Supplementary Information

Expanding the Scope of Bacterial CRISPR Activation with PAM-flexible dCas9 Variants

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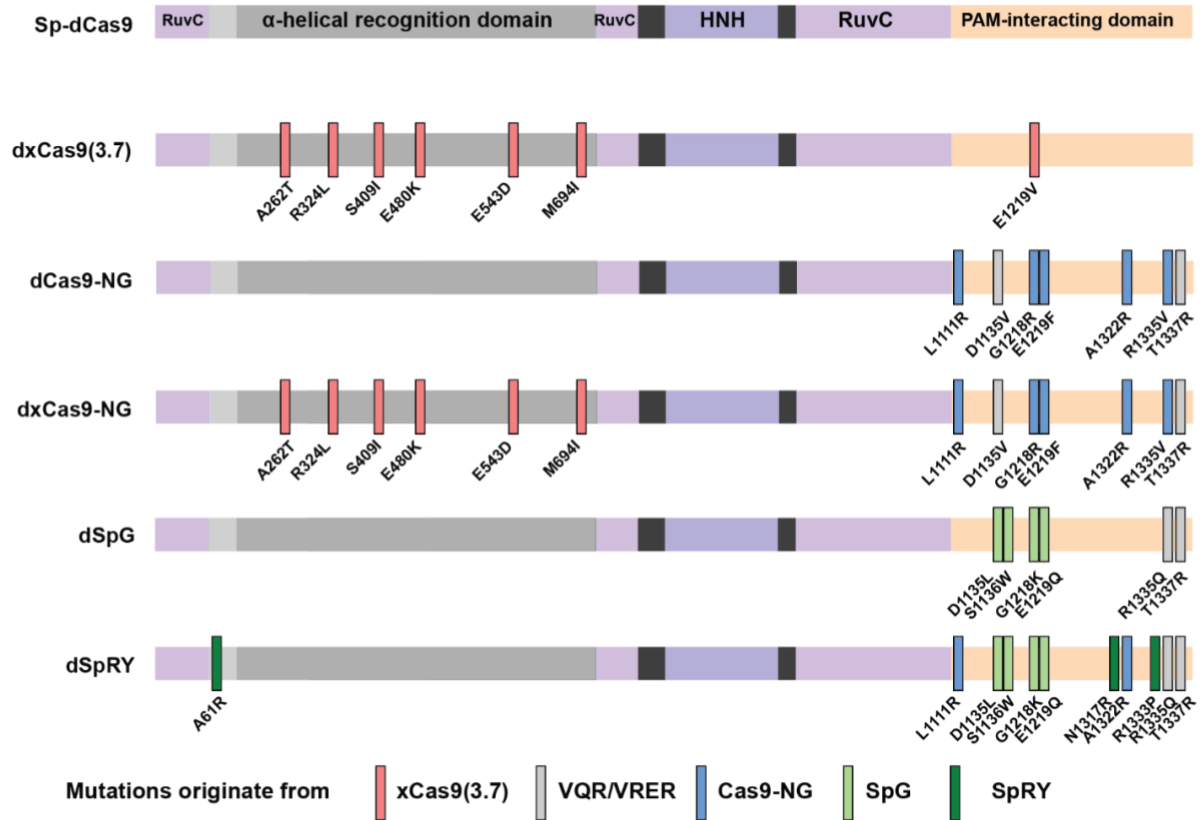

Figure S1: Mutations contributing to PAM-flexibility of engineered dCas9 variants

PAM-flexible dCas9 variants contain differing sets of mutations. All variants share the catalytically-inactivating D10A and H840A mutations of dCas9.[1] Mutations of dxCas9(3.7), from phage-assisted continuous evolution (PACE), were mostly at the α-helical recognition domain.[2] dCas9-NG engineering was guided by the crystal structure with mutations made in the PAM-interacting domain.[3] dxCas9-NG has combined mutations from dxCas9(3.7) and dCas9-NG.[4] dSpG and dSpRY were developed through structure-guided engineering with engineering trajectories monitored using a high-throughput PAM determination assay.[5,6] SpG was engineered using mutations from the VRQR variant as a starting point,[7,8] and SpRY contains mutations from Cas9-NG.[3] dSpRY is an evolved version of dSpG with a preference for NRN and some NYN PAMs (R is purine nucleotides, Y is pyrimidine nucleotides).[6] Mutation colors indicate the origin of each specific mutation. Complete sequences are provided in the DNA sequences section below.

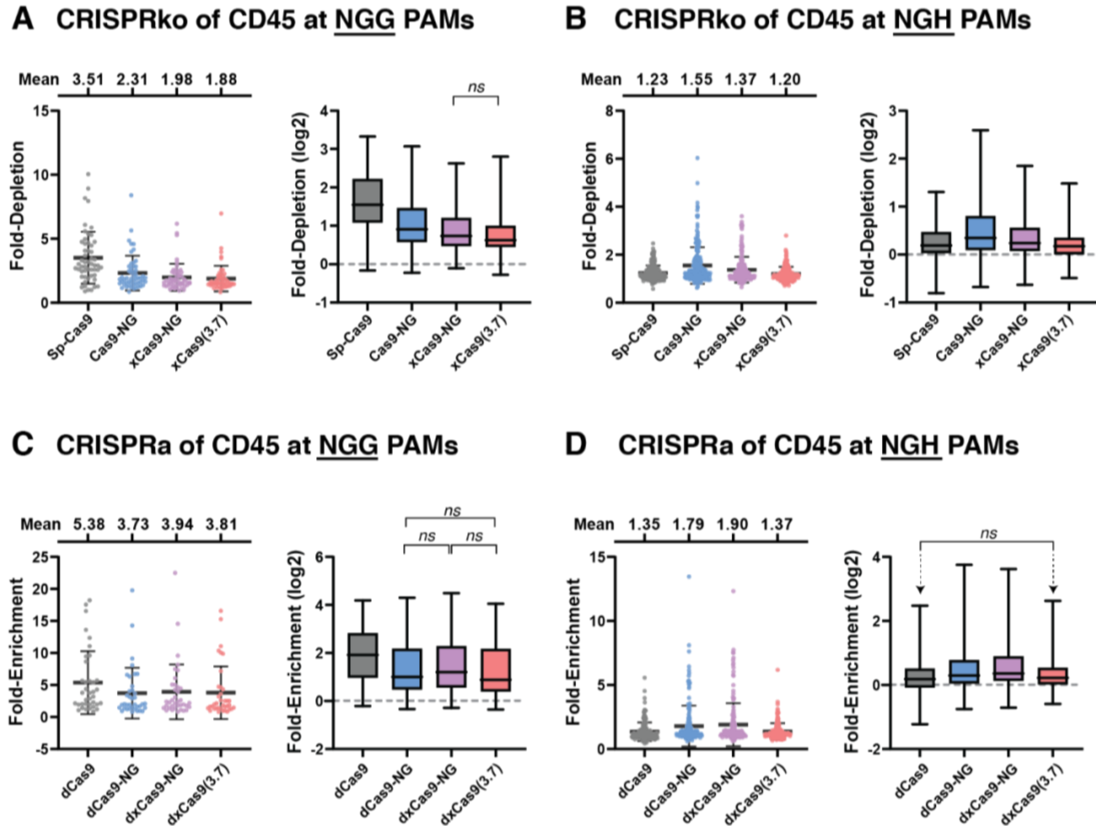

Figure S2: CRISPRko and CRISPRa in mammalian systems with PAM-flexible dCas9 variants

Cas9 variants were evaluated for CRISPRko (knockout) and CRISPRa (activation) at different PAMs. sgRNA-Cas9 effector complexes were targeted to the CD45 gene and changes in expression levels were evaluated by FACS-Seq (see Supplemental Methods). xCas9-NG and Cas9-NG exhibit comparable levels of PAM flexibility and both outperform xCas9(3.7). Data were plotted as fold-change scatter plots (left panel) or log2 fold-change box plots (right panel) for (A) CRISPRko at NGG PAMs, (B) CRISPRko at NGH PAMs, (C) CRISPRa at NGG PAMs, and (D) CRISPRa at NGH PAMs. In the scatter plots, bars and whiskers represent mean and standard deviation, respectively. In the box plots, two-tailed unpaired Welch's *t* test for each dCas9 pair were performed. Only non-significant comparisons (*ns*, $p > 0.05$) are indicated; all other differences (between proteins, within modalities) are significant.

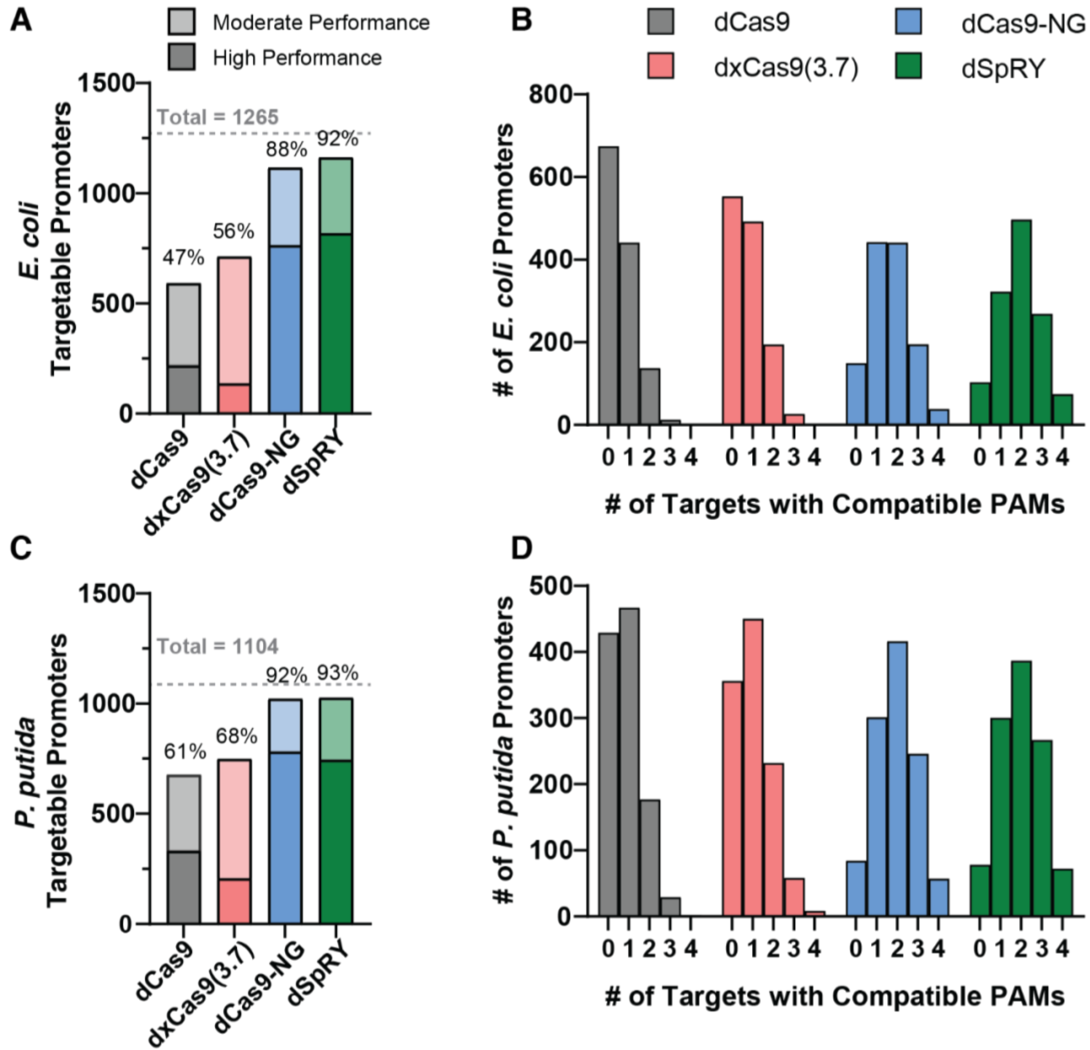

Figure S3: PAM availability and distribution in *E. coli* and *P. putida* promoters

Endogenous *E. coli* and *P. putida* promoters were analyzed for predicted targetable sites with different PAM-flexible dCas9 variants (see Methods section for further details). (A) Number of targetable *E. coli* promoters for different PAM-flexible dCas9 variants. 1265 *E. coli* promoters were analyzed for the presence of a compatible PAM at any of the four effective target site positions upstream of the TSS (see Methods). “High performance” PAMs are classified as PAMs with at least 50% efficiency relative to dCas9 at NGG PAMs. “Moderate performance” PAMs are classified as PAMs with 20-50% efficiency. (B) Distribution of promoters with 0 to 4 compatible PAMs with at least moderate performance in *E. coli*. (C) Number of targetable *P. putida* promoters for different PAM-flexible dCas9 variants. 1104 *P. putida* were analyzed with the same strategy. (D) Distribution of promoters with 0 to 4 compatible PAMs with at least moderate performance in *P. putida*. In both *E. coli* and *P. putida*, dxCas9-NG and dSpRY access more endogenous promoters than dCas9 and dxCas9(3.7). As *P. putida* has a higher GC content than *E. coli* and a correspondingly higher fraction of NGN PAMs, there is an almost identical number of targetable promoters for dCas9-NG and dSpRY.

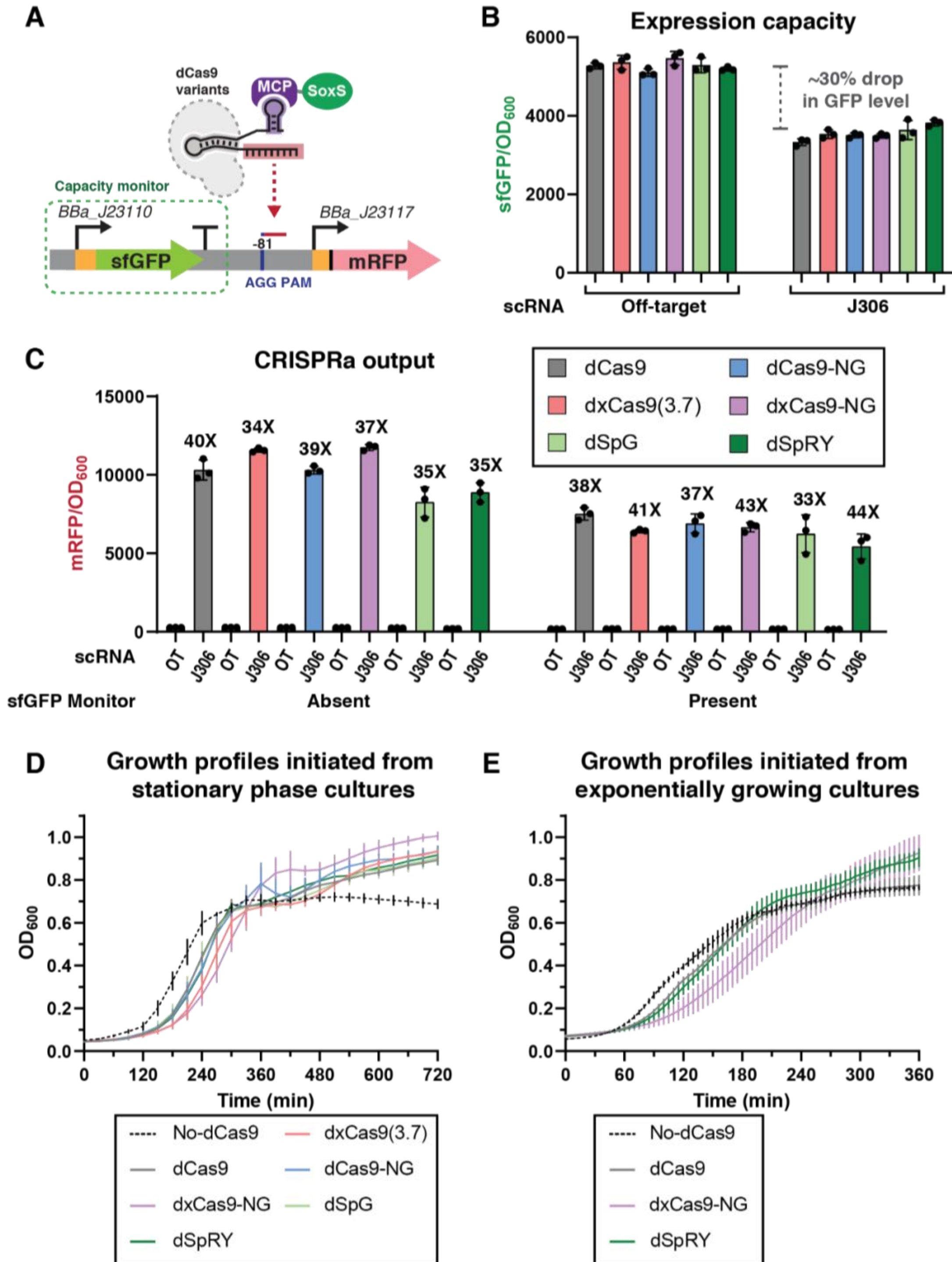

Figure S4: Expression capacity and growth burden associated with dCas9 variants

(A) A constitutive, medium-strength promoter (BBa_J23110) expressing sfGFP acts as a protein expression capacity monitor. (B) CRISPRa upregulation of mRFP (using J306 scRNA) produces a ~30% decrease in the sfGFP expression capacity monitor compared to an off-target scRNA control. Similar effects were observed with all dCas9 variants. (C) The presence of the sfGFP capacity monitor causes a modest decrease in CRISPRa-mediated mRFP expression. The basal expression levels (off-target controls) also decrease, leading to similar mRFP fold-changes

in the presence or absence of the sfGFP capacity monitor. Similar effects were observed with all dCas9 variants. (D&E) Expression of all dCas9 variants caused a modest lag in *E. coli* growth relative to cells that do not express dCas9. In (D), growth profiles were obtained from 1:100 subcultures from overnight, stationary-phase cultures. In (E), growth profiles were obtained by diluting exponentially growing cells back to OD_{600} = 0.1. This experiment was performed to evaluate if the growth lag from dCas9 expression observed in (D) was caused by slow recovery from stationary phase. We observed a similar growth lag from dCas9 expression in both (D) and (E). A recent paper describes comparable growth experiments with *E. coli* in microbioreactors; these experiments observed similar growth behavior with dCas9, dxCas9, and dSpRY.[9]

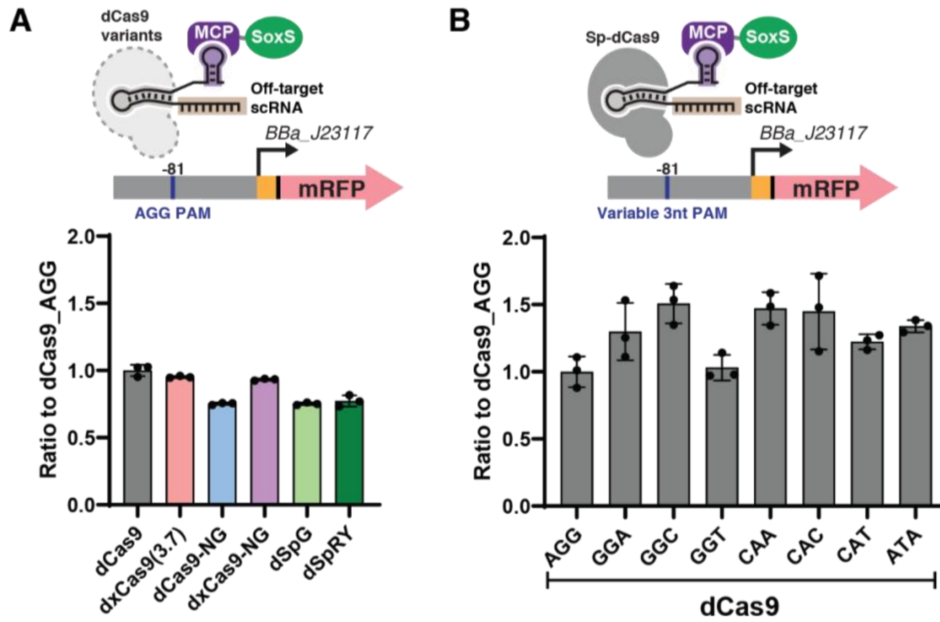

Figure S5: Basal expression of the mRFP reporter gene with different dCas9 variants and PAMs sequences

(A) Expression of different dCas9 variants produces less than 2-fold changes in basal reporter expression level. This experiment was performed with AGG PAM reporter and an off-target scRNA (hAAVS1). These off-target expression levels were used as a basal expression for fold-change calculation with each dCas9 variant in Figure 2. (B) Modified mRFP reporter genes with alternative upstream PAM sites produce less than 2-fold changes in basal reporter expression. This experiment was performed with Sp-dCas9 and an off-target scRNA (hAAVS1). Values in panel A and B represent the mean ± standard deviation calculated from n = 3.

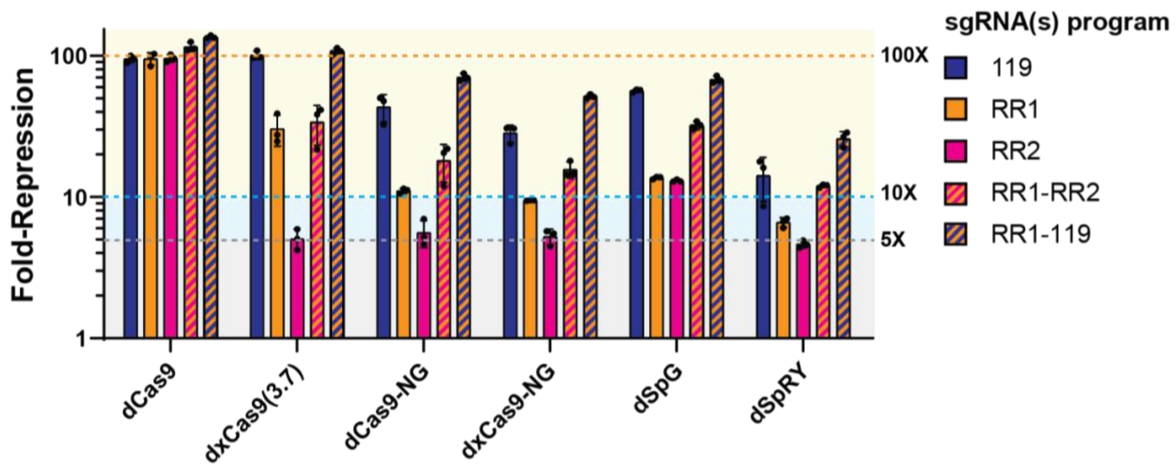

Figure S6: CRISPRi with PAM-flexible dCas9 variants

CRISPRi for PAM-flexible dCas9 variants was tested on an mRFP reporter gene with sgRNAs targeting the promoter (119) or coding sequence (RR1 and RR2) with one or two sgRNAs expressed, each targeting sites harboring NGG PAMs. dCas9 exhibited the highest repression efficiency (>90-fold) among all variants. When only a single sgRNA is expressed, the sgRNA targeting the promoter region (119) produces the largest repression effect with all dCas9 variants. The addition of the second sgRNA (RR1-RR2 or RR1-119) led to significant improvement in repression for all variants. Values represent the mean ± standard deviation calculated from n = 3.

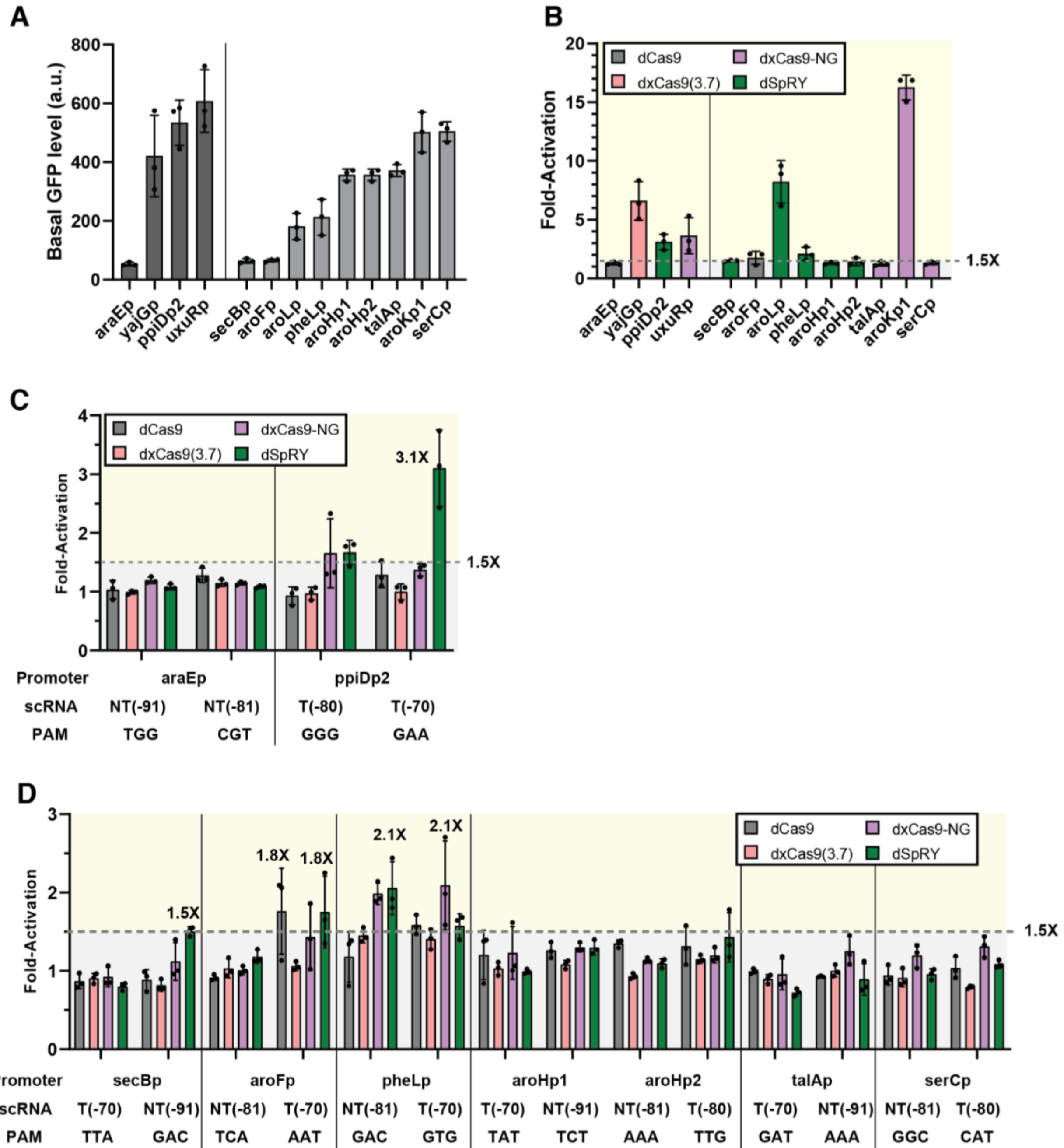

Figure S7: CRISPRa at endogenous promoters with PAM-flexible dCas9 variants

(A) Basal expression levels for endogenous promoters (left: promoters previously tested with dxCas9(3.7);[10] right: new promoters examined in this work). Basal expression levels were measured in a strain expressing the parent dCas9 and an off-target scRNA (hAAVS1). (B) Maximum fold-activation for each endogenous promoter. Bar color indicates the dCas9 variant with the highest fold-activation for the corresponding endogenous promoter. 8 out of 13 endogenous promoters can be activated (>1.5-fold) with PAM-flexible dCas9 variants. (C) CRISPRa at endogenous promoters previously tested with dxCas9(3.7) (araEp and ppiDp2) using NGG or non-NGG PAMs. See main text Figure 4B for the other two promoters from this set (yajGp and uxuRp). (D) CRISPRa at endogenous promoters involved in aromatic amino acid

biosynthesis using non-NGG PAMs (secBp, aroFp, pheLp, aroHp1, aroHp2, talAp, and serCp). See main text Figure 4C for the other two promoters from the aromatic amino acid biosynthesis set (aroLp and aroKp1). Data were collected by flow cytometry and fold-activation was calculated compared to the strain expressing the corresponding dCas9 variant and an off-target hAAVS1 scRNA. Values in panels A-D represent the mean ± standard deviation calculated from n = 3.

Supplementary Tables

Supplementary Table S1: *E. coli* strains.

Strain	Description	Genotype
MG1655	Wildtype *E. coli strain*	F- λ- ilvG- rfb-50 rph-1
CD38	Integrated BBa_J23119-mRFP used for CRISPRi (adapted from JF01[11])	MG1655 *rbsAR*::BBa_J23119-mRFP

Supplementary Table S2: Selected *E. coli* plasmids.

Plasmid	Marker	Origin	Promoter	Gene	Terminator	Reference
pJF043	CmR	p15A	None	None	None	This work
pCD442	CmR	p15A	1) Sp.pCas9 2) BBa_J23107	1) dCas9 2) MCP-SoxS	1) BBa_B0015 2) BBa_B1002	[10]
pCD564	CmR	p15A	1) Sp.pCas9 2) BBa_J23107	1) dxCas9(3.7) 2) MCP-SoxS	1) BBa_B0015 2) BBa_B1002	[10]
pCK668	CmR	p15A	1) Sp.pCas9 2) BBa_J23107	1) dCas9-NG 2) MCP-SoxS	1) BBa_B0015 2) BBa_B1002	This work
pCK669	CmR	p15A	1) Sp.pCas9 2) BBa_J23107	1) dxCas9-NG 2) MCP-SoxS	1) BBa_B0015 2) BBa_B1002	This work
pCK340	CmR	p15A	1) Sp.pCas9 2) BBa_J23107	1) dSpG 2) MCP-SoxS	1) BBa_B0015 2) BBa_B1002	This work
pCK341	CmR	p15A	1) Sp.pCas9 2) BBa_J23107	1) dSpRY 2) MCP-SoxS	1) BBa_B0015 2) BBa_B1002	This work
pCK085.scRNA	CmR	p15A	1) Sp.pCas9 2) BBa_J23107 3) BBa_J23119	1) dCas9 2) MCP-SoxS 3) scRNA	1) BBa_B0015 2) BBa_B1002 3) TrrnB	[12]
pCK281.scRNA	CmR	p15A	1) Sp.pCas9 2) BBa_J23107 3) BBa_J23119	1) dxCas9(3.7) 2) MCP-SoxS 3) scRNA	1) BBa_B0015 2) BBa_B1002 3) TrrnB	This work
pCK670.scRNA	CmR	p15A	1) Sp.pCas9 2) BBa_J23107 3) BBa_J23119	1) dCas9-NG 2) MCP-SoxS 3) scRNA	1) BBa_B0015 2) BBa_B1002 3) TrrnB	This work
pCK671.scRNA	CmR	p15A	1) Sp.pCas9 2) BBa_J23107 3) BBa_J23119	1) dxCas9-NG 2) MCP-SoxS 3) scRNA	1) BBa_B0015 2) BBa_B1002 3) TrrnB	This work
pCK363.scRNA	CmR	p15A	1) Sp.pCas9 2) BBa_J23107 3) BBa_J23119	1) dSpG 2) MCP-SoxS 3) scRNA	1) BBa_B0015 2) BBa_B1002 3) TrrnB	This work
pCK364.scRNA	CmR	p15A	1) Sp.pCas9 2) BBa_J23107 3) BBa_J23119	1) dSpRY 2) MCP-SoxS 3) scRNA	1) BBa_B0015 2) BBa_B1002 3) TrrnB	This work

pJF143. J3	AmpR	pSC101**	J3-BBa_J23117	mRFP	BBa_B0015	[10]
pCK760	AmpR	pSC101**	1) BBa_J23110 2) J3-BBa_J23117	1) sfGFP 2) mRFP	1) BBa_B0015 2) BBa_B0015	This work
pCK284. NNN	AmpR	pSC101**	J3(NNN-PAM)- BBa_J23117	mRFP	BBa_B0015	This work
pCD443. scRNA	AmpR	ColE1	BBa_J23119	scRNA	TrrnB	This work
pCK411. scRNA/ sgRNA	AmpR	ColE1	BBa_J23119	scRNA/sgRNA	BBa_K268040 5	This work
pCK590. XXX	KmR	pSC101	Endogenous (strand +)	GFPmut2		Same as [13]
pCK591. XXX	KmR	pSC101	Endogenous (strand –)	GFPmut2		Same as [13]

Supplementary Table S3: scRNA and sgRNA target sites.

sc/sgRNA	DNA sequence	Target strand[a]	Distance to TSS[b]	PAM
J306	TTGTGTCCAGAACGCTCCGT	NT	-81	AGG
hAAVS1	GGGGCCACTAGGGACAGGAT	Off-target	NA	–
RR1	AACTTTCAGTTTAGCGGTCT	NT	151[c]	GGG
RR2	TGGAACCGTACTGGAACTGC	NT	215[c]	GGG
119	AATTCAGATCTATTATACCT	NT	-15	AGG
yajGp_Y1	TTGACGAAATAATCGCCCCT	NT	-81	GGT
yajGp_Y2	CATCAGTGTTTCTTTTACCA	T	-79	GGG
uxuRp_U1	TGATTGACCAGTAAGTCTGT	NT	-81	AGG
uxuRp_U2	GATTACCCTACAGACTTACT	T	-70	GGT
ppiDp2_D1	ACTAAGCGTTGTCCCCAGTG	T	-80	GGG
ppiDp2_D2	GTCCCCAGTGGGGATGTGAC	T	-70	GAA
araEp_E1	TGCGACATGTCGTTATGTGA	NT	-91	TGG
araEp_E2	ATTAAATTGCTGCGACATGT	NT	-81	CGT
pheLp_X06	GCGATACACTCAATATAAAG	NT	-81	GAC
pheLp_X07	AGAGTAGTCCTTTATATTGA	T	-70	GTG
secBp_X07	CACCACGGTTCCCCAGATTT	T	-70	TTA
secBp_X08	AATCTGGGGAACCGTGGTGC	NT	-91	GAC
serCp_X06	ACCGTTGAGGGCAAAAATGT	NT	-81	GGC
serCp_X09	CTTTTGTGTGATGCAAGCCA	T	-80	CAT
talAp_X07	GGTAATAATCCTATAACACT	T	-70	GAT
talAp_X08	GTGTTATAGGATTATTACCA	NT	-91	AAA
aroFp_X06	CAGGCAATTTAGTCGCGCTT	NT	-81	TCA
aroFp_X07	AAGGGTTGAAAGCGCGACTA	T	-70	AAT
aroLp_X07	TGGTGGCTGGAAGTGCAACG	T	-70	TAG
aroLp_X08	GTTGCACTTCCAGCCACCAC	NT	-91	TTC
aroHp1_X07	ATTGCCACCAAGATCCTCGA	T	-70	TAT
aroHp1_X08	CGAGGATCTTGGTGGCAATC	NT	-91	TCT
aroHp2_X06	GTGGTTAGCATGATAACAAA	NT	-81	AAA
aroHp2_X09	TAGTGCATTAGCTTATTTTT	T	-80	TTG
aroKp1_X07	GAGTAAACAGCCGTAAAAGC	T	-70	GGT
aroKp1_X08	CTTTTACGGCTGTTTACTCA	NT	-91	CTG

[a] *Template strand (T) or non-template strand (NT).*

[b] *Distance from the 3' end of the guide site (PAM proximal) to the TSS. For synthetic promoters (BBa_J23117 or BBa_J23119, http://parts.igem.org), the TSS is immediately downstream.*

[c] *For RR1 and RR2 sgRNAs, the positive number refers to the distance downstream of the TSS.*

Supplementary Table S4: Predicted compatible PAMs for each dCas9 variants

PAM	Sp-Cas9	xCas9(3.7)	Cas9-NG	SpG	SpRY
AAA	0.18%	2.75%	19.51%	N.A.	27.97%
AAC	0.06%	1.52%	17.62%	N.A.	69.73%
AAG	46.32%	19.04%	43.10%	N.A.	28.35%
AAT	0.25%	3.80%	28.04%	N.A.	6.58%
ACA	0.61%	0.23%	5.03%	N.A.	19.84%
ACC	-0.50%	-0.15%	2.18%	N.A.	N.A.
ACG	17.85%	7.43%	12.10%	N.A.	N.A.
ACT	-0.45%	0.47%	7.07%	N.A.	N.A.
AGA	27.95%	29.33%	60.81%	65.89%	58.50%
AGC	7.82%	16.70%	52.14%	75.97%	56.19%
AGG	99.80%	62.37%	72.89%	38.75%	21.46%
AGT	1.82%	23.39%	68.90%	78.02%	59.55%
ATA	0.78%	0.21%	12.29%	N.A.	43.63%
ATC	-0.56%	-0.34%	5.07%	N.A.	3.20%
ATG	22.04%	5.45%	28.21%	N.A.	N.A.
ATT	-0.13%	0.55%	10.60%	N.A.	N.A.
CAA	-0.01%	6.21%	27.29%	N.A.	53.17%
CAC	-0.53%	2.69%	26.45%	N.A.	19.04%
CAG	44.62%	25.71%	47.15%	N.A.	18.17%
CAT	-0.63%	6.25%	32.92%	N.A.	22.44%
CCA	-0.13%	-0.21%	2.06%	N.A.	N.A.
CCC	-0.65%	-0.31%	0.89%	N.A.	1.01%
CCG	11.47%	3.99%	6.78%	N.A.	9.06%

CCT	-0.22%	-0.37%	3.09%	N.A.	N.A.
CGA	30.35%	36.77%	59.10%	78.85%	N.A.
CGC	10.29%	24.23%	52.31%	70.58%	N.A.
CGG	102.08%	68.15%	72.25%	48.14%	15.80%
CGT	2.47%	32.69%	69.50%	49.23%	23.77%
CTA	-0.23%	0.64%	9.53%	N.A.	20.11%
CTC	-0.52%	0.19%	4.65%	N.A.	25.22%
CTG	17.31%	3.94%	23.82%	N.A.	19.44%
CTT	-0.44%	-0.06%	9.93%	N.A.	2.30%
GAA	1.02%	11.96%	37.31%	N.A.	100.83%
GAC	-0.13%	4.71%	31.57%	N.A.	86.73%
GAG	53.38%	35.04%	57.91%	N.A.	55.14%
GAT	0.05%	10.29%	42.64%	N.A.	35.73%
GCA	-0.05%	0.76%	7.21%	N.A.	55.08%
GCC	-0.26%	0.28%	4.37%	N.A.	21.67%
GCG	13.01%	1.67%	11.22%	N.A.	N.A.
GCT	-0.58%	0.22%	10.36%	N.A.	43.08%
GGA	30.31%	37.93%	60.78%	62.42%	33.41%
GGC	12.24%	22.95%	53.55%	73.33%	41.75%
GGG	101.50%	70.07%	76.36%	67.74%	54.37%
GGT	7.16%	33.76%	69.40%	76.14%	65.25%
GTA	0.05%	1.79%	20.16%	N.A.	37.98%
GTC	-0.34%	0.56%	11.00%	N.A.	N.A.
GTG	23.36%	6.76%	37.60%	N.A.	50.91%
GTT	-0.15%	0.29%	16.20%	N.A.	4.66%
TAA	0.62%	1.36%	16.05%	N.A.	69.86%

TAC	-0.62%	0.15%	11.41%	N.A.	72.28%
TAG	36.56%	11.45%	34.32%	N.A.	30.23%
TAT	-0.22%	1.52%	19.26%	N.A.	44.40%
TCA	-0.36%	0.01%	1.27%	N.A.	N.A.
TCC	-0.81%	-0.28%	0.72%	N.A.	N.A.
TCG	10.87%	3.10%	5.79%	N.A.	N.A.
TCT	-0.15%	-0.37%	2.47%	N.A.	7.00%
TGA	28.62%	29.68%	61.71%	73.33%	45.34%
TGC	7.91%	15.40%	53.76%	88.36%	65.05%
TGG	96.62%	62.22%	72.08%	95.59%	69.12%
TGT	2.32%	24.78%	70.19%	103.69%	N.A.
TTA	0.06%	0.53%	3.71%	N.A.	N.A.
TTC	-1.16%	-0.09%	0.44%	N.A.	N.A.
TTG	9.19%	2.01%	11.05%	N.A.	0.14%
TTT	-0.46%	-0.14%	1.55%	N.A.	11.70%

The predicted PAM compatibility of each PAM-Cas9 pair was reported as indel frequency (in % units) relative to the benchmark efficiency of Sp-Cas9 at NGG PAMs from each experiment (see Supplementary Methods).[6,14] Each data point was color-coded based on predicted efficiency: Green (high efficiency, >50% of benchmark), blue (moderate efficiency, 20%–50%), and gray (low efficiency, less than 20%). NGG PAMs are highlighted in yellow. N.A. means data is not available. Some of the reported efficiency values are small negative numbers, likely due to negligible editing frequencies indistinguishable from the background. Comparable data for indel frequencies with xCas9-NG are not available; for this work we assumed Cas9-NG and xCas9-NG were similar based on their comparable performance in nuclease assays (Figure S2).

Supplementary Table S5: CRISPRa on endogenous *E. coli* promoters

Promoter	Max. FA	dCas9 variant	Position	PAM
yajGp	**6.6** 6.2 5.1	**dxCas9(3.7)** dxCas9-NG dSpRY	NT(-81)	GGT
uxuRp	**3.6** 2.6	**dxCas9-NG** dSpRY	T(-70)	GGT
araEp	1.3	dxCas9-NG	NT(-81)	CGT
ppiDp2	**3.1**	**dSpRY**	T(-70)	GAA
aroKp1	**16.3** 12.8	**dxCas9-NG** dxCas9(3.7)	T(-70)	GGT
aroLp	**8.2** 5.5	**dSpRY** dCas9	T(-70)	TAG
aroF	**1.8** **1.8**	**dCas9** **dSpRY**	T(-70)	AAT
aroHp1	1.3	dxCas9-NG	NT(-91)	TCT
aroHp2	1.4	dSpRY	NT(-81)	AAA
pheLp	**2.1** **2.1**	**dxCas9-NG** **dSpRY**	T(-70) NT(-81)	GTG GAC
secBp	**1.5**	**dSpRY**	NT(-91)	GAC
serCp	1.3	dxCas9-NG	T(-80)	CAT
talAp	1.2	dxCas9-NG	NT(-91)	AAA

The top two conditions were shown for each promoter, if >1.5-fold. The best conditions for each promoter is shown in bold. yajGp has three similar activation levels. Promoters with <1.5-fold activation were shaded in gray.

Supplementary Methods

Evaluating PAM accessibility from CRISPR knockout and CRISPR activation screens in mammalian systems

To predict the PAM accessibility for different Cas9 variants, we used data from CRISPR knockout (CRISPRko) and CRISPR activation (CRISPRa) screens in mammalian cells. Previously, screening data has been reported for three Cas9 variants (Sp-Cas9, xCas9(3.7), and Cas9-NG).[4] We report here a corresponding screen performed using the same approach with four Cas9 variants: Sp-Cas9, xCas9(3.7), Cas9-NG, and xCas9-NG. Lentiviral sgRNA libraries were constructed to target the CD45 gene, spanning the 3 kb region surrounding the TSS and constitutive CDS exons, using all possible 20-mer sequences upstream of an NG PAM sequence, as described before.[4] In addition to sgRNAs, each vector also contained a Cas9 variant (nuclease for CRISPRko and catalytically dead Cas9 fusion with VPR transcriptional activators for CRISPRa) and a short barcode downstream of the sgRNA to identify the Cas9 variant in the same Illumina read as the sgRNA. sgRNA library cloning, lentivirus production and cell transduction were done separately for each Cas9 variant. The transduced and selected cells were only pooled together prior to FACS sorting based on the CD45 protein expression. CRISPRko was performed in K562 cells and CRISPRa was performed in A375 cells; both cell lines were obtained from ATCC. The presort samples and 10% top/bottom bins were analyzed by high-throughput Illumina sequencing. To calculate fold-depletion for CRISPRko, the relative frequency (normalized to sequencing depth and median frequency of non-targeting sgRNAs for each Cas9 variant) of each sgRNA from the bottom 10% bin (lowest expression level) was divided by its corresponding frequency in the top 10% bin. To calculate fold-enrichment for CRISPRa, the relative frequency of each sgRNA from the top 10% bin (highest expression) was divided by its corresponding frequency in the bottom 10% bin. For CRISPRko, only sgRNAs targeting within the CDS were included in the analysis; for CRISPRa, only sgRNAs targeting within the core promoter region (as determined previously,[4] chr1:198638250-198639226) were included. The data were then filtered for designated PAMs (NGG or NGH) to visualize the data as shown in Figure S2.

PAM compatibility analysis

For each dCas9 variant, the ability to target each of the 64 possible 3-nucleotide PAMs was predicted based on previously reported CRISPR nuclease (CRISPRko) data in mammalian systems.[6,14] Genome editing efficiencies, measured as indel frequencies, have been reported for Sp-Cas9, xCas9(3.7), and Cas9-NG[14] and for Sp-Cas9, SpG, and SpRY.[6] For each Cas9 dataset, sgRNAs with the same 3-nt PAM were grouped together. We then calculated an average indel frequency for all sgRNAs with the same PAM. We assessed PAM compatibility by comparison to the benchmark indel frequency of Sp-Cas9 at NGG PAMs (Table S4). “High performance” PAMs were those with >50% indel frequency relative to the benchmark. “Moderate performance” PAMs were those with 20-50% efficiency relative to the benchmark. PAMs that were not tested in the previously-reported nuclease assays [6] were assumed to be incompatible.

Endogenous promoter and scRNA selection strategy

Gene candidates were selected from metabolic pathways related to aromatic amino acid biosynthesis, including the relevant genes in central metabolic pathways. We selected only

promoters that are regulated by sigma70 and are available in the previously characterized *E. coli* endogenous promoter library (obtained from Horizon Discovery).[13] To identify potentially activatable promoters with moderately-weak basal expression levels, we compared basal expression to yajGp, which was the strongest endogenous promoter that could be activated in previous work.[10] We eliminated any promoters with >10-fold higher expression levels than yajGp, yielding 9 promoters: secBp, aroFp, aroLp, pheLp, aroHp1, aroHp2, talAp, aroKp1, and serCp. Some of these promoters regulate multi-gene operons. Complete sequences of the endogenous promoters used in this study are available in the DNA sequences section below.

Out of selected genes, aroH contains two putative promoters — aroHp1/aroHp2. In Figure S6A, the basal expression levels for aroHp1 and aroHp2 were assumed to be the same value. Expression levels produced by each individual promoter cannot be determined from the fluorescent reporter used in this study because both promoters are upstream of the same fluorescent reporter gene.

In the secBp promoter constructed for the *E. coli* promoter library,[13] only the sequence to -7 bases from the TSS was included. To include enough sequence for scRNA target sites, we constructed a promoter extending to -137 bases from the TSS.

For each promoter, four scRNAs were identified according to the previously described target site preference. X06 and X08 represent the scRNAs targeting the non-template strand at -81 and -91 positions (Table S3). X07 and X09 represent the scRNAs targeting the template strand at -70 and -80 positions. One target site from the template strand and another from the non-template strand were selected for further analysis based on which PAM was predicted to be accessible to the highest number of dCas9 variants. Accessibility was assessed based on the moderate performance threshold cutoff (Supplemental Table S4).

DNA sequences

Reporter

>J3(**PAM**)-BBa_J23117-RBS-mRFP

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PAM location is bolded/underlined above

>BBa_J23110-RBS-sfGFP

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119, RR1, and RR2 targets are underlined above

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Cas9 variants (bolded/underlined mutations)

>*Sp*.pCas9-dCas9-dblTerm

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>dxCas9(3.7)

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>dCas9-NG

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>dxCas9-NG

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>dSpG

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>dSpRY

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scRNA/sgRNA expression cassettes

>BBa_J23119(SpeI)-scRNA.1xMS2.b2-TrrnB

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E. coli endogenous promoters (annotated by RegulonDB) as in pCK590 (+ strand) and pCK591 (– strand). Underlined sequences are 35bp minimal promoters. **Bolded** nucleotides are **TSS** according to RegulonDB (cite Santos-Zavaleta et al., 2019) or **start codon** of the GFPmut2.

>yajGp
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>uxuRp
CTCGAGACTTTGTTGGCGCAGTGACGGCGGCATATCAGCAGCTGTGCGAACGCGGTGCGCGCGAGTGTGTGGCTGCG
CTGTAACTAACTGATTACCCTACAGACTTACTGGTCAATCAAACTGATATTTGGTTGACCAGTTTTCGTTTTTTTGC
CCACCTGTACGTGCCAACTTCCAGT**G**CTAATGGTATAGTTTGAGATTAACGGGGGCCGTAAAATTGCCCGTTGTAGG
CCGGATAAGGCGTTCACGCCGCATCCGGCAAAAATTTGATTAACCGCACCTAACGGACACAACACCATGAAATCTGC
CACCTCTGCGCAAAGACCTTACCAGGAAGTCGGGGCGATGATCCGCGATCTGATCATAAAGACGCCGTACAATCCTG
GGGATCCTCTAGATTTAAGAAGGAGATATACAT**ATG**

>araEp
GGATCCCTGATGGGGCCGATAGTGTTCCTTGCCTCCAGCGCTTCAGATTATGTGAATGGTTATACCATTGCCGTGGA
TGGCGGTTGGCTGGCGCGTTAATTCATTCTTCTTACTTTTATGACCCTGCCGCATGGCAGGGTTTTTTATACCTGTA
GATCATCATAATCCATATCATGGTTATGAAATAATCCATATTAATTATCAATTAATGAACTTTATGAATTTTATCTG
CTGTAAAATTAGGTGGTTAATAATAATCTCAATAATTCAACTTAATTTGAAAATTGGAATATCCATCACATAACGAC
ATGTCGCAGCAATTTAATCCATATTTATGCTGTTTCCGACCTGACACCTGCGTGAGTTGTTCACGTATTTTTTCACT
AT**G**TCTTACTCTCTGCTGGCAGGAAAAAATGGTTACTATCAATACGGAATCTGCTTTAACGCCACGTTCTTTGCGGG
ATACGCGGCGTATGAATATGTTTGTTTCGGTAGCTGCTGCGCTCGAGAGATCCTCTAGATTTAAGAAGGAGATATAC
AT**ATG**

>ppiDp2
CTCGAGCCGCAGACCGGTAAAGAGATCACCATCGCTGCTGCTAAAGTACCGAGCTTCCGTGCAGGTAAAGCACTGAA
AGACGCGGTAAACTAAGCGTTGTCCCCAGTGGGGATGTGACGAAGTTCAAGGGCGCATCTACTGATGTGCCTTTTTT
ATTTGTATTCGGTGACTTTCTGCGTCTTGTGGGC**T**GACAATTGCCCCCGTTTCTTGTCACAATAGGCCTTTGCGCGC
ATCGATACGTTGCGTGAGGTACACAGTCATCTACAGCGGAGTGTTGTTACACCATGATGGACAGCTTACGCACGGCT
GGATCCTCTAGATTTAAGAAGGAGATATACAT**ATG**

>aroKp1 (aroKp2 is not included)
GGATCCTCGGGCAATTATTTCGTCATGACGGAAAAGAAGATGAACGACGCGAGTTAGTGGTGTTTATCACGCCACGA
CTGGTTTCCAGTGAGTAAACAGCCGTAAAAGCGGTAATGTTTTTACGCTGAACGTGTTTCATCTATTTGACGCGCGC
AGGTATTTAGCATACAAGGAGTACC**G**ATTTGAGAGTTGGTGCTCTTCGCTGCCTGCGTTCCATGATGATGATTTATC
ATTCAGGCGGCATTTTGCTGTCTTTTTTACGCTAATCTTACCCGGTGATTTATCGCCAGAGCGGTGGTAGCAAGGCA
GCGCGCTTGCAGCGACCAGATATGCAGAGGGATGGGTGATTTATTCAGTTGCCAAACCCGCTCGAGAGATCCTCTAG
ATTTAAGAAGGAGATATACAT**ATG**

>aroLp
CTCGAGGGCGGACCAGATAGCCTTTCACAACGTGACCGCCAGGCCTTTGCCGCGGAGCTGGAGAAGTGGTGGCTGGA
AGTGCAACGTAGTCGTGGCTAAATGTAATTTATTATTTACACTTCATTCTTGAATATTTATTGGTATAGTAAGGGGT
GT**A**TTGAGATTTTCACTTTAAGTGGAATTTTTTCTTTACAATCGAAATTGTACTAGTTTGATGGTATGATCGCTATT

CTCATGACACCGGCTTTCGCCGCATTGCGACCTATTGGGGAAAACCCACGATGACACAACCTCTTTTTCTGATCGGG
CCTCGGGGCTGTGGTAAAACAACGGTCGGAATGGCCCTTGCCGATTCGCTTAACCGGATCCTCTAGATTTAAGAAGG
AGATATACAT**ATG**

>aroFp
GGATCCCAACAAGGGGGCGATAAACTTTTTCATCATTTCTTTCTCCTTTTTCAAAGCATAGCGGATTGTTTTCAAAG
GGAGTGTAAATTTATCTATACAGAGGTAAGGGTTGAAAGCGCGACTAAATTGCCTGTGTAAATAAAAATGTACGAAA
TATGGA<u>TTGAAAACTTTACTTTATGTGTTATCGTTACGTC</u>**A**TCCTCGCTGAGGATCAACTATCGCAAACGAGCATAA
ACAGGATCGCCATCATGCAAAAAGACGCGCTGAATAACGTACATATTACCGACGAACAGGTTTTAATGACTCCGGAA
CAACTGAAGGCCGCTTTTCCATTCTCGAGAGATCCTCTAGATTTAAGAAGGAGATATACAT**ATG**

>aroHp (aroHp1 and aroHp2 are available in this reporter)
CTCGAGTCAGATCCCGTGGATTAACAGTACCAATTATTCGGTAGAAGAGATTGCCACCAAGATCCTCGATATCATGG
GCCTTAGTCGCCGAATGTACTAGAGAACTA<u>GTGCATTAGCTTATTTTTTTGTTATCATGCTA</u>**A**CCACCCGGCGAGGT
GTGACACACCTCGCAC<u>TTGAAATCAGCAGCGATTGGTTTATCGTGATGCGC</u>**A**TCACTTCCCGGCAGTCCTGCCGTAG
AAGCAACAAATTTCTGAGACTTGTAATGAACAGAACTGACGAACTCCGTACTGCGCGTATTGAGAGCCTGGTAACGC
CCGCCGAACTCGCGCTACGGTATCCCGTAACGCCTGGGGATCCTCTAGATTTAAGAAGGAGATATACAT**ATG**

>pheLp
CTCGAGACAAAGGCGAAGCACGTCGTGCCGCAACATCGGTGAAAGACGCCAACTTCGTCGAAGAAGTTGAAGAAGAG
TAGTCCTTTATATTGAGTGTATCGCCAACGCGCCTTCGGGCGCGTTTTTTG<u>TTGACAGCGTGAAAACAGTACGGGTA</u>
<u>CTGTACTAA</u>**A**GTCACTTAAGGAAACAAACATGAAACACATACCGTTTTTCTTCGCATTCTTTTTTACCTTCCCCTGA
ATGGGAGGATCCTCTAGATTTAAGAAGGAGATATACAT**ATG**

>secBp
GGATCCGATGGCAACGCCGCCAAGCGTGAAGAGATGATCAAACGCAGCGGTCGCACCACGGTTCCCCAGATTTTTAT
TGACGCACAGCACATTGGCGGCTGTGATGAC<u>TTGTATGCATTGGATGCACGTGGTGGACTGGATCC</u>**C**CTGCTGAAAT
AACGTGTGAACGTTGGCATTACATTGCGCAGTATTTAAGGACAACACTTAAGGGTTTTCTACACATGTCAGAACAAA
ACAACACTGAAATGACTTTCCAGATCCAACGTATTTATACCAAGGATATCTCTTTCGAAGCGCCGCTCGAGAGATCC
TCTAGATTTAAGAAGGAGATATACAT**ATG**

>serCp
CTCGAGTCATATGAAAGCGGGGGAAAAACAATTATGTCCGCGCTGTGCAAATCCAGAATGGACGAAGGCAAGTCGGG
CAAAACGGGTGACCTGACAGTAAAAACATCGGCTTTTTGCTAATAATCCGAGAGATTCTTTTGTGTGATGCAAGCCA
CATTTTTGCCCTCAACGGTTTTACTCATTGCGATGTGTGTCACTG<u>AATGATAAAACCGATAGCCACAGGAATAATGT</u>
<u>ATT</u>**A**CCTGTGGTCGCAATCGATTGACCGCGGGTTAATAGCAACGCAACGTGGTGAGGGGAAATGGCTCAAATCTTCA
ATTTTAGTTCTGGTCCGGCAATGCTACCGGCAGAGGTGCTTAAACAGGCTCAACAGGAACTGCGCGACTGGAACGGT
CTTGGGGATCCTCTAGATTTAAGAAGGAGATATACAT**ATG**

>talAp
CTCGAGGGGATCCAATATAGCCTCTGGCAACGCAGCCTCGAGGCTGTGTTGCCAGAGGCTTGGTTGGAGAAACCTGG
ATTTTCCCTGGAACTGGAAATTCATGGAAATCAAGTGCACTTTGTTTTAACTGGTCATCCATTTGGTTGTTCCTTTC
ACGTAACGTTCACAAATAAAGTGTGTGGGCAACAGCCCCTGCCCACAACGTGGCGCACATTATTACCCTGCCGGAGT
CTACAGACTTTGAGCAAGTCCAAACTCTCACCATTAATATAATGTTTTGGTAATAATCCTATAACACTGATGTTACC
TGCTTAATCCAGCAATACCATGCCTG<u>TCTGCTATGCTTTTTTGATGCGTTTAGCGAAATTT</u>**C**TCAGAAGTGTGAATT
AACGCACTCATCTAACACTTTACTTTTCAAGGAGTATTTCCTATGAACGAGTTAGACGGCATCAAACAGTTCACCAC
TGTCGTGGCAGACAGCGGCGATATTGGATCCTCTAGATTTAAGAAGGAGATATACAT**ATG**

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