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Supplemental Information

The Chromatin Structure of CRISPR-Cas9 Target DNA Controls the Balance between Mutagenic and Homology-Directed Gene-Editing Events

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SUPPLEMENTAL INFORMATION

Figure S1. Target sites of RGN complexes in the *TLR* construct. The target sequences for the RGN complexes Cas9:gTLR.1, Cas9:gTLR.2 and Cas9:gTLR.3 are indicated by horizontal lines linked to open boxes delimiting the respective PAM sequences. The positions of the site-specific DSBs generated by each RGN are marked (vertical open arrowheads). STOP, nonsense codon located within the *TLR* ORF.

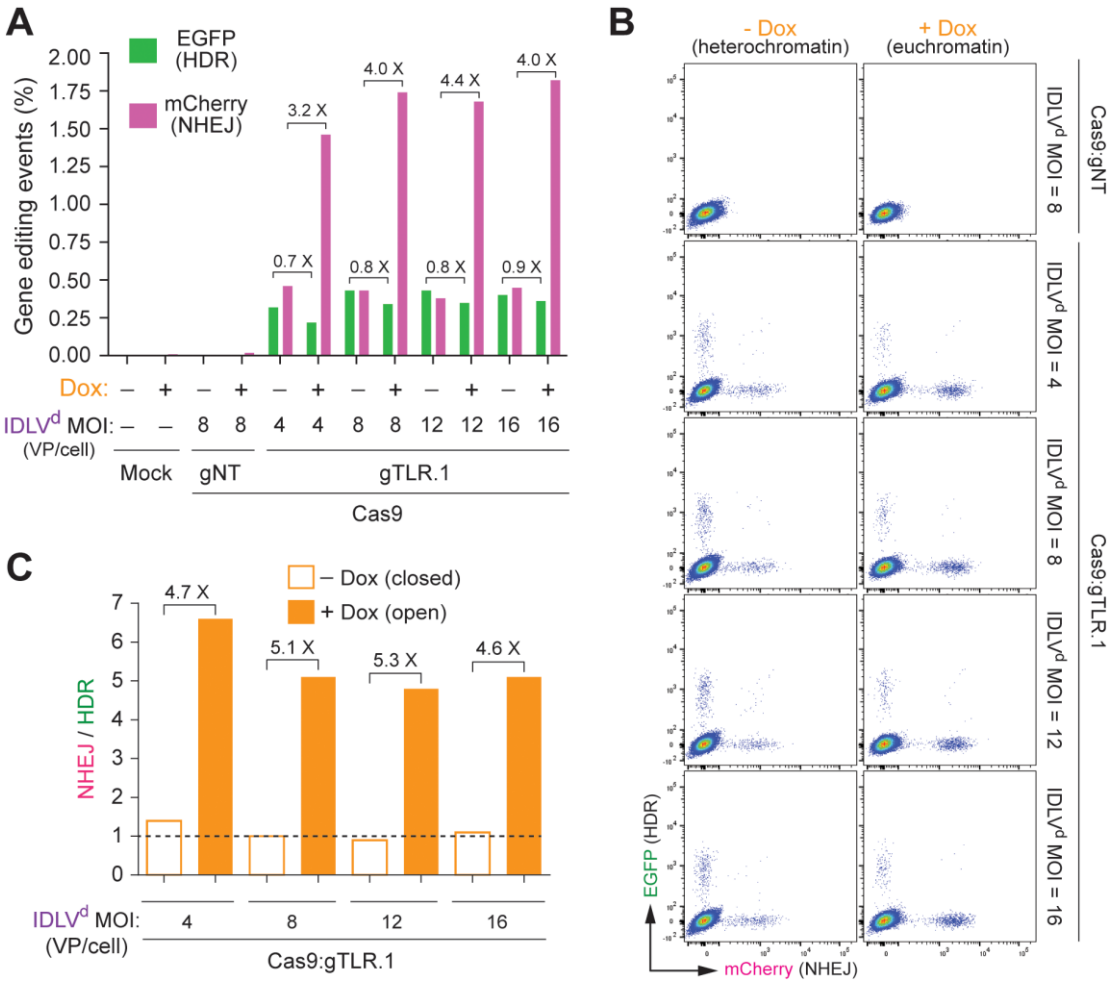


Figure S2. Gene editing endpoints at euchromatin versus heterochromatin after IDLV donor DNA delivery. (**A**) Dual-color flow cytometric quantification of HDR and NHEJ events in HER.TLR$^{TetO.KRAB}$ cells. HER.TLR$^{TetO.KRAB}$ cells were exposed to Cas9:gTLR.1 together with the indicated multiplicities of infection (MOI) of IDLV^{d}. Negative controls consisted of mock-treated cultures and of cultures exposed to a non-targeting gRNA (gNT), Cas9 and IDLV^{d} at an MOI of 8 vector particles per cell (VP/cell). The various experimental conditions were tested in HER.TLR$^{TetO.KRAB}$ reporter cells incubated in the absence (-) or in the presence (+) of doxycycline (Dox). The frequencies of HDR and NHEJ events in the various target cell populations were determined by measuring EGFP^{+} and mCherry^{+} cells, respectively. (**B**) Dot plots corresponding to HER.TLR$^{TetO.KRAB}$ cells transduced with different doses of IDLV^{d} particles and subjected to the indicated Dox regimens. (**C**) Relative participation of HDR and NHEJ pathways during IDLV-mediated repair of DSBs occurring at heterochromatin versus euchromatin. Data of panel **A** are presented as the ratios between the frequencies of NHEJ and HDR in HER.TLR$^{TetO.KRAB}$ cells not treated and treated with Dox.

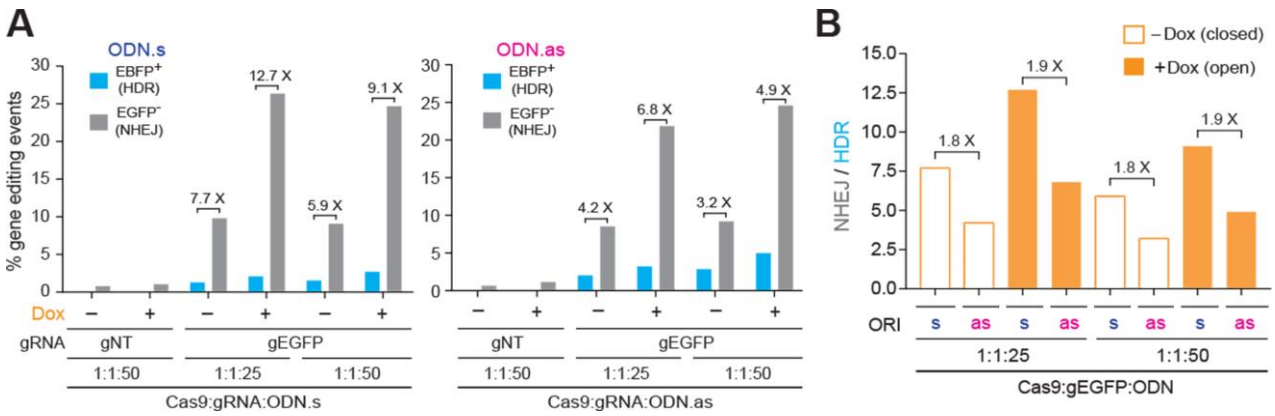


Figure S3. Gene editing endpoints at euchromatin versus heterochromatin in HEK.EGFP$^{TetO.KRAB}$ cells after transfection of ODNs with different polarities. (**A**) Testing the impact of chromatin structure on HDR-based gene editing with sense and antisense ODNs. HEK.EGFP^{TetKRAB} cells, incubated (+) or not incubated (-) with Dox, were exposed to the indicated experimental conditions. The frequencies of HDR and NHEJ were assessed by dual-color flow cytometry. (**B**) Relative participation of HDR and NHEJ pathways during the repair of euchromatic versus heterochromatic DSBs with ODNs with different polarities. Data of panel **A** displayed as the ratios between the

frequencies of NHEJ and HDR in HEK.EGFP$^{TetO.KRAB}$ cells treated and not treated with Dox.

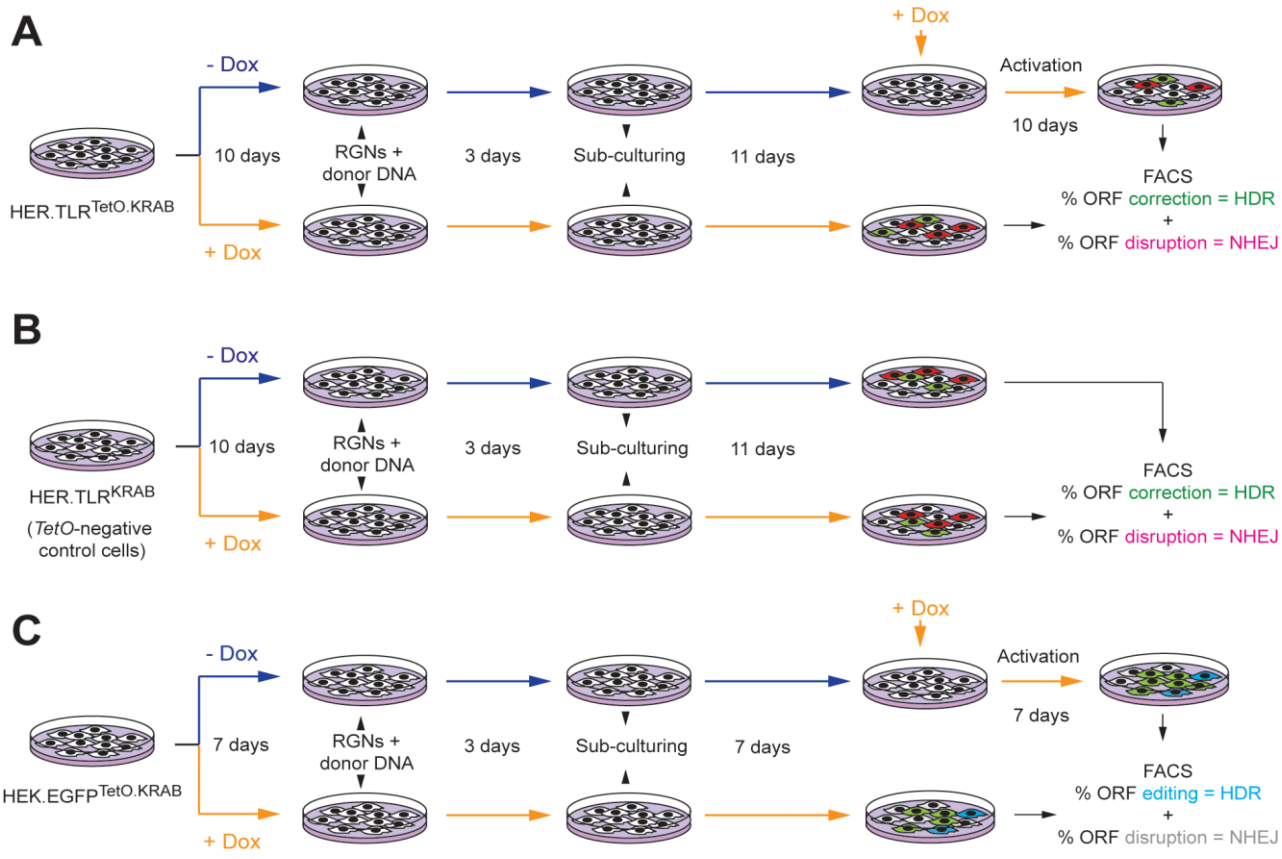


Figure S4. Schematic representation of the experimental settings used in the current study. The tTR-KRAB-expressing cells HER.TLR$^{TetO.KRAB}$ (**A**) contain the Dox-regulated *TLR*^{TetO} construct. The tTR-KRAB-expressing HER.TLR^{KRAB} reporter cells (**B**) have the Dox-insensitive *TLR* construct and were used as an isogenic control cellular system. The tTR-KRAB-expressing cells HEK.EGFP$^{TetO.KRAB}$ (**C**) contain the Dox-regulated *EGFP*^{TetO} construct. These reporter cells are transiently transfected in the presence or in the absence of Dox with different combinations of gene editing tools consisting of RGNs and donor DNA templates. After the generation of site-specific DSBs and the ensuing modification of target DNA sequences in cells subjected to both experimental settings (i.e. –Dox and +Dox), target gene expression is activated to quantifying by flow cytometry the frequencies of gene editing events resulting from the engagement of HDR and NHEJ pathways.

Table S1. Oligonucleotide pairs to generate the gRNA expression constructs expressing gTLR.1, gTLR.2, gTLR.3, gNT and gEGFP

Plasmids	Oligonucleotide pairs (5′- 3′)
Z42_pgTLR.1	5′-ACCGGTGAGCTCTTATTTGCGTA-3′ 5′-AAACTACGCAAATAAGAGCTCAC-3′
Z44_pTLR.2	5′-ACCGGGATAACAGGGTAATGTCG-3′ 5′-AAACCGACATTACCCTGTTATCC-3′
AW26_pTLR.3	5′-ACCGTAACAGGGTAATGTCGAGGC-3′ 5′-AAACGCCTCGACATTACCCTGTTA-3′
AM51_pgNT	5′-ACCGGTGAGCTCTTATTTGCGTAGCTAGCTGAC-3 5′-AAACGTCAGCTAGCTACGCAAATAAGAGCTCAC-3′
AX03_pgEGFP	5′-ACCGCTCGTGACCACCCTGACCTA-3′ 5′-AAACTAGGTCAGGGTGGTCACGAG-3′

Table S2. Experimental scheme corresponding to **Figure S2**

DONOR: IDLV^{d}	3.25 ×10^{5} HER.TLR$^{TetO.KRAB}$ cells per well of 24-well plates (500 µl medium per well with or without Dox)			
	PEI (1mg/ml)： 5.8 µl per well; <u>Ratio DNA / PEI equivalents = 6</u>			
Reagents	Cas9	gNT (Ctrl)	gTLR.1	Total (ng)
Construct length (bp)	9551	3056	3046	
DNA per well (ng)	**1327**	**423**		**1750**
	1327		**423**	**1750**
	1327			**1750**

Note 1: One day after transfecting plasmids expressing Cas9 and gTLR.1, IDLV^{d} particles were added at an MOI of 4, 8, 12 and 16 VP/cell; **Note 2**: One day after transfecting plasmids expressing Cas9 and gRNA^{NT}, IDLV^{d} particles were added at an MOIs of 8 VP/cell.

Table S3. Experimental scheme corresponding to **Figure 2** (**<u>Protocol A</u>**)

DONOR: IDLV^{d}	3.25 ×10^{5} HER.TLR$^{TetO.KRAB}$ cells per well of 24-well plates (500 µl medium per well with or without Dox)			
	PEI (1mg/ml)： 9.6 µl per well; <u>Ratio DNA / PEI equivalents = 10</u>			
Reagents	Cas9	gNT (Ctrl)	gTLR.1	Total (ng)
Construct length (bp)	9551	3056	3046	
DNA per well (ng)	**1327**	**423**		**1750**
	1327		**423**	**1750**
	1327			**1750**

Note: One day after transfection of the indicated plasmids, IDLVd particles were added at an MOI of 8 VP/cell.

Table S4. Experimental scheme corresponding to **Figure 2** (**Protocol B**)

DONOR: IDLV[d]	3.25 ×10[5] HER.TLR[TetO.KRAB] cells per well of 24-well plates (500 µl medium per well with or without Dox)				
	PEI (1mg/ml)： 5.8 µl per well; Ratio DNA / PEI equivalents = 6				
Reagents	Cas9	gNT (Ctrl)	gTLR.1	gTLR.2	Total (ng)
Construct length (bp)	9551	3056	3046	3046	
DNA per well (ng)	**1327**	**423**			**1750**
	1327		**423**		**1750**
	1327			**423**	**1750**

Note: One day after transfecting the indicated plasmids, IDLV[d] particles were added at an MOI of 8 VP/cell.

Table S5. Experimental scheme corresponding to **Figure 3** (**Protocol A**)

DONOR: Plasmid[d]	3.25 ×10[5] HER.TLR[TetO.KRAB] cells per well of 24-well plates (500 µl medium per well with or without Dox)						
	PEI (1mg/ml)： 9.6 µl per well; Ratio DNA / PEI equivalents = 10						
Reagents	Cas9	gNT (Ctrl)	gTLR.1	gTLR.2	gTLR.3	Plasmid[d]	Total (ng)
Construct length (bp)	9551	3056	3046	3046	3047	6194	
DNA per well (ng)	**890**	**284**				**577**	**1751**
	890		**284**			**577**	**1751**
	890			**284**		**577**	**1751**
	890				**284**	**577**	**1751**

Note: The same transfection conditions were applied in experiments carried out in control, Dox-unresponsive, HER.TLR[KRAB] cells (**Figure 4**).

Table S6. Experimental scheme corresponding to **Figure 3** (**Protocol B**)

DONOR: Plasmid[d]	3.25 ×10[5] HER.TLR[TetO.KRAB] cells per well of 24-well plates (500 µl medium per well with or without Dox)					
	PEI (1mg/ml)： 5.8 µl per well; Ratio DNA / PEI equivalents = 6					
Reagents	Cas9	gNT (Ctrl)	gTLR.1	gTLR.2	Plasmid[d]	Total (ng)
Construct length (bp)	9551	3056	3046	3046	6194	
DNA per well (ng)	**890**	**284**			**577**	**1751**
	890		**284**		**577**	**1751**
	890			**284**	**577**	**1751**

Table S7. Experimental scheme corresponding to **Figure 6**

DONOR: Plasmid[d]		3.25 ×10[5] HER.TLR[TetO.KRAB] cells per well of 24-well plates (500 µl medium per well with or without Dox)						
		PEI (1mg/ml)： 9.6 µl per well; Ratio DNA / PEI equivalents = 10						
Reagents	Cas9	gNT (Ctrl)	gTLR.1	gTLR.2	gTLR.3	Plasmid[d]	Plasmid[S]	Total (ng)
Construct length (bp)	8506	3056	3046	3046	3047	6194	6040	
DNA per well (ng)	**823**	**295**				**599**	**33**	**1750**
	823		**295**			**599**	**33**	**1750**
	823			**295**		**599**	**33**	**1750**
	823				**295**	**599**	**33**	**1750**

DONOR: Plasmid[d]	3.25 ×10[5] HER.TLR[TetO.KRAB] cells per well of 24-well plates (500 µl medium per well with or without Dox)						
	PEI (1mg/ml)： 9.6 µl per well; Ratio DNA / PEI equivalents = 10						
Reagents	Cas9[hGem(1/110)]	gNT (Ctrl)	gTLR.1	gTLR.2	gTLR.3	Plasmid[d]	Total (ng)
Construct length (bp)	8854	3056	3046	3046	3047	6194	
DNA per well (ng)	**856**	**295**				**599**	**1751**
	856		**295**			**599**	**1751**
	856			**295**		**599**	**1751**
	856				**295**	**599**	**1751**

Notes: The source of Cas9 and Cas9[hGem(1/110)] proteins were isogenic expression plasmids pX330.Cas9 and pX330.Cas9.hGem(1/110), respectively; Plasmid[S] refers to an irrelevant ("stuffer") construct to normalize the total amount of transfected DNA; The same transfection conditions were applied in experiments carried out in control, Dox-unresponsive, HER.TLR[KRAB] cells.

Table S8. Experimental scheme corresponding to **Figure 5**

DONOR: pTHG.Donor (Exp.1)	2.0 ×10[5] HEK.EGFP[TetO.KRAB] cells per well of 24-well plates (500 µl medium per well with or without Dox)				
	PEI (1mg/ml)： 6.2 µl per well; Ratio DNA / PEI equivalents = 9				
Reagents	eCas9	gEGFP	gNT (Ctrl)	pTHG.Donor	Total (ng)
Construct length (bp)	9360	3046	3056	3561	
DNA per well (ng)	**733**	**238**		**279**	**1250**
	733		**238**	**279**	**1250**
DONOR: pTHG.Donor (Exp.2)	2.0 ×10[5] HEK.EGFP[TetO.KRAB] cells per well of 24-well plates (500 µl medium per well with or without Dox)				
	PEI (1mg/ml)： 6.2 µl per well; Ratio DNA / PEI equivalents = 9				
Reagents	eCas9	gEGFP	gNT (Ctrl)	pTHG.Donor	Total (ng)
Construct length (bp)	9403	3046	3056	3561	
DNA per well (ng)	**733**	**238**		**279**	**1250**
	733		**238**	**279**	**1250**

Table S9. Experimental scheme corresponding to **Figure S3**

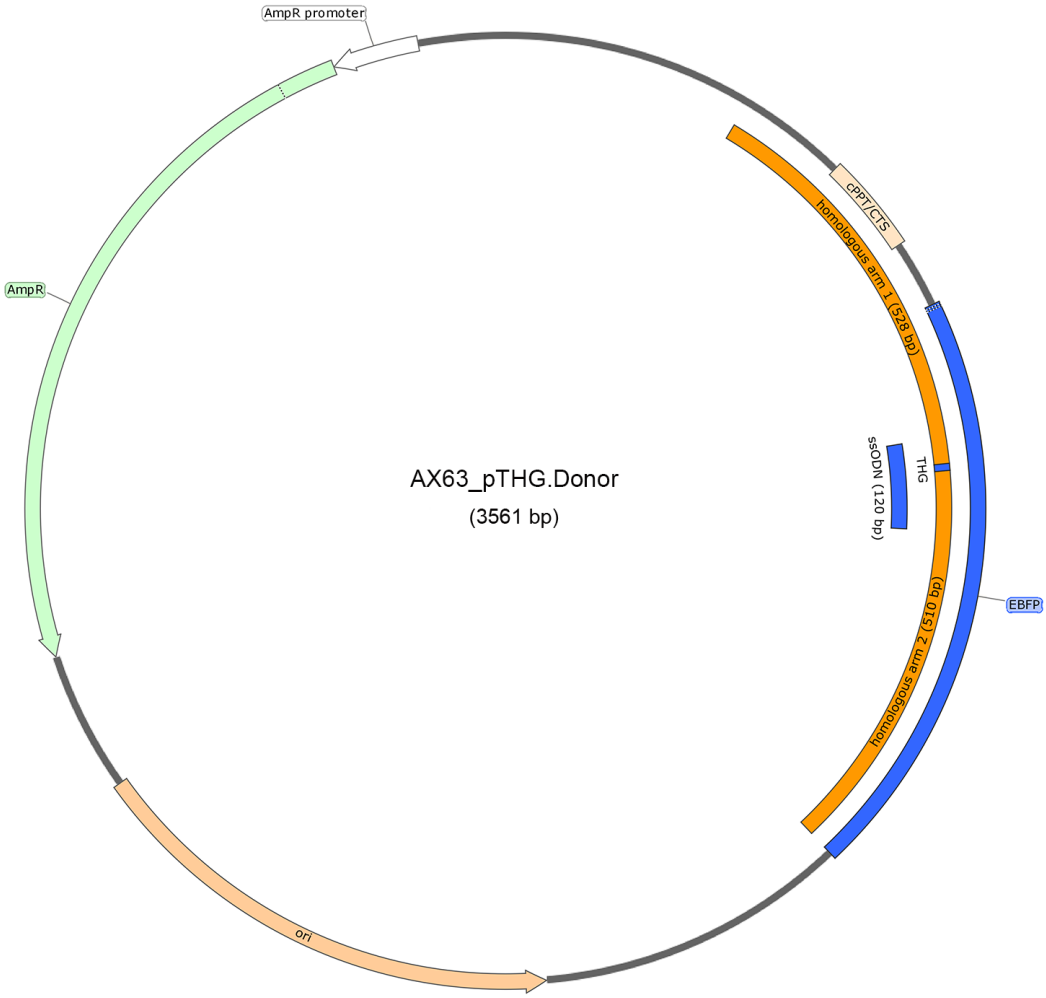
DONOR: ODN.s / ODN.as	2.5×10^5 HEK.EGFP$^{TetO.KRAB}$ cells per well of 24-well plates (500 µl medium per well with or without Dox)						
	PEI (1mg/ml)： 6.2 µl per well; Ratio DNA / PEI equivalents = 9						
Reagents	Cas9	gNT (Ctrl)	gEGFP	ODN.s	ODN.as	Total (ng)	Molar ratios
Construct length (bp)	9551	3056	3046	120	120		
DNA per well (ng)	**642**	**205**		**403**		**1250**	**1:1:50**
	766		**244**	**240**		**1250**	**1:1:25**
	642		**205**	**403**		**1250**	**1:1:50**
	642	**205**			**403**	**1250**	**1:1:50**
	766		**244**		**240**	**1250**	**1:1:25**
	642		**205**		**403**	**1250**	**1:1:50**

Table S10. Experimental scheme corresponding to **Figure 7C**

DONOR: ODN.s / ODN.as	2.5×10^5 HEK.EGFP$^{TetO.KRAB}$ cells per well of 24-well plates (500 µl medium per well with or without Dox)					
	PEI (1mg/ml): 6.2 µl per well; Ratio DNA / PEI equivalents = 9					
Reagents	Cas9	gNT (Ctrl)	gEGFP	ODN.as	Total (ng)	Molar ratios
Construct length (bp)	9551	3056	3046	120		
DNA per well (ng)	**642**	**205**		**403**	**1250**	**1:1:50**
	766		**244**	**240**	**1250**	**1:1:25**
	642		**205**	**403**	**1250**	**1:1:50**
	553		**176**	**521**	**1250**	**1:1:75**

Note: The molar ratios 1:1:50 of Cas9:gRNA:oligos were used in the experiments correponsing to the flow cytometry dot plots presented in Figure 6B.

Supplementary Notes



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Map and nucleotide sequence of pTHG.Donor for HDR-mediated editing of *EGFP* into *EBFP*. DNA sequences sharing identity to the target sequence in HEK.EGFP$^{TetO.KRAB}$ cells are indicated in orange; AmpR, *β-lactamase* ampicillin resistance gene; ori, high-copy number ColE1 prokaryotic origin of replication; cPPT/CTS, central polypurine tract and central termination sequence of HIV-1. As reference, the nucleotide sequences corresponding to the EBFP flurochrome (Thr-His-Gly) and the ssODNs are highlighted in blue and underlined, respectively.