

Figure S3. **Residence times of Cas9–guide RNA on targets.** (A) FRAP analysis was performed at target sites with C3-11 guide RNAs having single mismatches (red and underlined) at the -3 or -5 position of the seed region. The PAM sequence adjacent to the target is shown in orange. (B–H) Representative recovery rates observed with the guide RNAs are indicated in the insets of each panel; note the different time scales on the x axes. Data in all panels are representative of experiments performed at least three times.

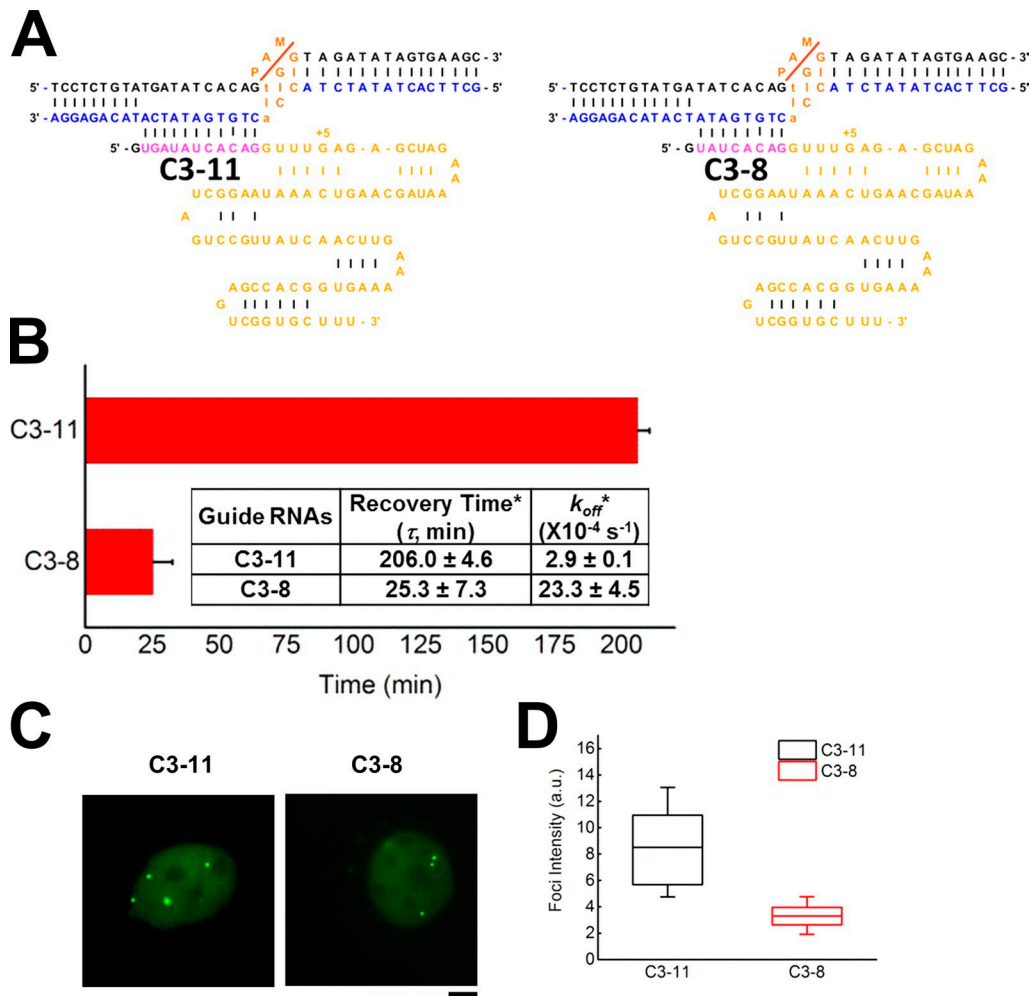


Figure S4. **Residence time for 8- versus 11-nt guide RNAs.** (A) The guide RNA was shortened to 8 nt (C3-8). Purple denotes guide sequences; yellow the guide RNA scaffold, blue the target sequence, and red the PAM. (B) Recovery time of dCas9-3XGFP/C3-8 guide RNA on target (C3). (inset table) The recovery times (τ) were measured by FRAP and dissociation rates (k_{off}) were calculated as described in Materials and methods. (*) The values represent the mean with standard errors from three independent experiments. (C) C3-11 or C3-8 guide RNAs were expressed in U2OS along with dCas9-3XGFP, and the foci brightness was examined under similar nuclear fluorescent backgrounds. Bar, 5 μm . (D) Plots for the foci brightness ($n = 26$ for C3-11 and $n = 24$ for C3-8). Box spans from first to last quartiles. Whiskers represent 10/90 percentiles, and the middle lines represent the mean position.

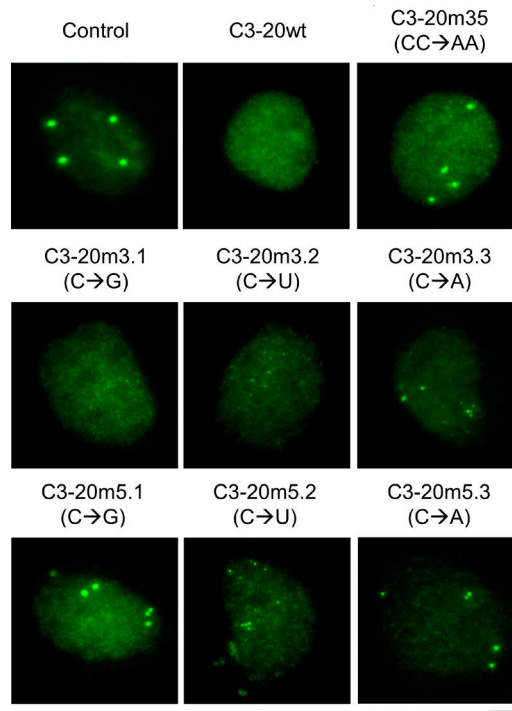


Figure S5. **Visualization of DNA cleavage in living cells.** U2OS cells were cotransfected with plasmids containing guide RNA pairs (C3-11 and C3-20 or mutants of C3-20) and Cas9-3XGFP and then fixed after 24 h and imaged by collecting z-stack images to capture all foci in each nucleus examined. Foci (control, noncleaved target), no foci (C3-20wt, highly cleaved target), or dispersed puncta (C3-20 mutants) are shown in representative images. Bar, 5 μ m. Data in all panels are representative of experiments performed at least three times.

Table S1. **Cas9 and guide RNAs used for CRISPR-Cas9 nuclear dynamics**

Cas9	Guide RNA1	Guide RNA sequence (5'-3')	Guide RNA2	Guide RNA sequence (5'-3')	Application
DD-dCas9-mCherry	C3-guide RNA-2XBroccoli	UGAUUACACAG	/	/	Imaging and FACS
dCas9-mCherry	C3-guide RNA-85-Broccoli	UGAUUACACAG	/	/	Imaging
dCas9-mCherry	C3-guide RNA-67-Broccoli	UGAUUACACAG	/	/	/
dCas9-mCherry	C3-guide RNA-54-Broccoli	UGAUUACACAG	/	/	/
dCas9-3XGFP	C3-11 (C3-11wt)	UGAUUACACAG	/	/	Imaging and FRAP
dCas9-3XGFP	C3-11m3.1	UGAUUACAGAG	/	/	/
dCas9-3XGFP	C3-11m3.2	UGAUUACAUAG	/	/	/
dCas9-3XGFP	C3-11m3.3	UGAUUACAAAG	/	/	/
dCas9-3XGFP	C3-11m5.1	UGAUUAGACAG	/	/	/
dCas9-3XGFP	C3-11m5.2	UGAUUUACAG	/	/	/
dCas9-3XGFP	C3-11m5.3	UGAUUAAACAG	/	/	/
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20 (C3-20wt)	UCCUCUGUAUGAUUACACAG	Imaging
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20m3.1	UCCUCUGUAUGAUUACAGAG	/
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20m3.2	UCCUCUGUAUGAUUACAUAG	/
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20m3.3	UCCUCUGUAUGAUUACAAAG	/
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20m5.1	UCCUCUGUAUGAUUAGACAG	/
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20m5.2	UCCUCUGUAUGAUUUACAG	/
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20m5.3	UCCUCUGUAUGAUUAAACAG	/
Cas9-3XGFP	C3-11	UGAUUACACAG	C3-20m35	UCCUCUGUAUGAUUAAAAG	/

The bold letters indicate the mutated nucleotide compared to the original guide RNA sequence. Slash (/) indicates not applicable.