

Supplementary Information

Chemically-inducible CRISPR/Cas9 circuits for ultra-high dynamic range gene perturbation

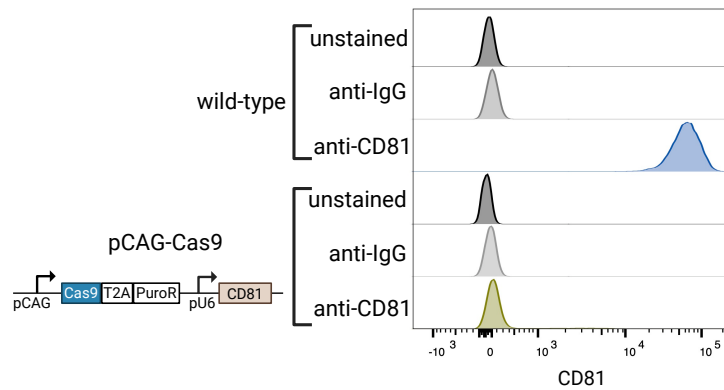
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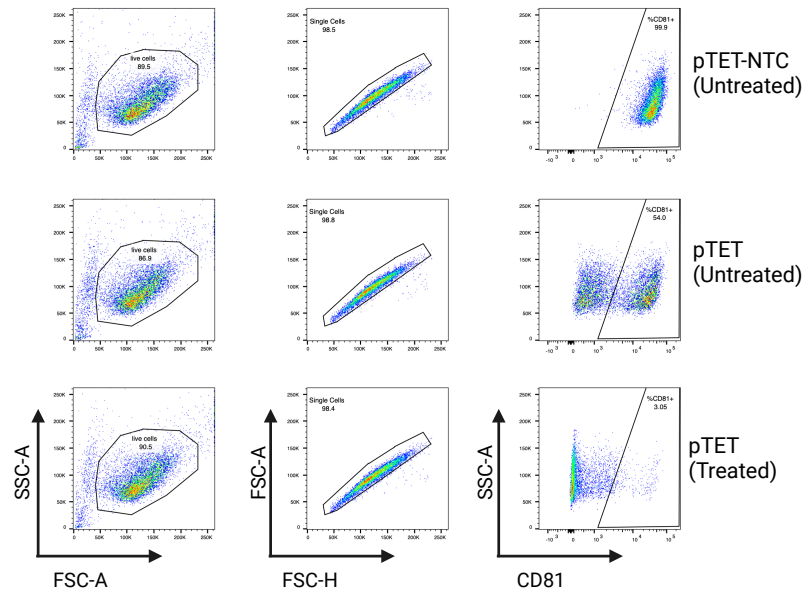
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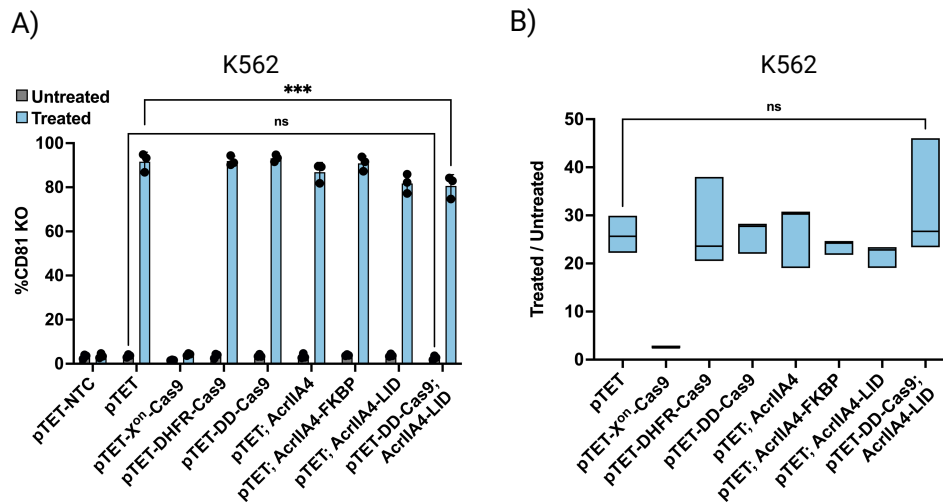
A)



B)

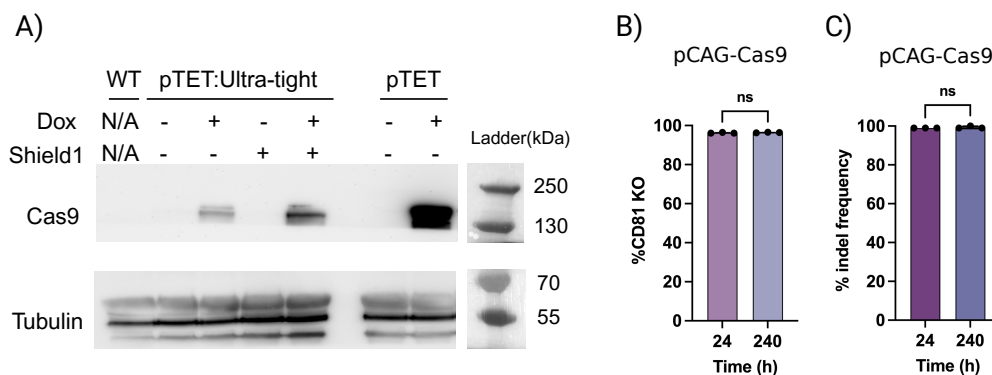


Supplementary Figure 1. Specificity controls and gating strategy for flow cytometric analysis. (A) Flow cytometry histogram plots of HEK293T wild-type and CD81 knockout cells stained with APC-conjugated CD81 antibody or an IgG isotype control antibody, with unstained samples included as negative controls. The knockout cell line was generated by stably integrating a plasmid (pCAG-Cas9) in which Cas9 and CD81 sgRNA are constitutively expressed from the CAG and U6 promoters, respectively (schematic shown). (B) Flow cytometry plots of pTET and pTET-NTC stable HEK293T cells with and without Dox treatment. SSC-A (side scatter) and FSC-A (forward scatter) profiles were used to isolate the live cell population. Doublets were excluded using FSC-A and FSC-H profiles. The pTET-NTC control line was stained in parallel to define the CD81 positive population. Plots and histograms are representative of 3 biological replicates per condition. Created in BioRender. Srinivasan, R. (2025) <https://BioRender.com/i6ic5ap>



Supplementary Figure 2. Comparison of various Cas9 activity control modules in K562 cells

(A, B) The different pTET constructs were stably transfected into K562 cells, followed by drug treatment for 10 days and flow cytometry ($n = 3$ biological replicates per stable line and treatment). (A) The percentage of CD81 negative cells in treated (colored bars) and untreated (gray bars) cells is shown. Bars show mean $\pm$ SD. (B) Floating bars display the dynamic range of Cas9 activity calculated as the ratio of percentage knockout in treated vs. untreated cells for each method; bars extend from the minimum to maximum values, and the line denotes the median. Significance by either Two-way ANOVA (A) or ordinary one-way ANOVA (B) adjusted with Tukey's multiple comparisons test. ns: $P > 0.9999$ (A), $P = 0.9232$ (B); *** $P < 0.001$ ($P = 0.0004$). Source data is provided as a Source Data file. Created in BioRender. Srinivasan, R. (2025) <https://BioRender.com/3riui06>

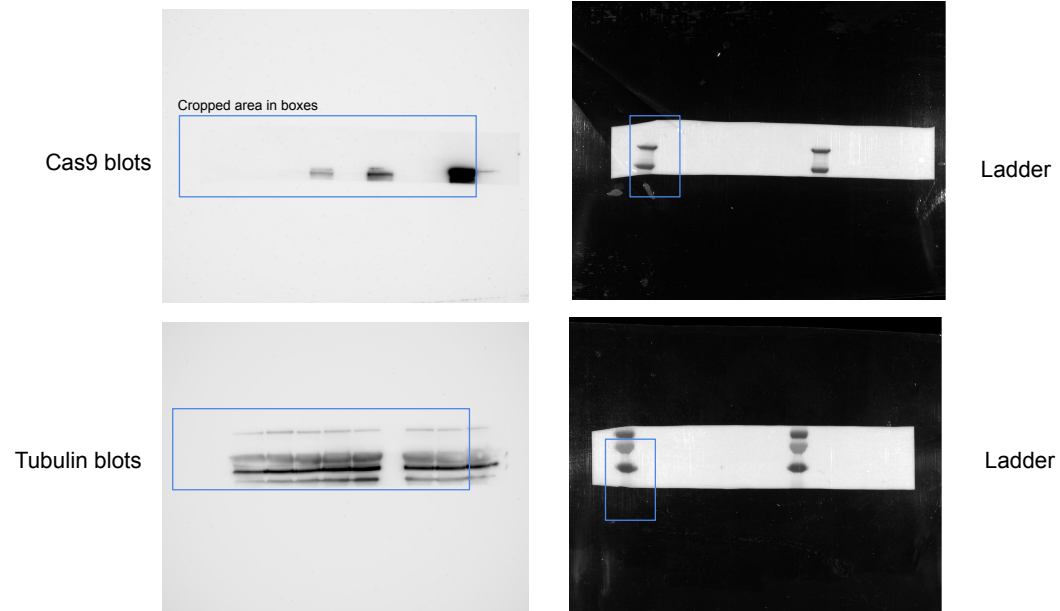


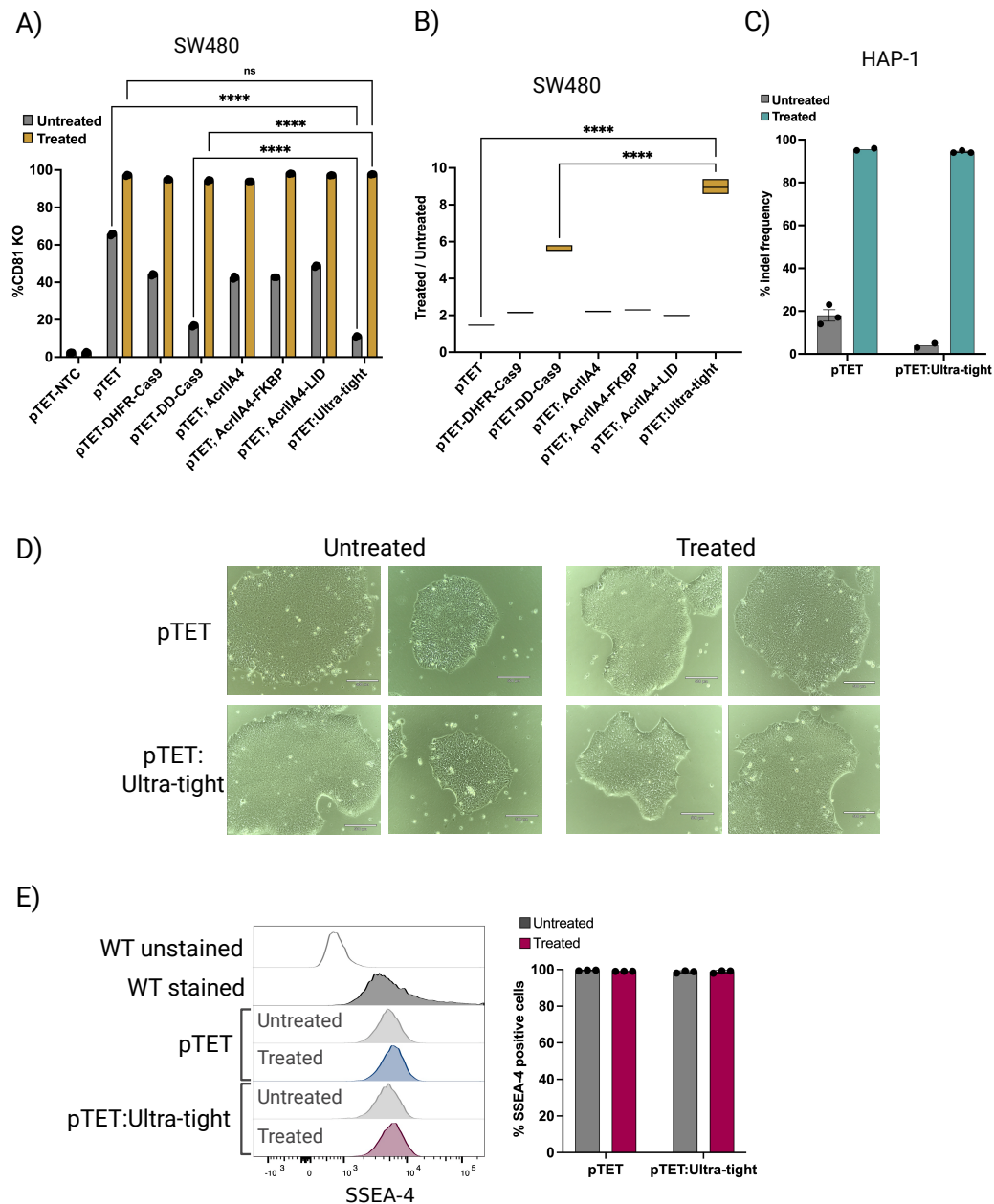
Supplementary Figure 3. Cas9 expression levels upon module induction and constitutive Cas9 disruption of CD81.

(A) Western immunoblotting was performed on HEK293T cells transfected with either pTET or pTET:Ultra-tight constructs, followed by 72-hour treatment with the indicated drugs. Data are representative of 2 replicates per condition. Untreated cells and wild-type parental (WT) controls were included. The upper panel shows Cas9 detection; the lower panel shows Tubulin as a loading control. (B, C) Bar graphs depict CD81 knockout by flow cytometry (B) and targeted amplicon sequencing (C) in the constitutive pCAG-Cas9 line at the indicated time points after stable line generation. Bars show mean $\pm$

SD ($n = 3$ biological replicates per time point). Significance by unpaired two-tailed Welch's t -test. ns: $P = 0.3413$ (B), $P = 0.4226$ (C). Source data is provided as a Source Data file. Created in BioRender. Sun, T. (2025) <https://BioRender.com/2f9gdf1>

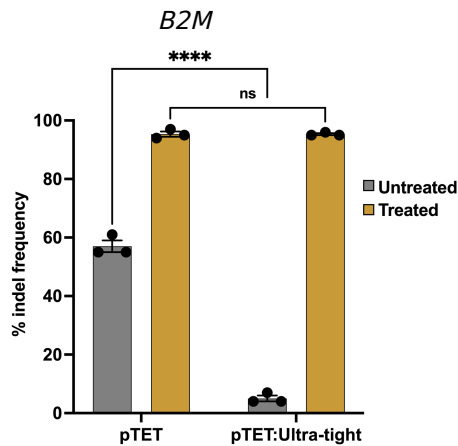
Supplementary Figure 3A



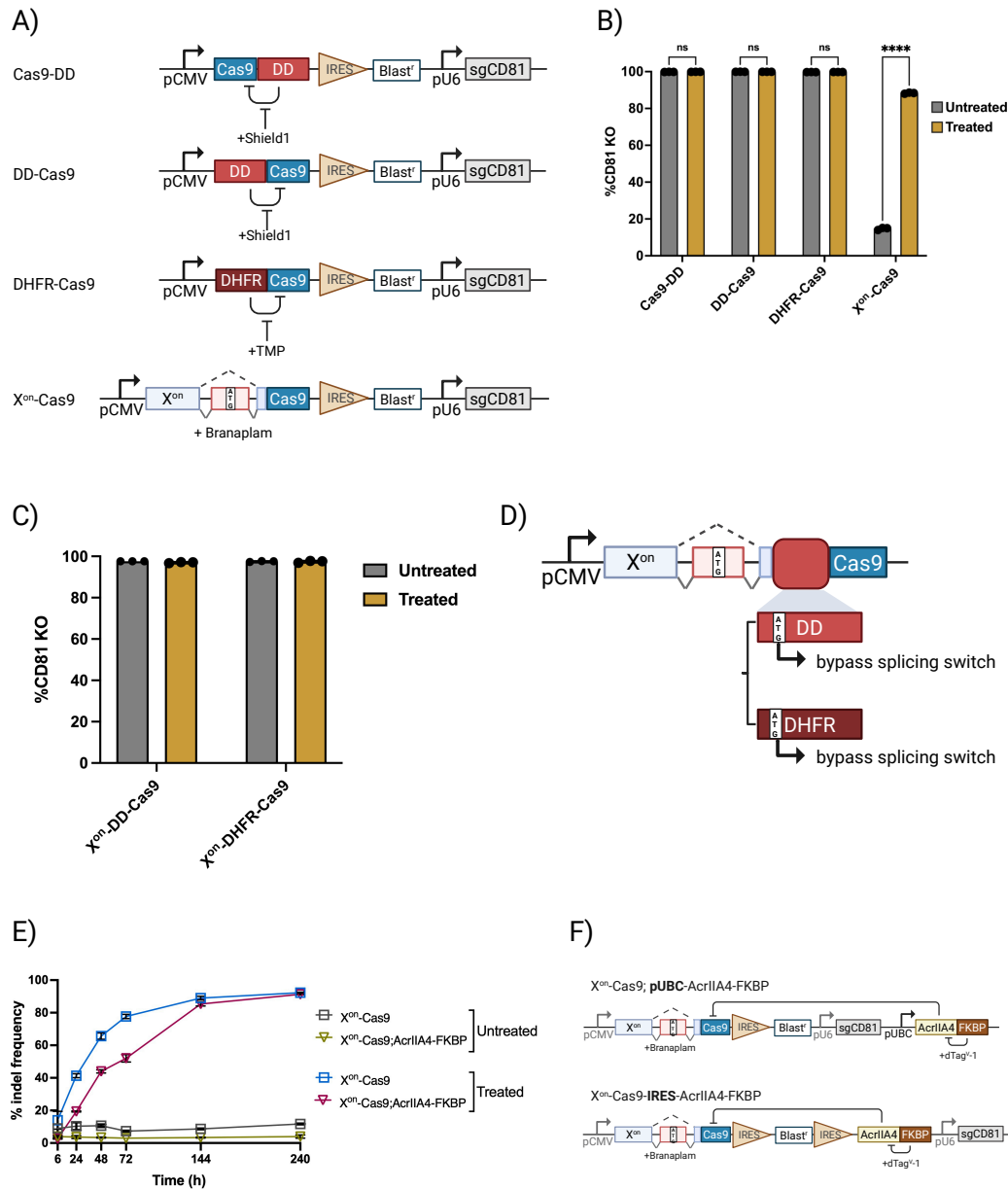


Supplementary Figure 4. pTET:Ultra-tight performance in transformed cell lines and assessment of basal iPSC phenotypes engineered with pTET or pTET:Ultra-tight (A, B) The different pTET constructs were stably transfected into SW480 cells, followed by drug treatment for 10 days and flow cytometry ($n = 3$ biological replicates per stable line and treatment). (A) The percentage of CD81 negative cells in treated (colored bars) and untreated cells (gray bars) is shown. Bars show mean $\pm$ SD. (B) Floating bars display the dynamic range of Cas9 activity calculated as the ratio of percentage knockout in treated vs. untreated cells for each method; bars extend from the minimum to maximum values, and the line denotes the median. (C) Bar graph depicts percentage indel frequency in untreated vs. treated HAP-1 cells stably expressing pTET or pTET:Ultra-tight transgenes ($n = 3$ biological replicates per stable line and treatment);

$n = 2$ biological replicates for pTET treated and pTET:Ultra-tight untreated conditions, error bars omitted for these conditions). Bars show mean $\pm$ SD. Significance by either Two-way ANOVA (A) or ordinary one-way ANOVA (B), adjusted with Tukey's multiple comparisons test. ns: $P = 0.9315$, **** $P < 0.0001$ (A, B). (D) Brightfield images depict colony morphology of KOLF2.1J iPSC stably expressing pTET or pTET:Ultra-tight transgenes, either left untreated or drug-treated for 10 days. Scale bars indicate 500 μ m. Images are representative of 3 biological replicates per condition. (E) Representative flow cytometry histogram plots display SSEA-4 marker expression in untreated and treated KOLF2.1J stable lines. Wild-type (parental, WT) unstained and FITC-conjugated SSEA-4 stained samples are included as controls. Quantified percentages of SSEA-4 positive cells are shown as bar graphs (mean $\pm$ SD; $n = 3$ biological replicates per stable line and treatment). Source data is provided as a Source Data file. Created in BioRender. Srinivasan, R. (2025) <https://BioRender.com/5x7hoy9>



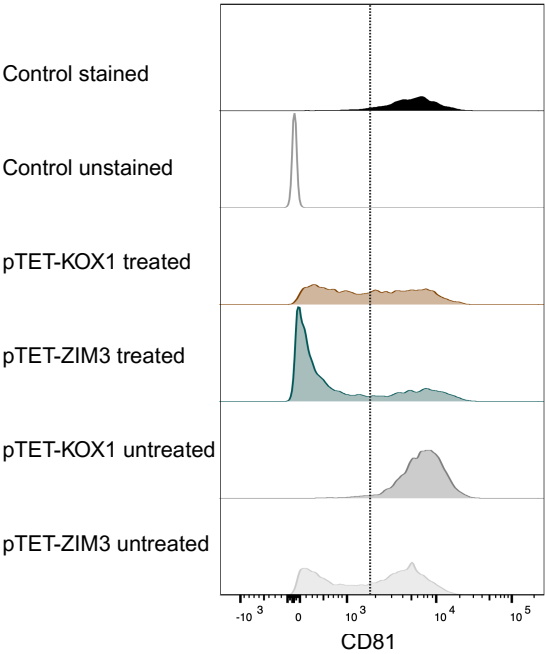
Supplementary Figure 5. Assessment of indel generation at the *B2M* locus in stable HEK293T cells before and after induction of Cas9 from pTET vs. pTET:Ultra-tight. pTET or pTET:Ultra-tight constructs expressing sgRNA targeting *B2M* were stably integrated into HEK293T cells. Stable cells were left untreated or treated for 10 days with 2 μ g/ml Dox (pTET) or 2 μ g/ml Dox + 1 μ M Shield1 (pTET:Ultra-tight) to induce Cas9 expression. Percentage indels at the *B2M* locus were measured by NGS ($n = 3$ biological replicates per stable line and treatment). Bars show mean $\pm$ SD. Significance by two-way ANOVA with Tukey's multiple comparisons test. ns: $P > 0.9999$, **** $P < 0.0001$. Source data is provided as a Source Data file. Created in BioRender. Srinivasan, R. (2025) <https://BioRender.com/ovogbnr>



Supplementary Figure 6. Comparison of Dox-independent Cas9 activity control modules in HEK293T cells

(A) Schematic diagram of dox independent inducible Cas9 systems. DD domain or DHFR domain is fused to Cas9 at the N- or C- terminus. In the X^{on} Cas9, ATG is deleted from the Cas9 coding sequence and provided in the alternatively spliced exon. (B) *CD81* knockout percentage in the untreated (gray bars) and treated (colored bars) HEK293T cells that stably express inducible Cas9 with a single regulatory module ($n = 3$ biological replicates per stable line and treatment). (C) *CD81* knockout percentage in the untreated (gray bars) and treated (colored bars) HEK293T cells with DD or DHFR in combination with X^{on} switch ($n = 3$ biological replicates per stable line and treatment). (B, C) Bars show mean $\pm$ SD. (D) Schematic diagram of the potential mechanism of Cas9 leaky expression observed in (C). Internal 'ATG' in the DD or DHFR domain bypassed the requirement of exon 2, rendering the X^{on} Cas9 constantly active. (E) Time-course

analysis of CD81 knockout in X^{on} -Cas9 vs. X^{on} -Cas9;AcrIIA4-FKBP HEK293T cells, that were left untreated or treated with either 20nM Branaplam (X^{on} -Cas9) or 20nM Branaplam + 1 μ M dTag^V-1 (X^{on} -Cas9;AcrIIA4-FKBP). Percentage indel frequencies at the *CD81* locus were quantified by targeted amplicon sequencing at the indicated time points ($n = 3$ biological replicates per stable line, treatment and timepoint; $n = 2$ biological replicates for X^{on} -Cas9-untreated 6 h timepoint, error bar omitted for this condition). Data represent mean $\pm$ SEM. (F) Schematic diagram of the two additional versions to control the anti-CRISPR module in the X^{on} system. Significance by two-way ANOVA adjusted with Tukey's multiple comparisons test. ns: $P > 0.9999$. **** $P < 0.0001$. Source data is provided as a Source Data file. Created in BioRender. Srinivasan, R. (2025) <https://BioRender.com/jm44nxh>



Supplementary Figure 7. *CD81* silencing with KOX1 vs. ZIM3 based inducible CRISPRi systems

Representative flow cytometry results of the CD81 stained cells with inducible CRISPRi systems from 3 biological replicates. DLD1 cells expressing *CD81 sgRNA* and pTET-KOX1 or pTET-ZIM3 were treated with 250 ng/ml Dox or untreated for 7 days. The cells were stained with CD81 antibody conjugated with PE. CD81 positive and negative cells were separated by the dashed line. Control: parental DLD1 cells. Created in BioRender. Srinivasan, R. (2025) <https://BioRender.com/9bz5w9s>

Supplementary Table 1. Cell culture and transfection conditions.

Parental cell line	Growth media	Cell type	PiggyBac transfection
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HEK293T	DMEM + 10% TET-free FBS + 1% NEAA + 2mM L-Glutamine; 1X pen-strep	adherent	Seed 3×10^5 cells/well (6-well plate) and the next day transfect with Lipofectamine 2000 (ThermoFisher Scientific); 2 μ g total DNA; 3:1 ratio of pB plasmid:pB transposase.
A549	RPMI 1640 + 10% TET-free FBS + 2mM L-Glutamine; 1X pen-strep	adherent	Seed 5×10^5 cells/well (6-well plate) and the next day transfect with GeneJuice (Novogene); 2 μ g total DNA; 10:1 ratio of pB plasmid:pB transposase.
SW480	RPMI 1640 + 10% TET-free FBS + 2mM L-Glutamine; 1X pen-strep	adherent	Seed 5×10^5 cells/well (6-well plate) and the next day transfect with GeneJuice (Novogene); 2 μ g total DNA; 3:1 ratio of pB plasmid:pB transposase.
HeLa	EMEM + 10% TET-free FBS + 2mM L-Glutamine; 1X pen-strep	adherent	Seed 5×10^5 cells/well (6-well plate) and the next day transfect with Lipofectamine 3000 (ThermoFisher Scientific); 2 μ g total DNA; 3:1 ratio of pB plasmid:pB transposase.
HAP-1	IMDM + 10% Tet-free FBS + 2mM L-Glutamine; 1X pen-strep	adherent	Seed 2×10^5 cells/well (6-well plate) and the next day transfect with GeneJuice (Novogene); 2 μ g total DNA; 3:1 ratio of pB plasmid:pB transposase.
HCT116	RPMI 1640 + 10% TET-free FBS + 2mM L-Glutamine; 1X pen-strep	adherent	Seed 5×10^5 cells/well (6-well plate) and the next day transfect with GeneJuice (Novogene); 2 μ g total DNA; 10:1 ratio of pB plasmid:pB transposase.
K562	RPMI 1640 + 10% TET-free FBS + 2mM L-Glutamine; 1X pen-strep	suspension	Transfect 1×10^6 cells with GeneJuice (Novogene); 2 μ g total DNA: 3:1 ratio of pB plasmid : pB transposase.
KOLF2.1J	StemFit Basic04 complete media (Nacalai, USA Cat. No. SF041-001) with iMatrix-511 (NacalaiUSA, Cat. No. 892012)	adherent	Nucleofect 6×10^5 cells in electroporation buffer using a Lonza 4D instrument with P3 CA-137 condition; 750ng total DNA: 2:1 ratio of pB plasmid : pB transposase. Stable cells were selected with 2 μ g/ml Puromycin

Supplementary Table 2. sgRNA sequences for CRISPR/Cas9 nuclease-based editing

Gene	Sequence 5' to 3'
NTC	GCTTATGGATTAGCCAATC

<i>CD81</i>	TTGGCTTCCTGGGCTGCTA
<i>B2M</i>	AGTAGCGCGAGCACAGCTA
<i>CD298</i>	CTTCAGACTCTCAACGATG
<i>CD9</i>	AATGGCGAATATCACCAAG
<i>PLK1</i>	CCGGCGAAAGAGATCCCGG
<i>H3-3A</i> sgRNA1	CCATCAACAGTCAGACGAA
<i>H3-3A</i> sgRNA2	AGGGATTGCCACTTACATG

Supplementary Table 3. sgRNA sequences for CRISPRi

Gene	Sequence 5' to 3'
NTC	AAACATCGACCGAAAGCGT
<i>CD81</i> sgRNA1	AGGCCTGGCAGGATGCGCG
<i>CD81</i> sgRNA2	CGGAGGCCTGGCAGGATGC
<i>PLK1</i> sgRNA1	GAGTGCTGCAGTGACTGCA
<i>PLK1</i> sgRNA2	GGAGGCTCTGCTCGGATCG

Supplementary Table 4. Small molecule drugs used in this study

Name	Source
Doxycycline	Clontech, Cat. No.: NC0424034
Shield1	Takara, Cat. No.: 632189
TMP (Trimethoprim)	Selleck, Cat. No.: S3129
dTag ^V -1	Bio-technie/Tocris, Cat No. 6914
Branaplam (LMI070)	MedChemExpress, Cat. No.: HY-19620

Supplementary Table 5: Primers used to generate amplicons for next-generation sequencing

Primer name	Primer sequence 5' to 3'
B2M Forward	ATTGGCTGGGCACGCG
B2M Reverse	GAGCGAGAGAGCACAGCG
CD81 Forward	GGCTGTGGCGAGTGTGTAG
CD81 Reverse	CCGGCCCAGGCACAG

Supplementary Table 6: List of plasmids used in this study. Refer to Supplementary Data 1 for information about the sequences used in this study

Transgenic circuit	Use/modality
pTET	Baseline inducible CRISPR knockout
pTET-DD-Cas9	Inducible CRISPR knockout with protein-stability control
pTET-DHFR-Cas9	Inducible CRISPR knockout with protein-stability control
pTET; AcrIIA4-FKBP	Anti-CRISPR-controlled knockout
pTET; AcrIIA4-LID	Anti-CRISPR-controlled knockout
pTET:Ultra-tight	Preferred tightly controlled CRISPR knockout
pTET:Ultra-tight (dual sgRNA)	Preferred inducible paired-cut deletion

Xon-Cas9;PGK-AcrIIA4-FKBP	Dox-independent CRISPR knockout with a strong Anti-CRISPR control
Xon-Cas9-IRES-AcrIIA4-FKBP	Dox-independent CRISPR knockout with a weak Anti-CRISPR control
pTET-KOX1	Inducible CRISPRi
pTET-DD-Zim3	Tightly controlled inducible CRISPRi with a potent ZIM3 repressor domain
pTET-Zim3	Inducible CRISPRi with a potent ZIM3 repressor domain
pCAG-Cas9	Constitutive knockout control

Supplementary Table 7: Reported performance of chemically inducible Cas9 systems compared with pTET:Ultra-tight at endogenous loci

Study (PMID)	Strategy	Inducer	Cell line	Max % editing in pools
27363581	Split Cas9	4OHT	HEK293	~27%
25643054	Split Cas9	Rapamycin	HEK293FT	~43%
35615005	Dual control (Dox + Shield1)	Dox + Shield1	H9 ESC	~25-40%
32994412	Safe-harbor integrated inducible Cas9	Dox	Multiple	~40-60%
25848930	Intein-Cas9	4OHT	HEK293	~10-20%
27618190	ERT2-Cas9	4OHT	HEK293	~15-25%

This study	pTET:Ultra-tight	Dox + Shield1	HEK293T, HAP1, KOLF2.1J iPSC, others	80–95%
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