# **Supporting Information**

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**Fig. S1.** Optimization of GFP-tagged Sp Cas9 expression. (*A*) Promoter optimization for CRISPR labeling using Sp Cas9-GFP. U2OS cells were transfected with 150 ng of dCas9-GFP expression plasmids under the control of a series of promoters:  $EF1\alpha$  (*Left*), SFFV (*Middle Left*), EFS (*Middle Right*), and CMV-TO (*Right*). Less than 5% of cells were GFP-positive when using the EF1 $\alpha$ , SSFV, and EFS promoters; however, the percentage of GFP-positive cells increased to 50% with use of the CMV-TO promoter. (Scale bar: 20 µm.) (*B*) Increased labeling by fusing 3XGFP to Sp Cas9. RPE-1 cells were cotransfected with 250 ng of sgRNA plasmid targeting to the MUC4 gene locus and 50 ng of plasmids encoding Sp dCas9-1XGFP, Sp dCas9-2XGFP, or Sp dCas9-3XGFP. The fluorescent images in the bottom row were obtained within the same range of linear sensitivity and scaled to the same threshold. The brightness of foci (arrows) gradually increased when Sp dCas9 was tagged with 1XGFP, 2XGFP, or 3XGFP. (Scale bar: 5 µm.)



**Fig. 52.** Optimizing Sp sgRNA length and PAM sequences. (*A*) U2OS cells were cotransfected with 150 ng of a plasmid encoding Sp dCas9-3XGFP and 750 ng of plasmids encoding sgRNAs of various lengths targeting the telomeric repeat. The sgRNAs of 10, 13, 17, and 21 nucleotides all effectively labeled the telomeres, whereas the 6-nucleotide sgRNA displayed only a few foci and substantially dispersed fluorescence, suggestive of decreased specificity for the target. (Scale bar: 5 μm.) (*B*) U2OS cells were cotransfected with 150 ng of plasmids encoding Sp dCas9-3XGFP and 750 ng of sgRNAs targeting to the telomeres and containing one of four different PAM sequences: TA, AG, GG, or GT. Phase-contrast (*Upper*) and fluorescent (*Lower*) images are shown. The sgRNA with the TA PAM sequence (*Left*) did not result in labeling of telomeres, whereas telomere labeling was observed when the sgRNAs had one of the other three PAM sequences: AG (*Middle Left*), GG (*Middle Right*), or GT (*Right*). (Scale bar: 5 μm.)



**Fig. S3.** Optimization of Nm sgRNAs and PAM sequences. (*A*) Two Nm sgRNAs, NmsgRNAm3 and NmsgRNA1.1, were designed, the latter with additional base-paired regions. (*B*) U2OS cells were cotransfected with 150 ng of a plasmid encoding Nm dCas9-3XGFP and 750 ng of plasmids encoding either NmsgRNAm3 (*Left*) or NmsgRNA1.1 (*Right*), with each sgRNA targeting to telomeres. Although no specific labeling was seen with nmsgRNAm3, the added base pairing in stem loop 1 and at the 3' end of nmsgRNA1.1 resulted in distinct labeling. (Scale bar: 5  $\mu$ m.) (*C*) U2OS cells were cotransfected with 150 ng of a plasmid encoding RNA targeting to telomeres. Although no specific labeling was seen with nmsgRNAm3, the added base pairing in stem loop 1 and at the 3' end of nmsgRNA1.1 resulted in distinct labeling. (Scale bar: 5  $\mu$ m.) (*C*) U2OS cells were cotransfected with 150 ng of a plasmid encoding RNA containing one of four different PAM sequences: AGGG, GGGT, or GTTA. No labeling was observed when the sgRNAs contained the PAM sequences AGGG (*Left*) or GGGT (*Middle Left*), whereas distinct labeling was observed with the sgRNAs containing the PAM sequences GGTT (*Middle Right*) and GTTA (*Right*). (Scale bar: 5  $\mu$ m.)



**Fig. 54.** Optimization of st1 dCas9 nuclear localization and St1 sgRNAs. (*A*) RPE-1 cells were transfected with 50 ng of plasmids encoding St1 dCas9-3XGFP fused to increasing numbers of NLS sequences. Very little nucleus-localized St1 dCas9-3XGFP was observed when fused to 2XNLS (*Left*); however, the degree of nuclear localization gradually increased when the St1 dCas9-3XGFP was fused to 3XNLS (*Middle Left*), 4XNLS (*Middle Right*), or 6XNLS (*Right*). (Scale bar: 5 µm.) (*B*) Five different St1 sgRNAs were designed, each targeting the pericentromeric C9-1 sequence on chromosome 9 (Fig. 3). St1sgRNAm1 is the canonical sgRNA for this target and St1 dCas9. In St1sgRNAm7 and St1sgRNA1.1, the UUUUU tract was mutated to UUUGU and UCUUU, respectively, shown in purple. St1sgRNA2.1 and St1sgRNA3.1 contained extensions in stem loop 1, shown in purple. (*C*) RPE-1 cells were cotransfected with 50 ng of a plasmid encoding any one of the five C9-1-targeting St1sgRNAs. No labeling was observed with St1sgRNA1.1 (*Middle Left*), whereas distinct labeling of the C9-1 sequence was observed with St1sgRNA1.1 (*Middle*), St1sgRNA2.1 (*Middle*), St1sgRNA3.1 (*Middle*), St3sgRNA3.1 (*Middle*), St3sgRNA3.1



Fig. S5. Karyotype of RPE-1 cells. Chromosome analysis of RPE-1 cells (American Type Culture Collection CRL-4000) was performed. Twenty metaphase cells were analyzed, and all showed a near-diploid karyotype as shown, with a 2N chromosome number of 46. As shown in the image, one copy of the X carries an additional element at Xq28 (arrow).



**Fig. S6.** Three-dimensional view of the chromosome 9 pericentromeric locus C9-1. U2OS cells were transfected with 150 ng of a plasmid encoding Sp dCas9-3XGFP and 750 ng of a plasmid encoding a SpsgRNA targeting to C9-1. Serial optical sections were then captured by confocal microscopy. The 3D images were reconstructed with Metamorph software. A representative example is shown in *x-y* (*Upper Left*) and *x-z* (*Lower Left*) projections. (*Upper Right*) The corresponding differential interference contrast (DIC) image. (Scale bar: 5 µm.)

#### Table S1. dCas9-FPs

Promoter	dCas9 fusion protein	dCas9 fusion protein NLS	
EF1α	NLS-Sp dCas9-NLS-sfGFP	2X	
SSFV	NLS-Sp dCas9-NLS-sfGFP	2X	
EFS	NLS-Sp dCas9-NLS-sfGFP	2X	
CMV-TetO	NLS-Sp dCas9-NLS-sfGFP	2X	
CMV-TetO	NLS-Sp dCas9-NLS-2XsfGFP	2X	
CMV-TetO	NLS-Sp dCas9-NLS-3XsfGFP	2X	
CMV-TetO	NLS-Sp dCas9-NLS-3XmCherry	2X	
CMV-TetO	NLS-Nm dCas9-NLS-3XsfGFP	2X	
CMV-TetO	NLS-Nm dCas9-NLS-3XmCherry	2X	
CMV-TetO	NLS-St1 dCas9-NLS-3XsfGFP	2X	
CMV-TetO	NLS-St1 dCas9-2XNLS-3XsfGFP	3X	
CMV-TetO	NLS-St1 dCas9-3XNLS-3XsfGFP	4X	
CMV-TetO	NLS-St1 dCas9-3XNLS-3XTagBFP2	4X	
CMV-TetO	NLS-St1 dCas9-3XNLS-3XsfGFP-NLS	5X	
CMV-TetO	NLS-St1 dCas9-3XNLS-3XsfGFP-2XNLS	6X	

## Table S2. sgRNA vectors

Vector name	sgRNA expression vector cassette
pLH-Sp sgRNA2	U6 promoter-BbsI-CcdB-BbsI-Sp sgRNA2
pLH-Nm sgRNAm3	U6 promoter-BbsI-CcdB-BbsI-Nm sgRNAm3
pLH-Nm sgRNA1.1	U6 promoter-BbsI-CcdB-BbsI-Nm sgRNA1.1
pLH-St1 sgRNAm1	U6 promoter-BbsI-CcdB-BbsI-St1 sgRNAm1
pLH-St1 sgRNAm7	U6 promoter-BbsI-CcdB-BbsI-St1 sgRNAm7
pLH-St1 sgRNA1.1	U6 promoter-BbsI-CcdB-BbsI-St1 sgRNA1.1
pLH-St1 sgRNA2.1	U6 promoter-BbsI-CcdB-BbsI-St1 sgRNA2.1
pLH-St1 sgRNA3.1	U6 promoter-BbsI-CcdB-BbsI-St1 sgRNA3.1

## Table S3. Single-guide RNA sequences

PNAS PNAS

dCas9	Target	Guide RNA sequence	PAM
S. pyogenes	MUC4	GTGGCGTGACCTGTGGATGCTG	GG
S. pyogenes	Telo-TA	GGTTAGGGTTAGGGTTAGGG	TA
S. pyogenes	Telo-GT	AGGGTTAGGGTTAGGGTTAG	GT
S. pyogenes	Telo-AG	GTTAGGGTTAGGGTTAGGGT	AG
S. pyogenes	Sp-Telo	TTAGGGTTAGGGTTAGGGTT	GG
S. pyogenes	Telo-17	GGGTTAGGGTTAGGGTT	GG
S. pyogenes	Telo-13	TAGGGTTAGGGTT	GG
S. pyogenes	Telo-10	GTTAGGGTT	GG
S. pyogenes	Telo-06	AGGGTT	GG
S. pyogenes	C9-1	TGGAATGGAATGGAATGGAA	GG
S. pyogenes	C9-2	TGTCTGTGAGGAAGCTCCCC	GG
S. pyogenes	C13-1	TAAGCATGGACCATTCCTTC	GG
S. pyogenes	C13-2	GGGCCAGGACCTCTAAAA	GG
		CCGGGGAAGTGCTGAGTC	GG
		TGGTGGGTGTAGACACGG	GG
N. meningitidis	Telo-AGGG	GGTTAGGGTTAGGGTTAGGGTTAG	AGGG
N. meningitidis	Telo-GGGT	GTTAGGGTTAGGGTTAGGGTTAGG	GGGT
N. meningitidis	Nm-Telo	TTAGGGTTAGGGTTAGGGTTAGGG	GGTT
N. meningitidis	Telo-GTTA	TAGGGTTAGGGTTAGGGTTAGGGT	GTTA
N. meningitidis	C13-1	CTCCATCCTGAAGGAATGGTCCAT	GCTT
S. thermophilus	St1-Telo	GGTTAGGGTTAGGGTTAGGG	AGGG
S. thermophilus	C9-1	ATGGAATGGAATGGAATGGA	GGAA