SUPPORTING INFORMATION

Multidimensional control of Cas9 by evolved RNA polymerase-based biosensors

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Table S1 | List of plasmids used in this work.

Vector name	Antibiotic resistance	Origin	Purpose	Map
p2-22	carb	sc101	P_{T7} luciferase reporter plasmid	a
p3-7	spec	P15A	T7 $RNAP_N$ (wt)-linker-ZA expression plasmid	b
p2-55	chlr	CloDF13	T7 $RNAP_C$ expression plasmid	c
p2-39	chlr	CloDF13	ZB-linker-T7 $RNAP_C$ expression plasmid	d
p5-74	spec	P15A	T7 $RNAP_N$ (N-29-1)-linker-ZA (L13I, L20I) expression plasmid	b
p8-15	spec	P15A	T7 $RNAP_N$ (N-29-1)-linker-ABI expression plasmid	e
p8-17	chlr	CloDF13	PYL-linker-T7 $RNAP_C$ expression plasmid	f
pJin 274	kan	pBR322	P_{T7}-GFP mRNA expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (d5-14)-linker-ABI	g
pJin 278	kan	pBR322	P_{T7}-GFP mRNA expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (d5-19)-linker-ABI	g
pJin 286	kan	pBR322	P_{T7}-GFP mRNA expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (N-29-1)-linker-ABI	g
pJin 287	kan	pBR322	P_{T7}-GFP mRNA expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (d5-6)-linker-ABI	g
pJin 288	kan	pBR322	P_{T7}-GFP mRNA expression, PYL-linker-T7 RNAPC, T7 $RNAP_N$ (d5-13)-linker-ABI	g
pJin 239	kan	pBR322	pT7-gRNA GFP expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (N-29-1)-linker-ABI	h
pJin 264	kan	pBR322	P_{T7}-gRNA GFP expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (d5-19)-linker-ABI	h
pJin 272	kan	pBR322	P_{T7}-gRNA GFP expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (d5-13)-linker-ABI	h
pJin 263	kan	pBR322	P_{T7}-gRNA GFP expression, PYL-linker-T7 $RNAP_C$, T7 $RNAP_N$ (d5-14)-linker-ABI	h
pJin 290	kan	pBR322	P_{CGG}-gRNA GFP-off expression, FKBP-linker-T7 $RNAP_C$, T7 $RNAP_N$ (d5-19)-linker-FRB	i
pJin 310	kan	pBR322	P_{T7}-gRNA GFP expression, Bcl-2-linker-T7 $RNAP_C$	j
p12-34	kan	pBR322	T7 $RNAP_N$ (d5-19)-linker-tBID mammalian expression plasmid	k
p12-33	kan	pBR322	T7 $RNAP_N$ (d5-19)-linker-dBID mammalian expression plasmid	k
pJin 178	carb	sc101	Positive AP (RBS1 – SD8, RBS2 – sd6)	l
pJin 179	carb	sc101	Positive AP (RBS1 – SD8, RBS2 – sd5)	l
pJin 179	kan	pBR322	Negative AP (RBS3 – SD4, RBS4 – sd6)	m
p9-18	carb	CloE1	*Staphylococcus aureus* Cas9 expression vector	n
p5-54	carb	CloE1	P_{U6} driven gRNA GFP expression vector	o
P3-62	kan	pUC	P_{CMV}-driven RFP expression vector	p

Table S2 | ABA-induced gRNA production from pJin 264. HEK293T cells transfected with pJin 278 (**Figure 1D**, variant d5-19). During transfection, the cells were treated with 0, 1, 10, or 100 μM ABA. After 40 h of growth, the cells were lysed, total RNA isolated, and the levels of gRNA production analyzed by RT-qPCR. Ct values shown. GAPDH RNA levels analyzed as a control for RNA isolation.

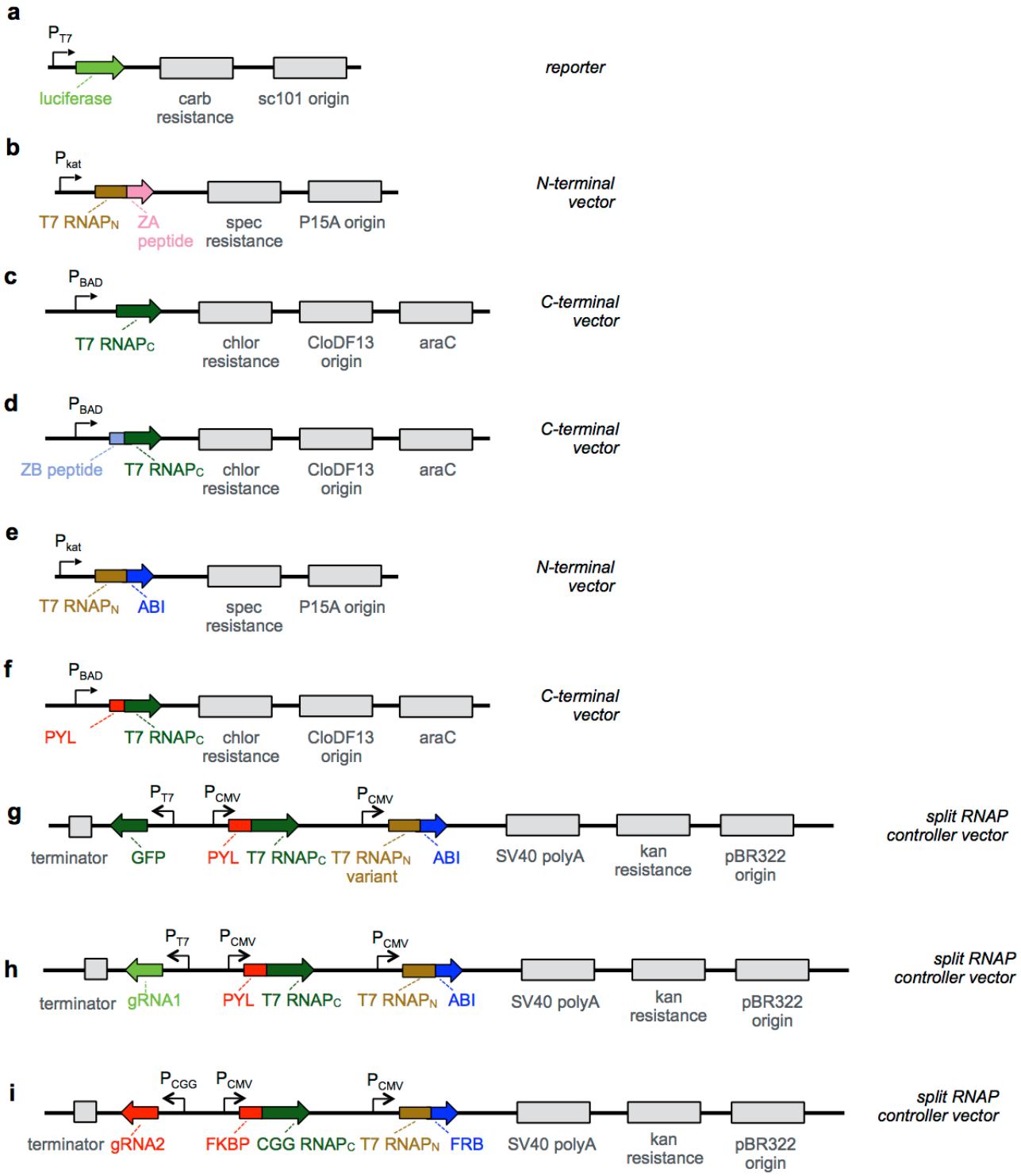
[ABA]	0	1 µM	10 µM	100 µM
GAPDH	20.29	20.22	20.31	20.17
gRNA-1	21.12	16.6	16.72	16.61

Table S3 | Concentration-dependence of ABT199. HEK293T cells transfected with pJin 310 and p12-34 (**Figure 3A**). During transfection, the cells were treated with 0, 50, 250, 500 or 1,000 nM ABT199. After 40 h of growth, the cells were lysed, total RNA isolated, and the levels of gRNA production was analyzed by RT-qPCR. Ct values shown. GAPDH RNA levels analyzed as a control for RNA isolation.

[ABT-199]	0	50 nM	250 nM	500 nM	1,000 nM
GAPDH	19.95	19.67	20.06	19.88	19.94
gRNA-1	20.12	22.89	22.7	21.99	23.11

Table S4 | saCas9 gRNA and RT-qPCR primer sequences.

Name	DNA or oligo sequences
gRNA-1 (GFP)	GCCCTCGAACTTCACCTCGGCGTTTTAGTACTCTGTAATGAAAATTA CAGAATCTACTAAAACAAGGCAAAATGCCGTGTTTATCTCGTCAAC TTGTTGGCGAGATTTTTTT
gRNA-2 (off switch)	AGTTCGAGGGCTCTCCCTATAGTTTTAGTACTCTGTAATGAAAATTA CAGAATCTACTAAAACAAGGCAAAATGCCGTGTTTATCTCGTCAAC TTGTTGGCGAGATTTTTTT
gRNA-1-for	CCTCGAACTTCACCTCGGCGT
gRNA-2-for	AGTTCGAGGGCTCTCCCTATAGT
gRNA-rev	CTCGCCAACAAGTTGACGAGATAAACAC
GAPDH-for	TGCACCACCAACTGCTTAGC
GAPDH-rev	GGCATGGACTGTGGTCATGAG


a
P T7
luciferase
carb resistance
sc101 origin
reporter
b
P kat
T7 RNAP N
ZA peptide
spec resistance
P15A origin
N-terminal vector
c
P BAD
T7 RNAP C
chlor resistance
CloDF13 origin
araC
C-terminal vector
d
P BAD
ZB peptide
T7 RNAP C
chlor resistance
CloDF13 origin
araC
C-terminal vector
e
P kat
T7 RNAP N
ABI
spec resistance
P15A origin
N-terminal vector
f
P BAD
PYL
T7 RNAP C
chlor resistance
CloDF13 origin
araC
C-terminal vector
g
P T7
P CMV
P CMV
terminator
GFP
PYL
T7 RNAP C
T7 RNAP N variant
ABI
SV40 polyA
kan resistance
pBR322 origin
split RNAP controller vector
h
P T7
P CMV
P CMV
terminator
gRNA1
PYL
T7 RNAP C
T7 RNAP N
ABI
SV40 polyA
kan resistance
pBR322 origin
split RNAP controller vector
i
P CGG
P CMV
P CMV
terminator
gRNA2
FKBP
CGG RNAP C
T7 RNAP N
FRB
SV40 polyA
kan resistance
pBR322 origin
split RNAP controller vector

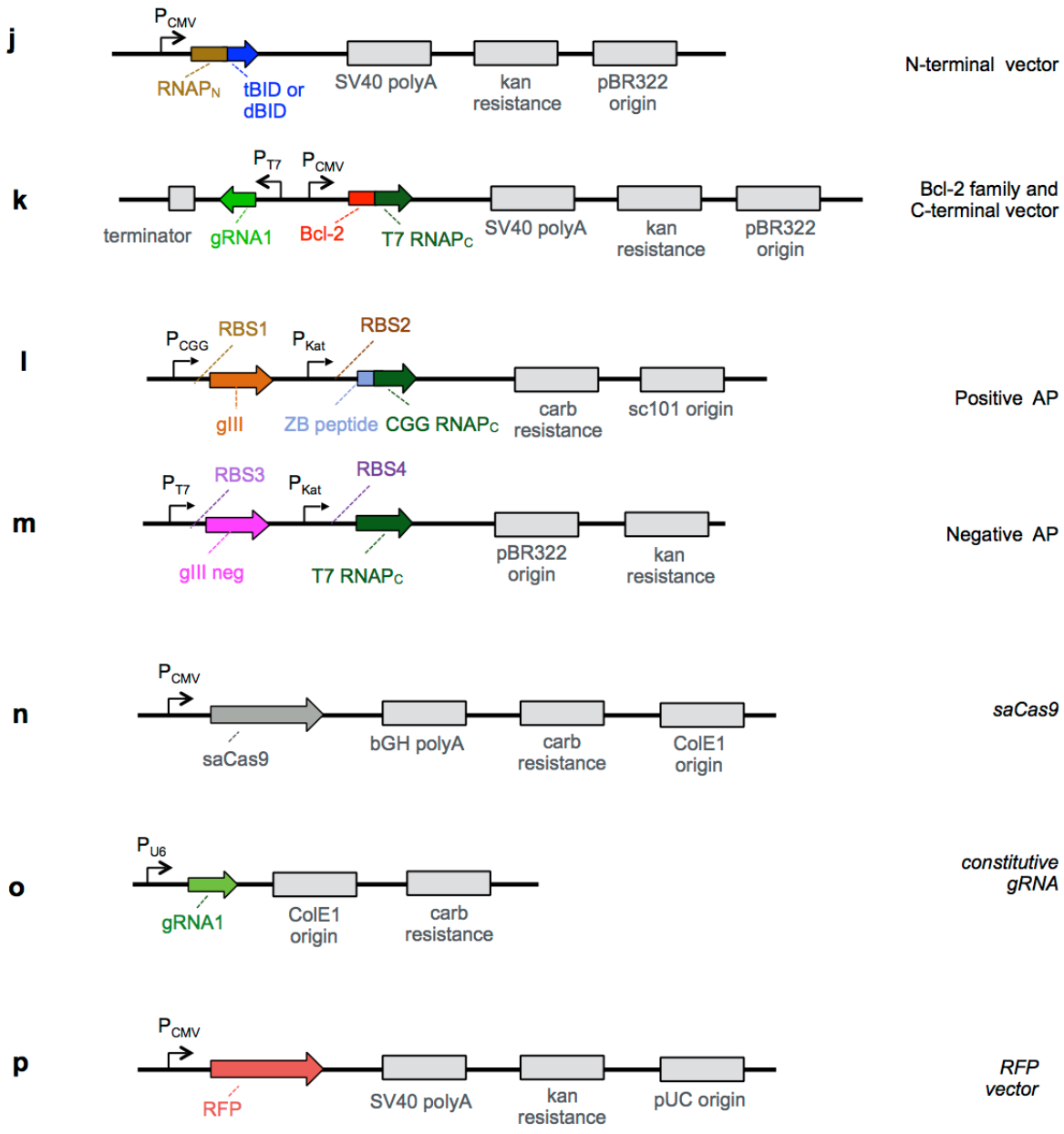


Figure S1 | Vector maps for all constructs used in this work. Vector maps corresponding to the vectors listed in **Table S1** shown.

evolutionary date	positive AP					negative AP				
	Vector name	Map	origin	RBS1	RBS2	Vector name	Map	origin	RBS3	RBS4
day1	pJin178	l	sc101	SD8	sd6	pJin153	m	pBR322	SD4	sd6
day2	pJin178	l	sc101	SD8	sd6	pJin153	m	pBR322	SD4	sd6
day3	pJin178	l	sc101	SD8	sd6	pJin153	m	pBR322	SD4	sd6
day4	pJin178	l	sc101	SD8	sd6	pJin153	m	pBR322	SD4	sd6
	pJin179	l	sc101	SD8	sd5	pJin153	m	pBR322	SD4	sd6
day5	pJin179	l	sc101	SD8	sd5	pJin153	m	pBR322	SD4	sd6

Figure S2 | Evolutionary protocol for PACE experiment. Vector names and details are provided for each day of PACE. Vector maps for the posAP and negAP vectors are shown in **Figure S1,** l and m. Two sets of posAP/negAP vectors listed on the same day indicates that a mixed selection pressure was utilized, in which two types of host cells, each containing one set of the posAP/negAP plasmids were added to a lagoon simultaneously. The relative RBS strengths were obtained from previous studies.[1]

day3																									
d3-1				L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d3-2				L32S		E35G				E63K					K98R		Q107K		T122S					A144T	
d3-4				L32S		E35G		R57Y		E63K					K98R		Q107K		T122S					A144T	
d3-5				L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d3-7				L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d3-8				L32S		E35G				E63K					K98R		Q107K		T122S					A144T	
d3-9				L32S		E35G				E63K					K98R		Q107K		T122S					A144T	
d3-10				L32S		E35G		R57C		E63K					K98R		Q107K		T122S		T127A			A144T	
d3-11				L32S		E35G				E63K					K98R		Q107K		T122S				A136S	A144T	
d3-12				L32S		E35G				E63K					K98R		Q107K		T122S				A136S	A144T	
d3-14				L32S		E35G				E63K					K98R		Q107K		T122S					A144T	
d3-15				L32S		E35G		R57C		E63K					K98R		Q107K		T122S		T127A			A144T	
d3-16				L32S		E35G				E63K					K98R		Q107K		T122S					A144T	
day5																									
d5-1				L32S		E35G		R57C		E63K	N67T				K98R		Q107K		T122S					A144T	
d5-2				L32S		E35G		R57C		E63K	N67T	T76S		R96S	K98R		Q107K		T122S					A144T	
d5-3				L32S		E35G		R57C	A61	E63K	N67T		L77I		K98R		Q107K		T122S					A144T	
d5-4				L32S		E35G		R57C		E63K					K98R		Q107K		T122S	L123M				A144T	
d5-5			D26Y	L32S		E35G	M46I	R57C		E63K					K98R		Q107K		T122S					A144T	
d5-6				L32S		E35G		R57C		E63K					K98R		Q107K	K120N	T122S			V134I		A144T	
d5-8				L32S		E35G		R57C		E63K	N67				K98R		Q107K		T122S					A144T	
d5-9				L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d5-10				L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d5-12				L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d5-13				L32S	A33T	E35G		R57C		E63K					K98R		Q107K		T122S					A144T	A159S
d5-14		L24R		L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d5-15			D26Y	L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d5-16				L32S		E35G		R57C		E63K					K98R		Q107K		T122S					A144T	
d5-18		L24R		L32S		E35G		R57C		E63K					K98R	Q104K	Q107K		T122S					A144T	
d5-19	F21L			L32S		E35G		R57C		E63K					K98R	Q104K	Q107K		T122S					A144T	

Figure S3 | Mutational analysis of evolving split RNAP. Single phage sequenced during the course of the PACE experiment and coding mutations are shown for a set of variants assayed at each time point. The final variant selected for further assay (d5-19) is highlighted yellow.

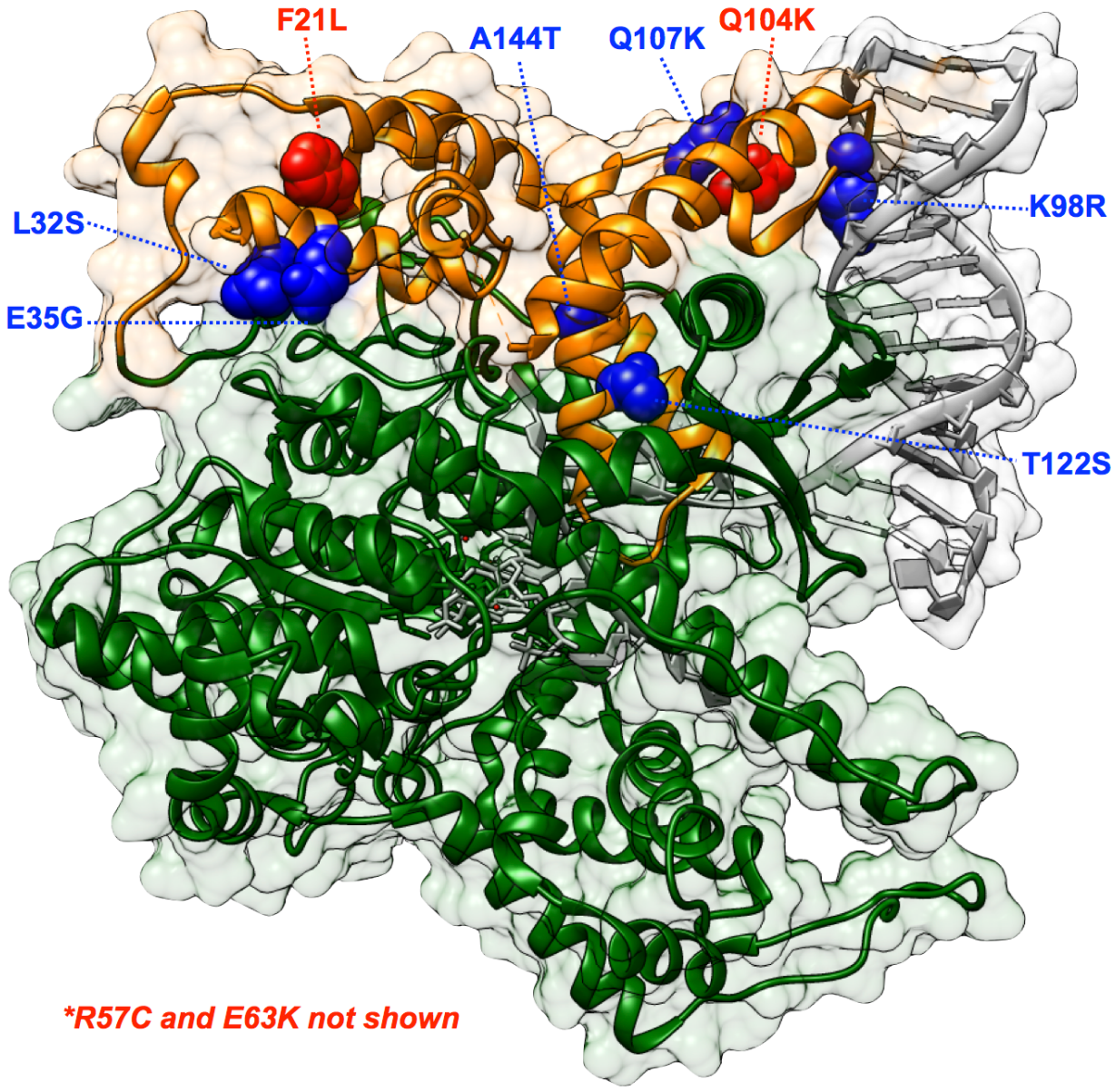


Figure S4 | Mutations from d5-19 variant mapped onto the T7 RNAP initiation complex crystal structure. Mutations and split site mapped onto T7 RNAP crystal structure. $RNAP_N$ shown in orange and $RNAP_C$ shown in green. Mutations from previous evolution (N-29-1) shown in blue and new mutations from d5-19 shown in red. Mutations in regions of the protein that do not show up in the structure omitted. (PDB 1QLN[2]).

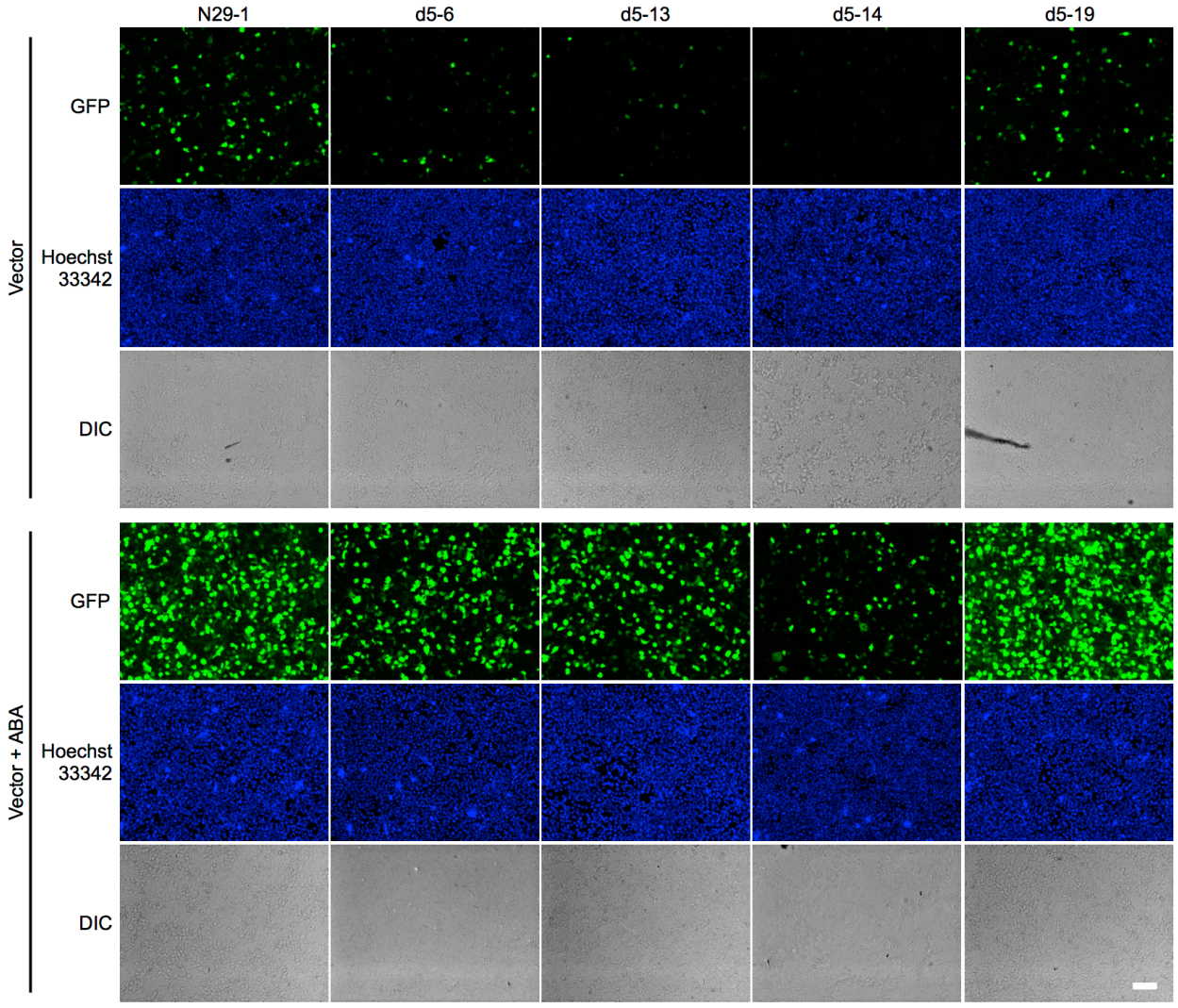


Figure S5 | Complete imaging series of Figure 2E. HEK293T cells transfected with the plasmids shown in Figure 2D. 7 h after transfection, the cells were treated with either nothing or 10 μM ABA. After an additional 23 h of growth, the cells were loaded with 1 μM Hoechst 33342 and analyzed by fluorescence microscopy. 100 μm scale bar shown.

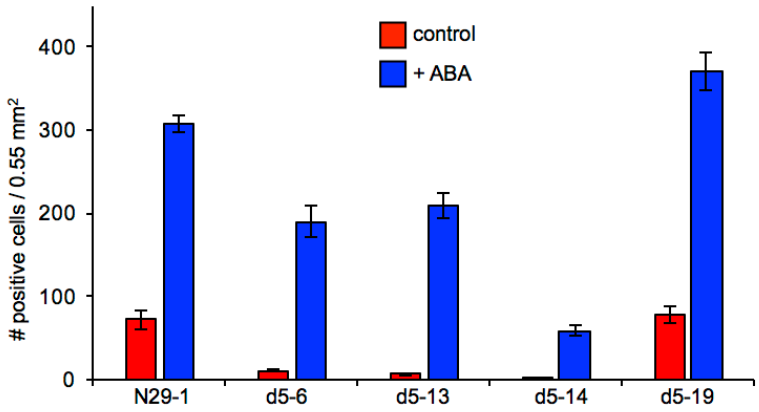


Figure S6 | Quantification of experiment shown in Figure 2E and S5. Error bars are ± SEM, n = 5.

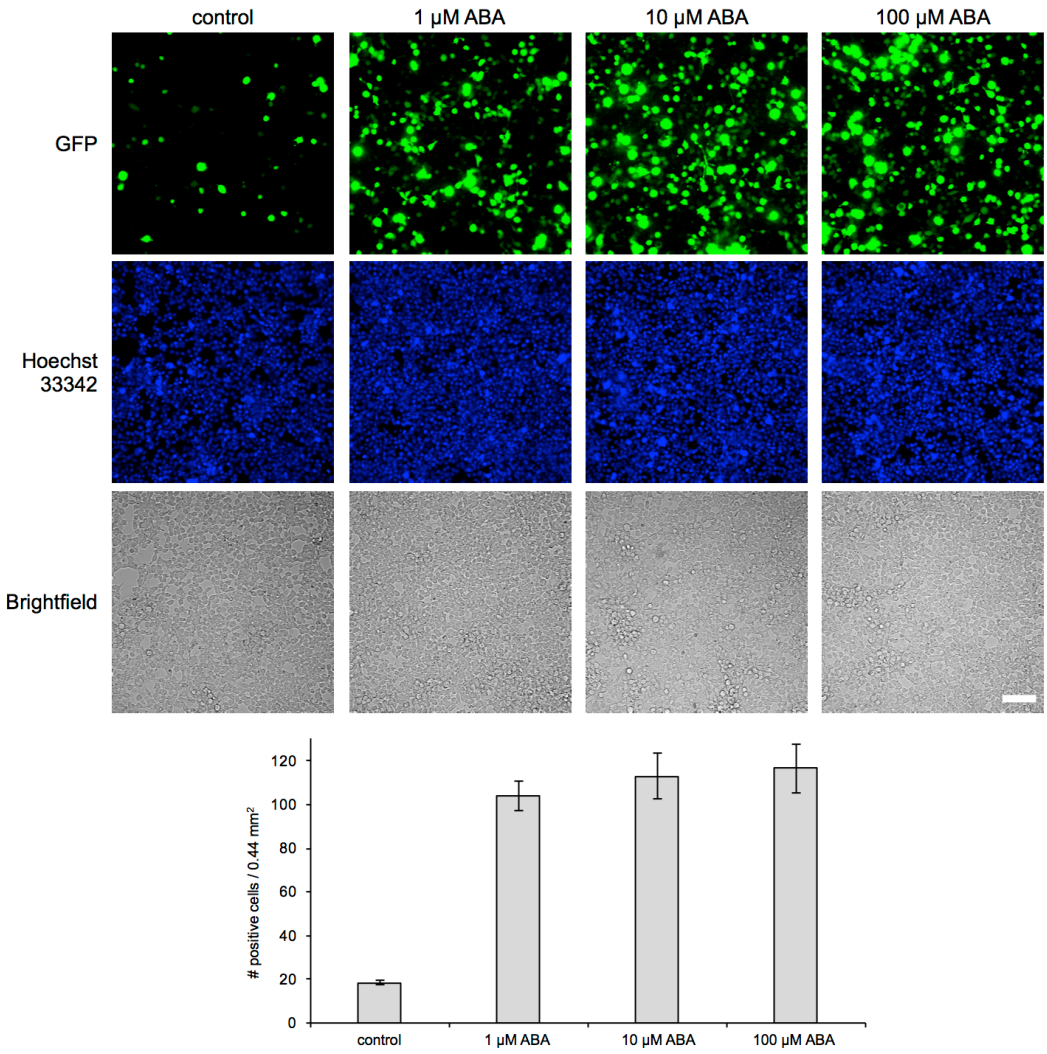


Figure S7 | Dose response to ABA. HEK293T cells transfected with pJin 278. During transfection, the cells were treated with 0, 1, 10, or 100 μM ABA. After 46 h of growth, the cells were loaded with 1 μM Hoechst 33342 and analyzed by fluorescence microscopy. 100 μm scale bar shown. For quantification of imaging, error bars are ± SEM, n = 4.

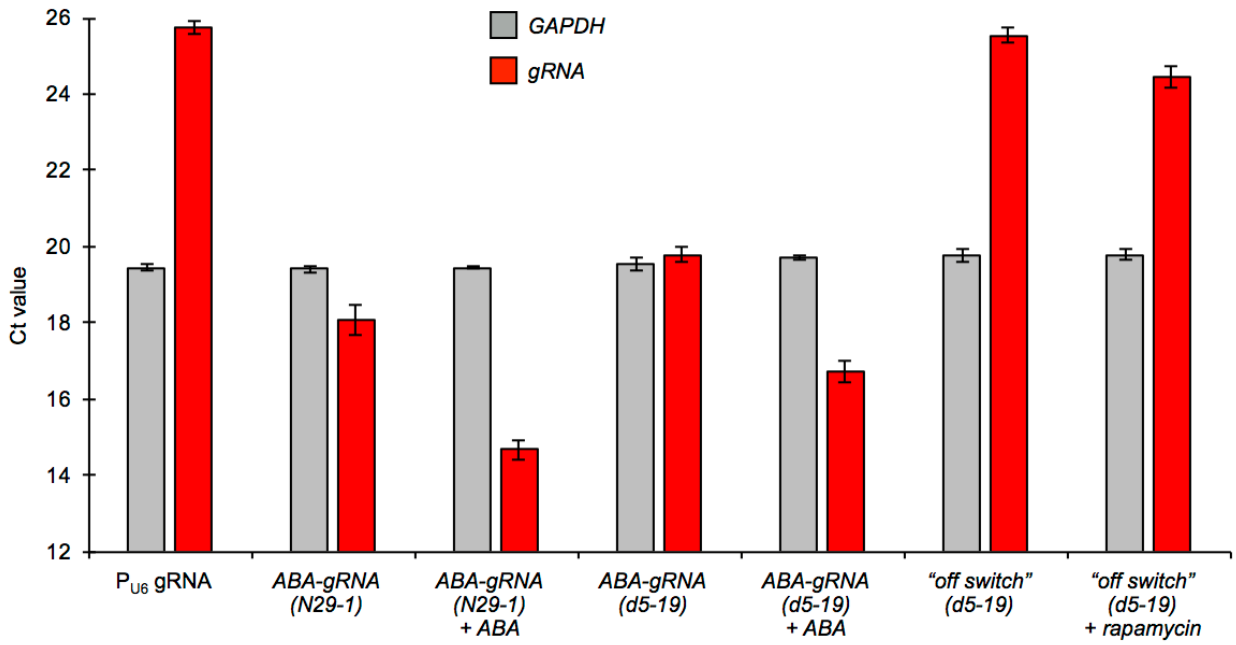


Figure S8 | Analysis of amount of gRNA produced by split RNAP vectors compared to constitutive gRNA vectors. HEK293T cells transfected with either a P_{U6}-driven gRNA vector (p5-54), the N-29-1 ABA-inducible vector shown in **Figure 1D** (pJin 239), the "on switch" d5-19 ABA-inducible vector shown in **Figure 4A** (pJin 264), or the rapamycin-inducible "off switch" vector shown in **Figure 4A** (pJin 290). 15 h after transfection, the cells were treated with DMSO control, 10 μM ABA, or 10 nM rapamycin. 26 h after treatment, total RNA was collected from the cells and the amount of gRNA was analyzed by RT-qPCR. GAPDH was also analyzed as a control for RNA isolation. As seen in the plot, both the N-29-1 and d5-19 produce more gRNA than the constitutive P_{U6}-driven vector, even though they display lower background and lower levels of target knockout. Additionally, the rapamycin-inducible vector shows a very low background of gRNA production, but also modest rapamycin-induced gRNA production, providing an explanation as to why the "off switch" vector did not completely block the Cas9 response. This is possibly due to the diminished activity of the CGG-$RNAP_C$ variant. Error bars are ± SEM, n = 3.

References

1. Ringquist, S., Shinedling, S., Barrick, D., Green, L., Binkley, J., Stormo, G. D., and Gold, L. (1992) Translation initiation in Escherichia coli: sequences within the ribosome-binding site, *Mol. Microbiol. 6*, 1219-1229.
2. Cheetham, G. M., and Steitz, T. A. (1999) Structure of a transcribing T7 RNA polymerase initiation complex, *Science 286*, 2305-2309.