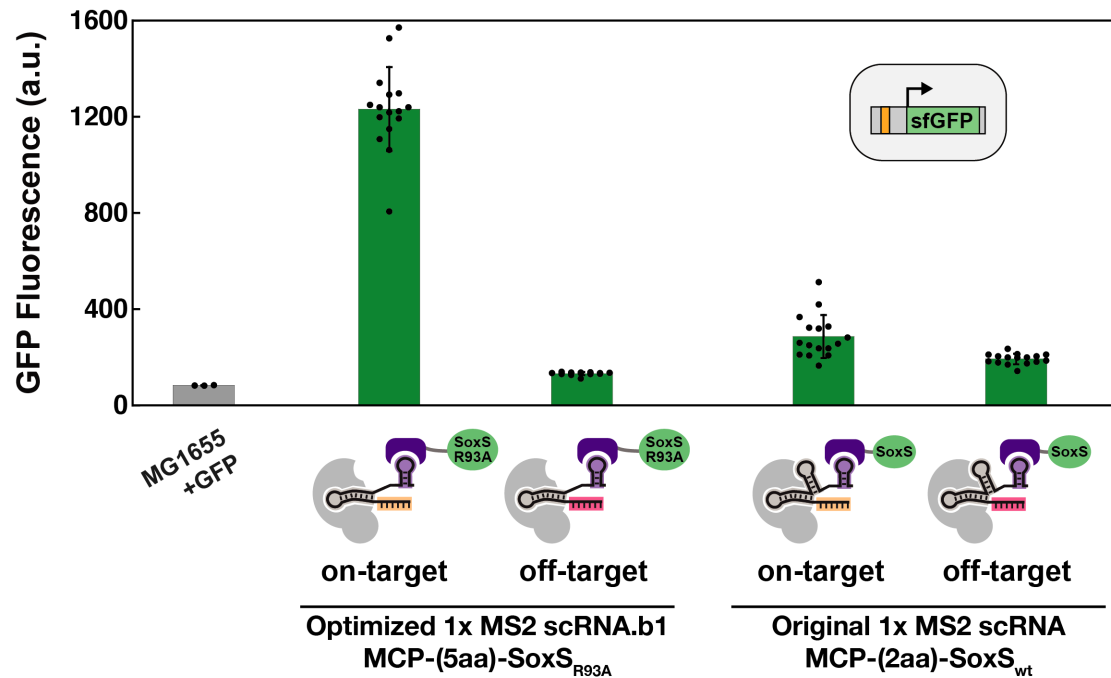
Supplementary Figure 3. Mutations in SoxS reduce activity at endogenous SoxS promoters.

A) Plasmids containing *zwf*-LacZ and *fumC*-LacZ fusion constructs were used as reporters of endogenous SoxS transcriptional activity⁷. The *zwf* promoter is representative of SoxS class I promoters in which the SoxS target site (soxbox) is upstream of the -35 site. The *fumC* promoter is representative of SoxS class II promoters in which the soxbox overlaps the -35 site. Complete sequences of the reporter constructs are included in Supplementary Methods.

B) LacZ (β-Gal) activity was measured in *E. coli* strain CD06 (Supplementary Table 1), an MG1655 strain modified to include a GFP reporter for CRISPRa activity. This strain was further transformed with a plasmid containing either the *zwf*-LacZ or *fumC*-LacZ reporter, along with a plasmid with the CRISPRa system components dCas9, a 1x MS2 scRNA.b1, and an MCP-SoxS fusion protein. Point mutants of SoxS that disrupt the DNA binding interface lead to reduced LacZ activity, and the results are consistent with previously reported activity trends for these mutants⁷. LacZ activity was measured as previously described⁸, and values are reported in Miller units

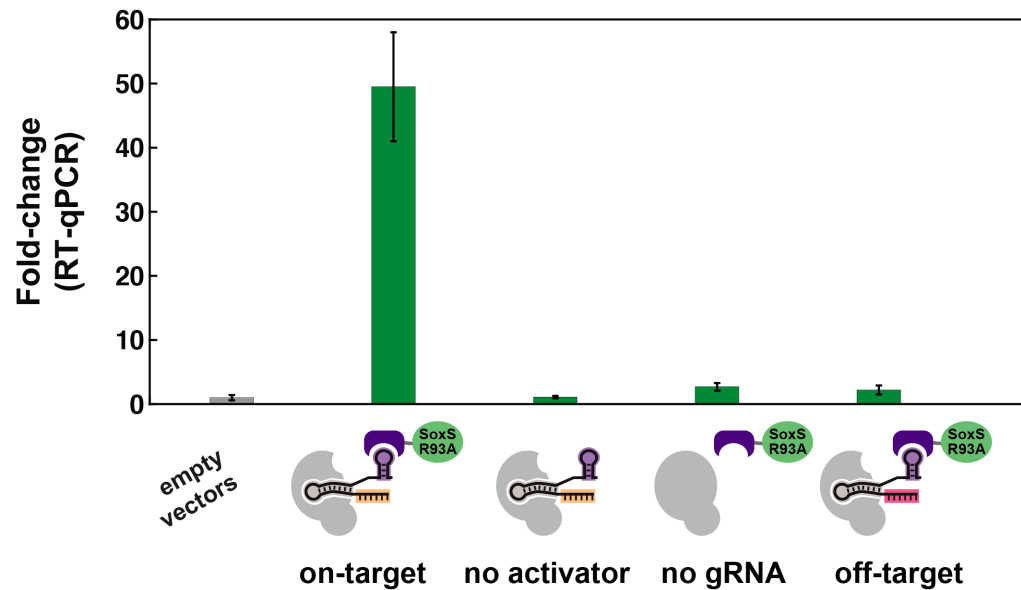
normalized to the value obtained in the MG1655 parent strain with no reporter plasmids. Values are median $\pm$ s.d. for three measurements (specific values are indicated by black dots).

C) Plots of CRISPRa activity vs endogenous activity for wild type (wt) and mutant SoxS proteins indicate that transcriptional activation can be decoupled from binding to endogenous targets. In the main text Fig. 3D, CRISPRa activity values were measured in strains that did not contain LacZ reporters (Fig. 3C). The CRISPRa activity values plotted here were obtained in the same strains used to measure LacZ activity. Similar trends were observed in both cases. CRISPRa activity values were normalized to the value obtained for wt SoxS. Endogenous activity values are LacZ activity (Supplementary Fig. 3B), corrected for background reporter activity in the absence of SoxS and normalized to the value obtained for wt SoxS. Values are median $\pm$ s.d. for at least three measurements.



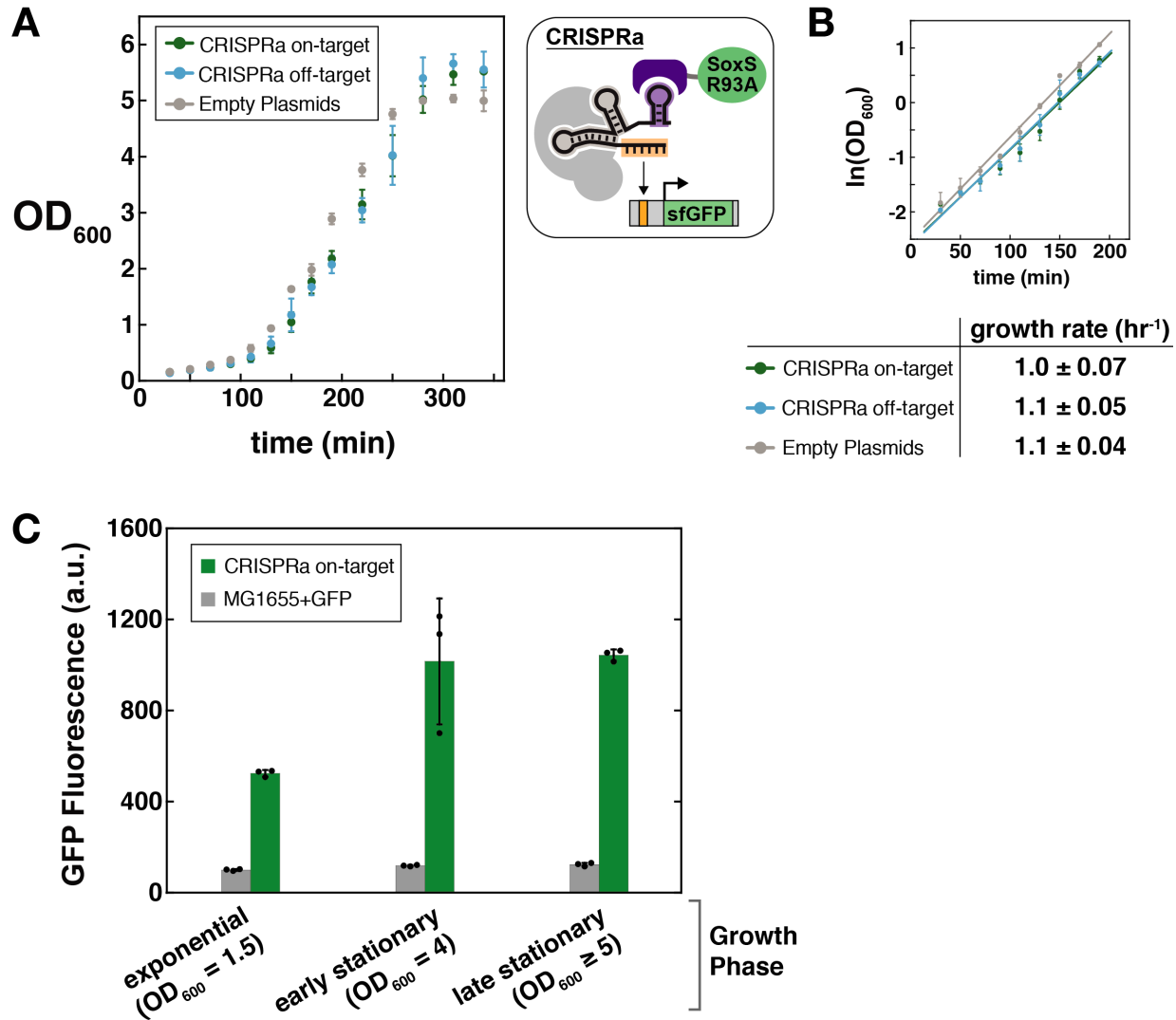
Supplementary Figure 4. An optimized CRISPRa system improves activity.

The optimized CRISPRa system with 1x MS2 scRNA.b1 and MCP-(5aa)-SoxS_{R93A} produces significantly more on-target GFP expression and less off-target GFP expression than the original system with 1x MS2 scRNA with MCP-(2aa)-SoxS. The on-target site is W108 and the off target site is RR2 (Supplementary Table 2). Values reported are GFP fluorescence levels measured by flow cytometry. Values are median $\pm$ s.d. for at least three biological replicates (specific values are indicated by black dots).



Supplementary Figure 5. CRISPRa significantly increases GFP mRNA levels.

GFP expression with a fully optimized scRNA.b1, MCP-(5aa)-SoxS_{R93A} CRISPRa system increases 50-fold relative to an empty vector control, as measured by RT-qPCR. Negative controls without MCP-(5aa)-SoxS_{R93A} (no activator) or scRNA.b1 produce 1.1-fold and 2.7-fold changes in GFP mRNA levels, respectively, relative to the empty vector control. When an off-target scRNA is expressed (RR2, Supplementary Table 2), there is a 2.2-fold change in GFP mRNA relative to the empty vector control. For comparison to the 50-fold increase in GFP mRNA levels with CRISPRa, we observe a 30-fold increase in GFP fluorescence in the same strain (Fig. 3E). The fluorescence change may be an underestimate of the true change in protein levels, as there is a significant autofluorescence background (see for example the observed GFP levels in the MG1655 parental strain versus MG1655+GFP in Fig. 2A). Expression levels from RT-qPCR were calculated from at least three replicates using the $\Delta\Delta C_T$ method⁹ with three independent measurements each of the target gene (GFP) and reference gene (16S rRNA). Error bars represent s.e.m. Because the mean and error of fold changes in the $\Delta\Delta C_T$ method are calculated from the average and error of the individual C_T values, individual data points are not plotted.

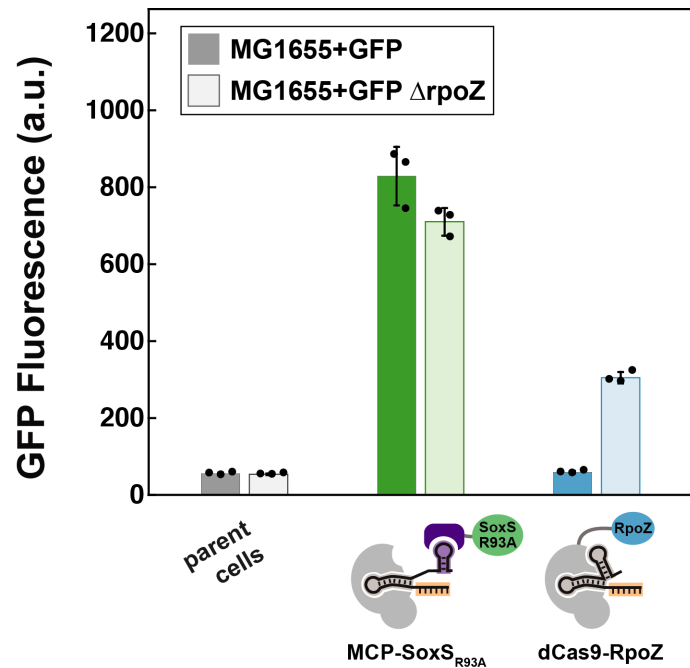


Supplementary Figure 6. Relationship between cell growth and CRISPRa.

A) The CRISPR-Cas system does not introduce a significant growth burden. Cell growth (measured by OD₆₀₀) versus time for *E. coli* cells transformed with empty plasmids or with CRISPRa system components, using 1x MS2 scRNAs with either on or off-target sequences. The growth curves are similar in all cases. Experiments were performed in biological triplicate, and error bars represent the standard deviation from at least three measurements.

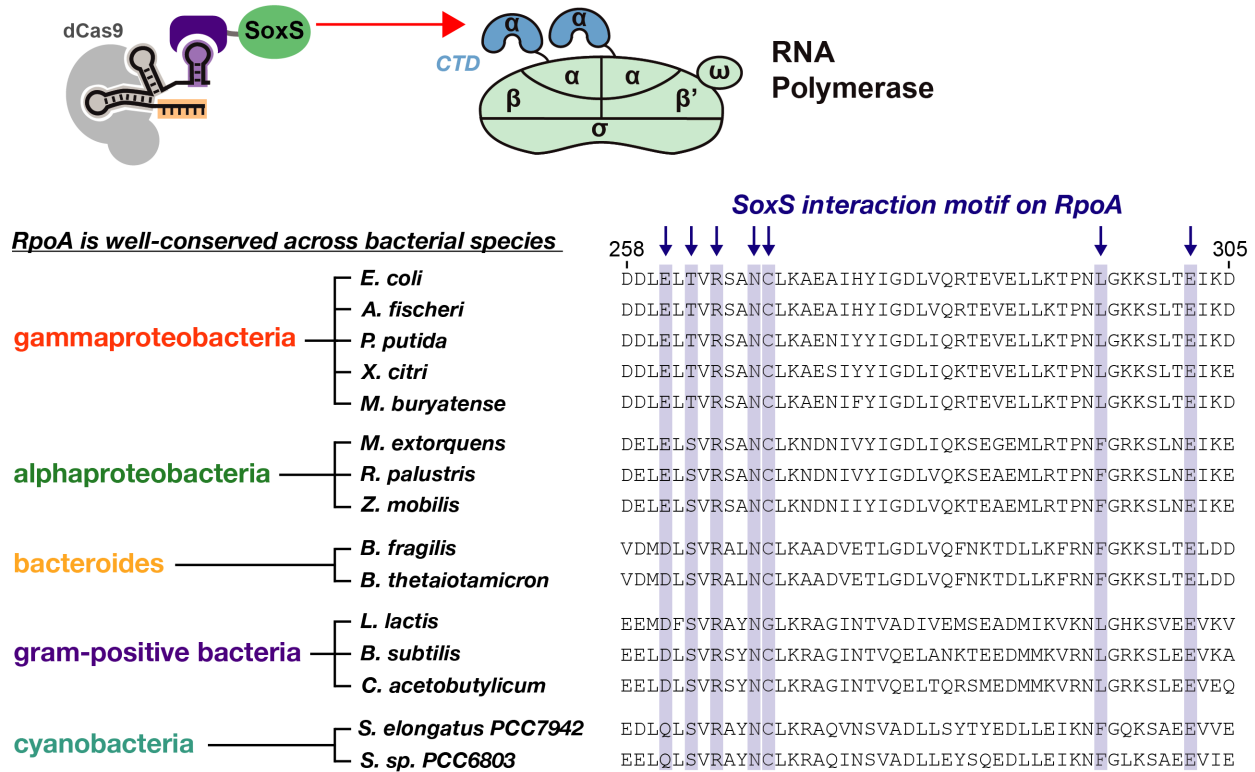
B) Quantification of growth rates using exponential phase data only (up to 200 minutes). The slope of the plot of ln(OD₆₀₀) versus time gives the growth rate. The observed growth rates are identical within error.

C) CRISPRa-mediated GFP expression increases as cells enter stationary phase, although gene expression is still detectable in exponential phase. Values reported are GFP fluorescence levels measured by flow cytometry. Values are median ± s.d. for at least three biological replicates (specific values are indicated by black dots).



Supplementary Figure 7. CRISPRa with fully optimized MCP-SoxS_{R93A} is more effective than dCas9-RpoZ, a previously-described bacterial CRISPRa system¹. MCP-SoxS_{R93A} activates gene expression in an MG1655 strain, where dCas9-RpoZ is ineffective. In a $\Delta rpoZ$ strain, where dCas9-RpoZ is effective, the SoxS-based system outperforms dCas9-RpoZ by >2-fold. Values reported are GFP fluorescence levels measured by flow cytometry. Values are median $\pm$ s.d. for at least three measurements (specific values are indicated by black dots).

SoxS interacts with the C-terminal domain (CTD) of the RNA polymerase α subunit (RpoA)

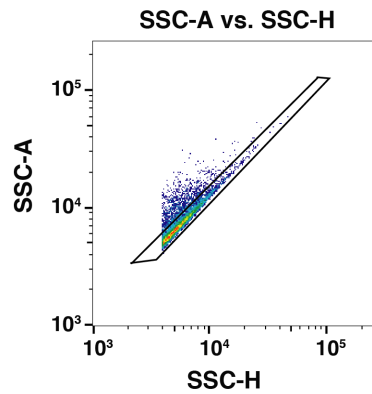


Supplementary Figure 8. The SoxS recruitment site on RNA polymerase is highly conserved. *E. coli* SoxS interacts with the C-terminal domain of RpoA, the polymerase α -subunit (α CTD), and specific amino acids on RpoA important for interacting with SoxS have been identified¹⁰. These amino acids in RpoA are largely conserved across a broad range of bacterial species. The observed conservation suggests that *E. coli* SoxS may be able to recruit RNA polymerase in other bacteria in addition to *E. coli*. Complete RpoA sequences were aligned using Clustal Omega¹¹, and the sequences corresponding *E. coli* residues 258-305 are shown.

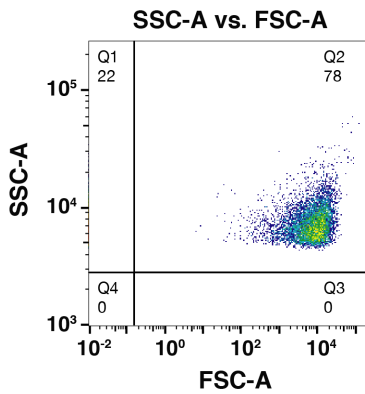
At least seven residues have been reported to affect the *E. coli* SoxS- α CTD interaction upon alanine substitution¹⁰. Alanine substitutions at E261, T263, and R265 have significant detrimental effects on the SoxS- α CTD interaction. R265 is absolutely conserved among the species examined here, and E261 and T263 are largely conserved with a few homologous substitutions (i.e. E- $\rightarrow$ D/Q or T- $\rightarrow$ S). Alanine substitutions at L295 and E302 have modest detrimental effects. L295 is largely conserved with some homologous substitutions (L- $\rightarrow$ F), and E302 is absolutely conserved. Alanine substitutions at N268 and C269 have modest beneficial effects on the SoxS- α CTD interaction; both residues are absolutely conserved except for a C- $\rightarrow$ G substitution in *L. lactis*.

1) Apply a threshold trigger on SSC-H during data collection

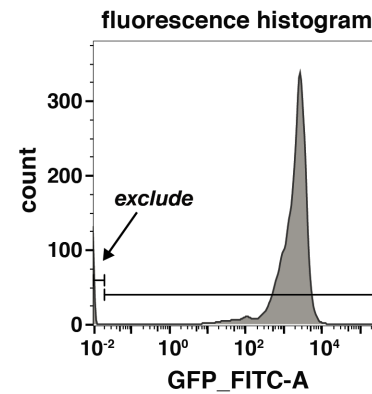
2) Gate along diagonal



3) Exclude edges



4) Exclude lower edge



Supplementary Figure 9. Flow cytometry gating strategy. To select for single bacterial cells, we (1) applied a side scatter threshold trigger (SSC-H) during data collection. We then gated for single cells by (2) selecting events along the diagonal of the SSC-H vs. SSC-A plot¹², (3) excluding events that appeared on the edges of the SSC-A vs. FSC-A plot, and (4) excluding events that appeared on the edges of the fluorescence histogram.

Supplementary Tables

Supplementary Table 1. *E. coli* Strains

Strain	Description	Genotype
MG1655	parent <i>E. coli</i> strain	F- λ - ilvG- rfb-50 rph-1
CD03	MG1655/ Δ rpoZ	MG1655 Δ rpoZ
CD06	MG1655/sfGFP (weak promoter)	MG1655 <i>W1-BBa_J23117-sfGFP KanR::nfsA</i>
CD08	MG1655/sfGFP (weak promoter)/ Δ rpoZ	MG1655 <i>W1-BBa_J23117-sfGFP KanR::nfsA ΔrpoZ</i>
CD10	MG1655/sfGFP (weak promoter)/ mRFP1 (strong promoter	MG1655 <i>W1-BBa_J23117-sfGFP::nfsA,</i> <i>BBa_J23119-mRFP1 KanR::rbsAR</i>

Supplementary Table 2. gRNA Target Sites

sgRNA target	DNA Sequence	Target Strand ^c	Distance to TSS ^d
W108 ^a	GAAGATCCGGCCTGCAGCCA	NT	91
RFP R2 ^b	TGGAACCGTACTGGAAGTGC	NT	-215
J101	TGGGTTCCACCGGATACCTC	T	40
J103	AGGCGTCCTTTGGGTTCCAC	T	50
J105	CGGTTACCAAAGGCGTCCTT	T	60
J107	CGGTGTCCTGCGGTACCAA	T	70
J109	AGGTATCCTGCGGTGTCCTG	T	80
J111	GGGCGACCTCAGGTATCCTG	T	90
J113	GGGCCACCACGGGCGACCTC	T	100
J115	TGGTGACCATGGGCCACCAC	T	110
J117	GGGTGACCTATGGTGACCAT	T	120
J119	TGGTTGCCAAGGGTGACCTA	T	130
J121	AGGACACCTTTGGTTGCCAA	T	140
J102	AGGTATCCGGTGGAAACCCAA	NT	61
J104	TGGAACCCAAAGGACGCCTT	NT	71
J106	AGGACGCCTTTGGTAACCGC	NT	81
J108	TGGTAACCGCAGGACACCGC	NT	91
J110	AGGACACCGCAGGATACCTG	NT	101
J112	AGGATACCTGAGGTCGCCCCG	NT	111
J114	AGGTCGCCCCGTGGTGGCCCA	NT	121
J116	TGGTGGCCCATGGTCACCAT	NT	131
J118	TGGTCACCATAGGTCACCCCT	NT	141
J120	AGGTCACCCCTTGGCAACCAA	NT	151

^a The W108 site for GFP activation was described previously using a 30 base gRNA targeting site (i.e. spacer sequence)¹. We use a 20 base gRNA targeting site for the same site (i.e. the same PAM site).

^b The RFP R2 site for RFP repression by CRISPRi was described previously¹³.

^c Template strand (T) or non-template strand (NT).

^d Distance to TSS is the distance from the 3' end (PAM proximal) of the guide target site to the transcription start site. For synthetic promoters driven by BBa_J23117 or BBa_J23119 (<http://parts.igem.org>), the TSS is immediately downstream of the BBa sequence (see complete maps below).

Supplementary Table 3. *E. coli* Expression Plasmids^a

Plasmid	Marker	origin	Promoter	Gene	Terminator
pCD067	1) <i>AmpR</i> 2) <i>KanR</i>	<i>R6K</i>	<i>BBa_J23117</i>	sfGFP	<i>BBa_B0015</i>
pJF023	1) <i>AmpR</i> 2) <i>KanR</i>	<i>R6K</i>	<i>BBa_J23119</i>	mRFP1	<i>BBa_B0015</i>
p_gRNA_bact_RR2 ^b	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	sgRNA (RR2)	<i>TrrnB</i>
pCD005	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	1x MS2 scRNA (W108 target)	<i>TrrnB</i>
pCD006	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	2x MS2 scRNA (W108 target)	<i>TrrnB</i>
pCD015	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	sgRNA (W108 target)	<i>TrrnB</i>
pCD061	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	1x MS2 scRNA.b1 (W108 target)	<i>TrrnB</i>
pJF67-2	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	1x MS2 scRNA.b2 (W108 target)	<i>TrrnB</i>
pCD034	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	1x MS2 scRNA (RR2)	<i>TrrnB</i>
pCD315	<i>AmpR</i>	<i>ColE1</i>	<i>pBAD</i>	1x MS2 scRNA.b1 (W108 target)	<i>TrrnB</i>
pCD325	<i>AmpR</i>	<i>ColE1</i>	<i>pBAD</i>	sgRNA (RR2)	<i>TrrnB</i>
pCD326	<i>AmpR</i>	<i>ColE1</i>	1) <i>pBAD</i> 2) <i>pBAD</i>	1) 1x MS2 scRNA.b1 (W108 target) 2) sgRNA (RR2)	1) <i>TrrnB</i> 2) <i>TrrnB</i>
pCD372	<i>AmpR</i>	<i>ColE1</i>	1) <i>BBa_J23119</i> 2) <i>BBa_J23119</i>	1) 1x MS2 scRNA.b1 (W108 target) 2) sgRNA (RR2)	1) <i>TrrnB</i> 2) <i>TrrnB</i>
pCD403	<i>AmpR</i>	<i>ColE1</i>	<i>BBa_J23119</i>	1x MS2 scRNA.b1 (RR2)	<i>TrrnB</i>
pCD017	<i>CmR</i>	<i>p15A</i>	<i>Sp.pCas9</i>	dCas9	<i>BBa_B0015</i>
pCD018	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-rpoZ	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD037	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) αNTD-MCP	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD064	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-rpoD	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD087	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-N4SSB (Y75A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD123	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(2aa)-SoxS (wt)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD134	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(2aa)-MarA	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD141	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(2aa)-Rob	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD146	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(2aa)-TetD	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD151	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(2aa)-λcII	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>

pCD175	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23107</i>	1) dCas9 2) MCP-AsiA 3) rpoD (F563Y)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>BBa_B1002</i>
pCD226	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23107</i>	1) dCas9 2) MCP-GP33	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD351	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-CAP	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD156	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(5aa)-SoxS (wt)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD157	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(10aa)-SoxS (wt)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD185	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(10aa)-SoxS (R93A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD186	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD404	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(5aa)-SoxS (F88A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD406	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(5aa)-SoxS (R40A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pCD408	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i>	1) dCas9 2) MCP-(5aa)-SoxS (S101A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pJF093	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>TetR-pTet</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pJF094	<i>CmR</i>	<i>p15A</i>	1) <i>TetR-pTet</i> 2) <i>Bba_J23107</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pJF104B	<i>CmR</i>	<i>p15A</i>	1) <i>TetR-pTet</i> 2) <i>TetR-pTet</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i>
pJF121	<i>AmpR</i>	<i>ColE1</i>	<i>TetR-pTet</i>	1x MS2 scRNA (W108 target)	<i>TrrnB</i>
pCD227	<i>CmR</i>	<i>p15A</i>	1) <i>pBAD</i> 2) <i>BBa_J23107</i> 3) <i>araC</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A) 3) <i>AraC</i>	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>N/A</i>
pJF077.1~21°	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A) 3) 1x MS2 scRNA.b2 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD294.1~21	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) PCP-(5aa)-SoxS (R93A) 3) 1x PP7 scRNA.b1 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD296.1~21	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-TetD 3) 1x MS2 scRNA.b2 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD297.1~21	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) αNTD-MCP 3) 1x MS2 scRNA.b2 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD298.1~21	<i>CmR</i>	<i>p15A</i>	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(2aa)-λcII (R42A) 3) 1x MS2 scRNA.b2 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>

pCD416.1~21	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(2aa)-SoxS (R93A) 3) 1x MS2 scRNA.b2 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD417.1~21	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(10aa)-SoxS (R93A) 3) 1x MS2 scRNA.b2 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD441.1~21	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(20aa)-SoxS (R93A) 3) 1x MS2 scRNA.b2 (J101~121 targets)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD290	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A) 3) 1x MS2 scRNA.b2 (J106 target)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD477	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-SoxS (wt) 3) 1x MS2 scRNA.b1 (W108)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD478	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-SoxS (R93A) 3) 1x MS2 scRNA.b1 (W108)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD479	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-SoxS (S101A) 3) 1x MS2 scRNA.b1 (W108)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD481	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-SoxS (F88A) 3) 1x MS2 scRNA.b1 (W108)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pCD482	CmR	p15A	1) <i>Sp.pCas9</i> 2) <i>BBa_J23107</i> 3) <i>BBa_J23119</i>	1) dCas9 2) MCP-(5aa)-SoxS (R40A) 3) 1x MS2 scRNA.b1 (W108)	1) <i>BBa_B0015</i> 2) <i>BBa_B1002</i> 3) <i>TrrnB</i>
pJF076	<i>AmpR</i>	pSC101	<i>J1_BBa_J23117</i>	mRFP1	<i>BBa_B0015</i>
pCD469	<i>AmpR</i>	pSC101	<i>zwf</i>	<i>zwf</i> _{N-term} -LacZ	<i>BBa_B0015</i>
pCD470	<i>AmpR</i>	pSC101	<i>fumC</i>	<i>fumC</i> _{N-term} -LacZ	<i>BBa_B0015</i>
pCD355	<i>AmpR</i>	pSC101	<i>J1_BBa_J23117</i>	<i>Z. mobilis</i> <i>pdh/adhB</i> (single operon)	<i>BBa_B0015</i>
pJF009 ^d	<i>AmpR</i>	<i>ColE1</i>	n/a	n/a	n/a
pJF043 ^d	CmR	p15A	n/a	n/a	n/a

^a BBa sequences are from the Repository of Standard Biological Parts (<http://parts.igem.org>). dCas9 is the catalytically inactive form of *S. pyogenes* Cas9¹³. Sp.pCas9 is the endogenous Cas9 promoter from *S. pyogenes*. pBAD is an arabinose-inducible promoter, described previously¹⁴. Complete sequences of the GFP, RFP, and LacZ reporter constructs are included in the Supplementary Experimental Procedures.

^b The plasmid with the RFP R2 gRNA for RFP repression was described previously¹³.

^c Plasmid designations such as pJF077.1~21 indicate a set of plasmids (pJF077.1, pJF077.2...) where the final number corresponds to guide RNA target sites (J101, J102..., Supplementary Table 2) used for the J1 mRFP1 reporter (Fig. 4).

^d pJF009 and pJF043 are empty vector control plasmids with expression cassettes removed from p_gRNA_bact_RR2 and pCD017, respectively. These control plasmids were used in RT-qPCR experiments (Supplementary Fig. 5) and growth burden experiments (Supplementary Fig. 6).

Supplementary Table 4. Primer Sequences for RT-qPCR

Primer	Sequence
qCD010_sfGFP_f	GAGGGTGAAGGTGATGCTACAA
qCD011_sfGFP_r	GGTCAGAGTAGTGACAAGTGTGG
qCD012_16S_f	AAAGTTAATACCTTTGCTCATTGACGTT
qCD013_16S_r	GACTACCAGGGTATCTAATCCTGTTT

Supplementary Methods

Scaffold RNA (scRNA) Sequence Designs

gRNA and 1x MS2 scRNA sequences with RNA recruitment hairpins were initially constructed following previous designs^{4,13}. 1x MS2 scRNA.b1 removes the final tracr RNA terminator hairpin, based on secondary structure predictions of the hairpin structure¹⁵. 1x MS2 scRNA.b2 removes the final tracr RNA terminator hairpin, based on the x-ray crystal structure of gRNA in complex with Cas9 and a DNA target¹⁶. Compared to scRNA.b1, the scRNA.b2 design includes one additional G from the tracr sequence and removes the two base GC linker to the MS2 hairpin.

Parent sgRNA

GAAGATCCGGCCTGCAGCCA GTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGC TTTTTT

1x MS2 scRNA

GAAGATCCGGCCTGCAGCCA GTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGC GCGACATGAGGATCACCCATGTGC TTTTTT

2x MS2 scRNA

GAAGATCCGGCCTGCAGCCA GTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGC GGGAGCACATGAGGATCACCCATGTGCCACGAGCGACATG
AGGATCACCCATGTCTCGTCTGTTCC TTTTTT

1x MS2 scRNA.b1

GAAGATCCGGCCTGCAGCCA GTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGCGACATGAGGATCACCCATGTGC TTTTTT

1x MS2 scRNA.b2

GAAGATCCGGCCTGCAGCCA GTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTG GCGACATGAGGATCACCCATGTGC TTTTTT

1x PP7 scRNA.b1

GAAGATCCGGCCTGCAGCCA GTTT TAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGT AACATAAGGAGTTTATATGGAAACCCATTATG TTTTTT

Annotations:

20 base target site (W108), 1x MS2, tracr RNA terminator, 1x PP7

Candidate activator sequences

Sequences shown below are those candidate activators that gave detectable GFP expression in initial experiments. RNA binding proteins MCP (MS2 coat protein) or PCP (PP7 coat protein) were fused to the N-termini of candidate activator proteins, except for the RpoA α NTD. RpoA has a native flexible linker connecting its N and C terminal domains¹⁷, so we cloned the α NTD with its flexible linker and fused this sequence to the N-terminus of MCP. The MCP and PCP proteins are modified versions (MCP_{ΔFG, V29I} and PCP_{ΔFG}) to prevent oligomerization and improve RNA binding activity⁴.

> MCP-(2aa)-SoxS (wtSoxS with 2aa linker used in Fig. 2)

MGPASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTIKVEVPGAWRSYLN
MELTIPIFATNSDCELIVKAMQGLLKDGNIPIPSAIAANSIYGGGSM^{SHQKI IQDLIAWIDEHIDQPLNIDVVAKSGY}
SKWYLQRMFRTVTHQTLGDYIRQRRLLLA^{AVELRTTERPIFDIAMDLGYVSQQTFSRVFRRQFDRTPSDYRHRL}

> MCP-(5aa)-SoxS_{R93A} (fully optimized version, Fig. 3 and subsequent)

MGPASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTIKVEVPGAWRSYLN
MELTIPIFATNSDCELIVKAMQGLLKDGNIPIPSAIAANSIYGGGGSM^{SHQKI IQDLIAWIDEHIDQPLNIDVVAK}
SGYSKWYLQRMFRTVTHQTLGDYIRQRRLLLA^{AVELRTTERPIFDIAMDLGYVSQQTFSRVFARQFDRTPSDYRHRL}

> MCP-(5aa)-TetD (optimized linker for Fig. 5; 2aa linker used in Fig. 2)

MGPASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTIKVEVPGAWRSYLN
MELTIPIFATNSDCELIVKAMQGLLKDGNIPIPSAIAANSIYGGGGSM^{YIEQHSRYQNKANNIQLRYDDKQFHTTVI}
KDVLWIEHNLDQSLLLDDVANKAGYTKWYFQRLFKKVTGVTLAS^{YIRARLTKAAVELRLTKKTILEIALKYQFDS}
QQSFTRRFKYIFKVTPSYRRNKLWELEAMH

> MCP- λ cII

MGPASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTIKVEVPGAWRSYLN
MELTIPIFATNSDCELIVKAMQGLLKDGNIPIPSAIAANSIYGSM^{VRANKRNEALRIESALLNKIAMLGTEKTAEAV}
GVDKSQISRWKRDWIPKFSMLLAVLEWGVVDDDMARLARQVAAILTNKKRPAATERSE^{QIQMEF}

> MCP-RpoZ

MGPASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTIKVEVPGAWRSYLN
MELTIPIFATNSDCELIVKAMQGLLKDGNIPIPSAIAANSIYGS^{ARVTVDQAVEKIGNRFDLVLAARRARQMVG}
KDPLVPEENDKTTVIALREIEEGLINNQILDVRERQEQQEQEAAELQAVTAIAEGR

> α NTD-MCP

MQGSVTEFLKPRLV^{DI}EQVSSTHAKVTLEPLERGF^{GHT}LGNALRRILLSSMPGCAVTEVEIDGVLHEYSTKEGVQED
ILEILLNLKGLAVRVQ^{GK}DEVILT^{LN}KSIGPVTAA^{DITH}DGDVEIVK^{PQH}VICH^{LT}DENASISMRIK^{VQR}GRGYVP
ASTRIHSEEDERPIGRLLVDACYS^{PVER}IAYNVEAARVEQ^{RTDL}DKLVIEMETNGTIDPEEAIRRAATILAEQLEAF
VDLRDVRQPEVKEEKPEASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTI
KVEVPGAWRSYLN
MELTIPIFATNSDCELIVKAMQGLLKDGNIPIPSAIAANSIY

> MCP-AsiA

MGPASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTIKVEVPGAWRSYLN
MELTIPIFATNSDCELIVKAMQGLLKDGNIPIPSAIAANSIYGSM^{NKNIDTVREIITVASILIKFSREDIVENRANF}
IAFLNEIGVTHEGRKL^{NQNS}FRKIVSELTQEDK^{KT}LID^{EF}NEGFEGVRYLEMYTNK

> PCP-(5aa)-SoxS_{R93A}

MGPSKTI^{VL}SVGEATRTLTEIQSTADRQIFEEKVG^{PLV}GRRLTASLRQNGAKTAYRVNLKLDQADVVD^{SGL}PKVRY
TQVWSDVTIVANSTEASR^{SLY}DLTKSLVATSQVEDLVVNLVPLGRGGGGS^{MSHQKI IQDLIAWIDEHIDQPLNID}
VVAKSGYSKWYLQRMFRTVTHQTLGDYIRQRRLLLA^{AVELRTTERPIFDIAMDLGYVSQQTFSRVFARQFDRTPSD}
YRHRL

Fluorescent Protein Reporter Sequences

GFP Reporter (Fig. 2, 3, & 5)

This construct reports on gene activation using a GFP reporter driven by a weak promoter. The promoter was constructed following the design by Bikard et al., 2013¹, driving expression of superfolder GFP (sfGFP)¹⁸. BbA sequences are from the Repository of Standard Biological Parts (<http://parts.igem.org>).

W108 target site, BbA_J23117 promoter, Bujard RBS, sfGFP, BbA_B0015 terminator

```
GCATGCCCAGTCAACGTCTCATTTTCGCCAGATATCAAGCAGAGGAGCAAAAGCTCATTTCTGAAGAGGACTTGTTG
CGGAAACGACGAGAACAGTTGAAACACAACTTGAACAGCTACGGAACCTTGTGCGTAAGGAAAAGTAAGGAAAAC
GATTCCTTTCTAACAGAAATGTCCTGAGCAATCACCTATGAAGTGTGACTCGAGCCTCTATGGATTATCACCTTGGC
TGCAGGCCGGATCTTCACAAACACGCACGGTGTTACATTAGGCATACCGGTCttgacagctagctcagtcctagggg
ttgtgctagcGAATTCATTAAAGAGGAGAAAGGTACCATGAGCAAAGGAGAAGAAGCTTTTCACTGGAGTTGTCCAA
TTCTTGTGAATTAGATGGTGTATGTTAATGGGCACAAATTTTCTGTCCGTGGAGAGGGTGAAGGTGATGCTACAAAC
GGAAACTCACCTTAAATTTATTTGCACTACTGGAAACTACCTGTTCCGTGGCCAACACTTGTCACTACTCTGAC
CTATGGTGTTCATGCTTTTCCCGTTATCCGGATCACATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAG
GTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGACCTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGT
GATACCTTGTGAATCGTATCGAGTTAAAGGGTATTGATTTTAAAGAAGATGGAACATTCTTGGACACAAACTCGA
GTACAACCTTAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTC
GCCACAACGTTGAAGATGGTTCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTC
CTTTTACCAGACAACCATTACCTGTGACACAATCTGTCTTTTCGAAAGATCCCAACGAAAAGCGTGACCACATGGT
CCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAGCTCTACAAAtaaggatccaaactcgagt
aaggatctccaggcatcaataaaaacgaaaggctcagtcgaaagactgggcctttcgcttttatctgttgtttgtcg
tgaacgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttata
```

RFP Reporter (Fig. 5)

This construct reports on gene silencing using an RFP reporter driven by a strong promoter. The reporter was constructed following the design from Qi et al., 2013^{1,13}. The RFP R2 gRNA target site is underlined.

BbA_J23119 promoter, Bujard RBS, mRFP1, BbA_B0015 terminator

```
GCCCTCTAGAGGTGCAAAACCTTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATtt
gacagctagctcagtcctaggtataatagatctGAATTCATTAAAGAGGAGAAAGGTACCATGGCGAGTAGCGAAGA
CGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCCGTTAACGGTCACGAGTTCGAAATCGAAGGTG
AAGGTGAAGGTCGTCCGTACGAAGGTACCCAGACCGCTAAACTGAAAGTTACCAAGGTGGTCCGCTGCCGTTTCGCT
TGGGACATCCTGTCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGTTAAACACCCGGCTGACATCCCGGACTACCT
GAAACTGTCTTCCCGGAAGGTTTCAAATGGGAACGTGTTATGAAGTTCGAAGACGGTGGTGTGTTACCGTTACCC
AGGACTCCTCCCTGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGTCCGACGGTCCG
GTTATGCAGAAAAAACCATGGGTTGGGAAGCTTCCACCGAACGTATGTACCCGGAAGACGGTGCTCTGAAAGGTGA
AATCAAAATGCGTCTGAAACTGAAAGACGGTGGTCACTACGACGCTGAAGTTAAACACCTACATGGCTAAAAAAC
CGTTTCAGCTGCCGGGTGCTTACAAAACCGACATCAAACCTGGACATCACCTCCCAACGAAGACTACACCATCGTT
GAACAGTACGAACGTGCTGAAGGTCGTCACTCCACCGGTGCTTAaggatccaaactcgagtaaggatctccaggcat
caataaaaacgaaaggctcagtcgaaagactgggcctttcgcttttatctgttgtttgtcggtgaacgctctctacta
gagtcacactggctcaccttcgggtgggcctttctgcgtttata
```

J1 RFP reporter (Fig. 4)

This construct reports on gene activation using an RFP reporter driven by a weak promoter. The J1 upstream region includes PAM sites on both strands every 10 bases to systematically map out the relationship between gRNA target site position and gene activation.

J1 upstream region, BBA_J23117 promoter, Bujard RBS, mRFP1, BBA_B0015 terminator

```
actcttcctttttcaatattattgaagcatttatcaggggttattgtctcatgagcggatatacatatttgaatgtattt
agaaaaataaacaataggggttccgcgcacatttccccgaaaagtgccacctgtggcaattccgacgtcGCCTACG
GTATCCACCGGAGACCTATGGCAGCCTCCGGCCGCCATAGGACACCTTTGGTTGCCAAGGGTGACCTATGGTGACCA
TGGGCCACCACGGGCGACCTCAGGTATCCTGCGGTGTCCTGCGGTTACCAAAGGCGTCCTTTGGGTTCCACCGGATA
CCTCCGGACTtgacagctagctcagtcctagggattgtgctagcGAATTCATTAAAGAGGAGAAAGGTACCATGGCG
AGTAGCGAAGACGTTATCAAAGAGTTCATGCGTTTCAAAGTTCGTATGGAAGGTTCCGTTAACGGTCACGAGTTCGA
AATCGAAGGTGAAGGTGAAGGTGTCGTCGTACGAAGGTACCCAGACCGCTAAACTGAAAGTTACCAAAGGTGGTCCGC
TGCCGTTTCGCTTGGGACATCCTGTCCCCGCAGTTCCAGTACGGTTCCAAAGCTTACGTTAAACACCCGGCTGACATC
CCGGACTACCTGAAACTGTCCTTCCCGGAAGGTTTCAAATGGGAACGTGTTATGAACTTCGAAGACGGTGGTGTGT
TACCGTTACCCAGGACTCCTCCCTGCAAGACGGTGAGTTCATCTACAAAGTTAAACTGCGTGGTACCAACTTCCCGT
CCGACGGTCCGGTTATGCAGAAAAAACCATGGGTGGGAAGCTTCCACCGAACGTATGTACCCGGAAGACGGTGCT
CTGAAAGGTGAAATCAAATGCGTCTGAAACTGAAAGACGGTGGTCACTACGACGCTGAAGTTAAAACACCTACAT
GGCTAAAAAACCGGTTTCAGCTGCCGGGTGCTTACAAAACCGACATCAAAGTGGACATCACCTCCACAACGAAGACT
ACACCATCGTTGAACAGTACGAACGTGCTGAAGGTCGTCACTCCACCGGTGCTTAaggatccaaactcgagtaagga
tctccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgtttgttcggtgaac
gctctctactagagtcacactgggtcaccttcgggtgggcctttctgcgtttata
```


fumC-LacZ reporter

This construct reports on activation of the endogenous Class II SoxS target gene *fumC* using a *fumC*-LacZ operon fusion. The construct includes 46 bases upstream of the TSS, the 5' UTR, and coding sequence corresponding to *fumC* amino acids 1-56 fused in frame to LacZ. This construct is similar but not identical to a previously described *fumC*-LacZ reporter gene²². The TSS and the soxbox were experimentally identified previously^{21,23}. Bba sequences are from the Repository of Standard Biological Parts (<http://parts.igem.org>).

fumC promoter, *fumC*₁₋₅₆, *LacZ*, Bba_B0015 terminator. The soxbox is underlined.

```
ATGGCACGAAAGACCAAAACATTTGTTATCAAATGGTAAATAATAAGTGAGCTAAAAGTTGCTTAACGAAAGCAAAACAGAAAGAAA
AAATTAATCAGGTGAGGAGCAGGTCATGAATACAGTACGACGCGAAAAAGATTTCGATGGGGGCGATTGATGTCCCGGCAGATAAGC
TGTGGGGCGCACAACTCAACGCTCGCTGGAGCATTTCCGCATTTTCGACGGAGAAAATGCCACCTCACTGATTTCATGCGCTGGCG
CTAACCAAGCGTGCAGCGGCCATGACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCTGGCGT
TACCCAACCTAATCGCCTTGACGACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC
AGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCTTGGTTTCCGGCACCAGAAGCGGTGCCGAAAGCTGGCTGGAGTGCATCTT
CCTGAGGCCGATACGTGTCGTGCTCCCTCAAACCTGGCAGATGCACGGTTACGATGCGCCCATCTACACCAACGTGACCTATCCCAT
TACGGTCAATCCGCCGTTTGTTCACGAGAAATCCGACGGGTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGG
AAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCCTTTCATCTGTGGTGCAACGGGCGCTGGGTGGTTACGGCCAGGAC
AGTCGTTTGGCGTGTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGA
CGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCGCTGACGTCTCGTTGCTGCATAAACCGACTACACAAA
TCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTTCAGCCGCGCTGTACTGGAGGCTGAAGTTTCAGATGTGCGGCGAGTTG
CGTGACTACCTACGGGTAACAGTTTCTTTATGGCAGGGTGAAACGACAGGTGCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTTAT
CGATGAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCGAAAACCCGAAACTGTGGAGCGCCGAAATCCCGAATC
TCTATCGTGCGGTGGTTGAAGTGCACACCGCCGACGGCAGCGTGATTGAAGCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGG
ATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTCACGAGCATCATCTCTGCATGGTCA
GGTCATGGATGAGCAGACGATGGTGAGGATATCCTGCTGATGAAGCAGAACAACCTTAACGCCGTGCGCTGTTTCGCATTATCCGA
ACCATCCGCTGTGTGACACGCTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATGGTGCCA
ATGAATCGTCTGACCGATGATCCGCGCTGGCTACCGGCGATGAGCGAACGCGTAACGCGAATGGTGACGCGCATCGTAATCACCC
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Ethanol Production Pathway Cassette (pCD355)

This construct includes the *Z. mobilis* *pdh-adhB* gene cluster driven by a weak promoter. In Fig. 6, the CRISPRa complex targets the J106 site upstream of the promoter. J106 is the site with maximal activity in reporter assays (Fig. 4B).

J1 upstream region (J106 site underlined), BBa_J23117 promoter, Bujard RBS, *Zm.pdc*, *Zm.adhB*, BBa_B0015 terminator

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