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Supplementary information

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Programmable promoter editing for precise control of transgene expression

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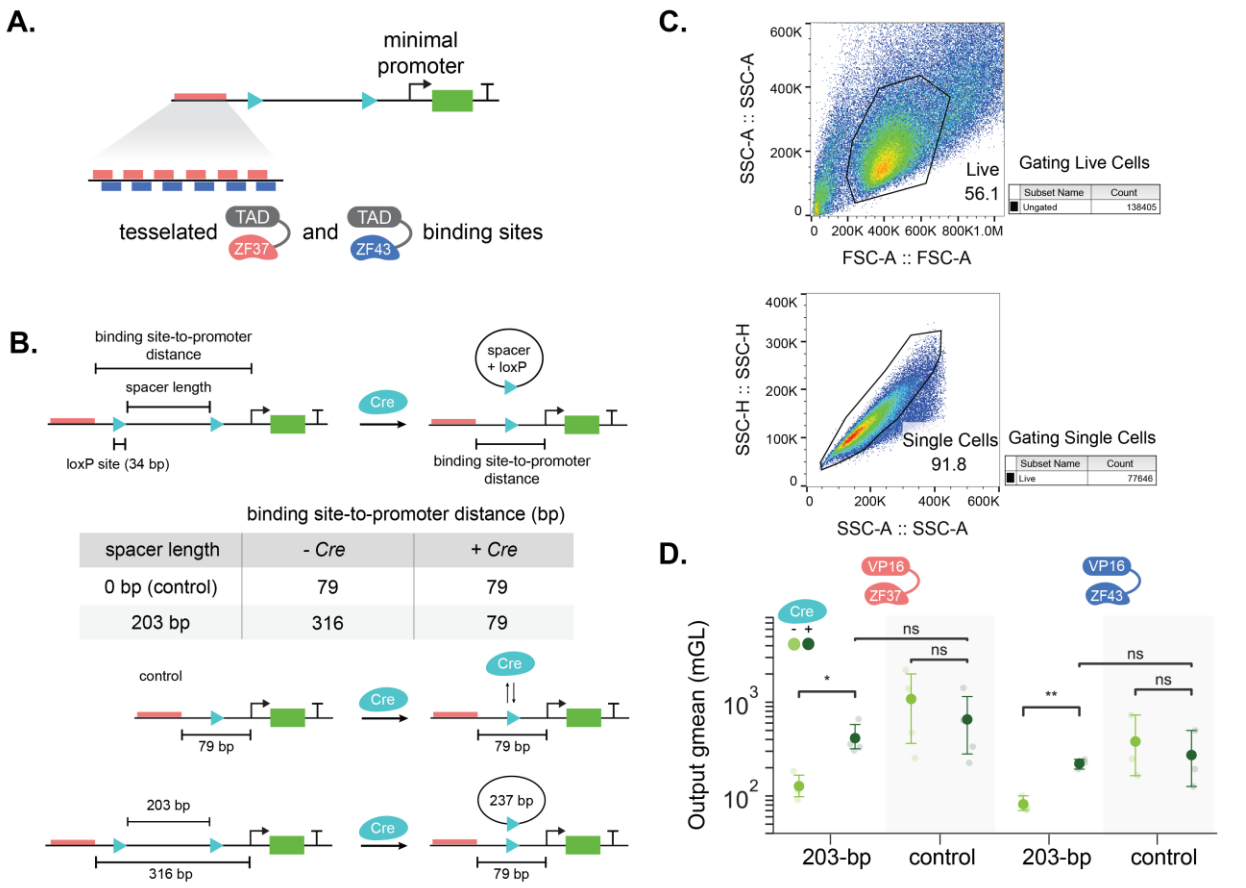


Figure S1. DIAL promoter changes the setpoint by reducing the distance between the binding sites and minimal promoter. | A. Schematic of tessellated hybrid binding sites for ZF37 and ZF43 placed upstream of the floxed spacer to build the DIAL promoter. The DIAL system is composed of the DIAL promoter, recombinase, and the cognate ZFa that induces expression of the gene of interest. **B.** Schematic of the pre- and post-excision states of the DIAL promoter. Spacer length is defined as the distance between the loxP recognition sites. The binding site-to-promoter distance is defined as the total distance between the last binding site and the minimal promoter. Cre excises a loxP site (34 bp) along with the spacer. The control statically encodes the post-excision promoter state. In the control, the binding site-to-promoter distance is 79 bp. While Cre may bind to the remaining loxP site on the post-excision constructs, editing does not occur. **C.** Representative single cell distributions of ungated cells (top) and live cells (bottom) to demonstrate gating strategy of live and single cells, respectively. Live cells were gated based on FSC-A and SSC-A using FlowJo. Subsequently, single cells were gated via SSC-H and SSC-A. As described previously, multiple cells will appear as a larger area at the same height.[1–4] Single cells were then exported and all plots and statistics were generated from pandas, matplotlib, statannot, and seaborn packages in Python. **D.** Output reporter mGL geometric mean (gmean) fluorescence intensity expressed from the 203-bp DIAL promoter or control transfected on plasmids into HEK293T cells (n≥3). In the presence of Cre for both ZFas, the output of the DIAL promoter converges to the output level of the control. Addition of Cre to the control promoter slightly reduces output expression. All units for fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n≥3). Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01.

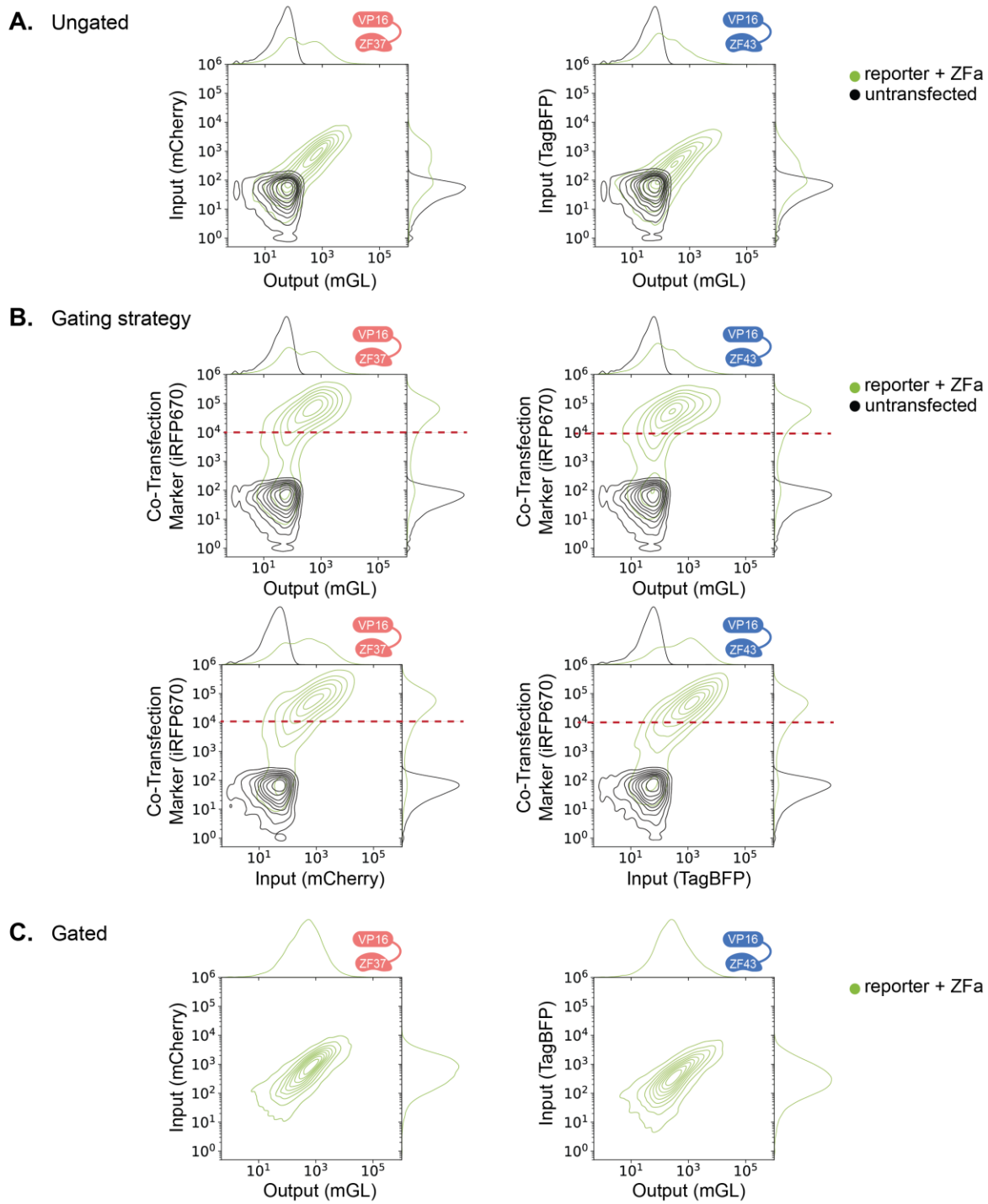


Figure S2. Gating strategy for isolating transfected cells based on the co-transfection marker. | Representative gating strategy based on co-transfection marker. For transient transfection experiments in HEK293T cells at the 96-well scale, live and single cells were additionally gated by a co-transfection marker iRFP670 as shown. For transient transfection of all iPSCs, cells were gated by co-transfection marker TagBFP with a similar method, at TagBFP-A>3,000. For RNA FISH experiments, cells were gated at mRuby2>700. Further gating methods for other experiments are described in relevant legends. (A-C) Histograms and joint contour plots of mGL expressed from the 203-bp DIAL promoter, co-transfected with VP16-ZF37 or VP16-ZF43 (co-expressed with mCherry or TagBFP, respectively), and separate transfection marker iRFP670 on plasmids into HEK293Ts**. A.** Single cell distributions of populations gated for live, single cells. The ZFa protein expression levels are proxied by the fluorescence intensity of mCherry (co-expressed with VP16-ZF37) or TagBFP (co-expressed with VP16-ZF43). Output level of the DIAL promoter is represented by

mGL fluorescence intensity. **B.** Gating strategy of live, single cells based on iRFP670 marker. Gates are shown as dashed lines at the threshold of the co-transfection marker iRFP670 > 10,000. In other experiments, iRFP670>20,000. Gate for co-transfection marker is always consistent within each experiment. C. Single cell distributions of live single cells gated to be positive for iRFP670. When controlling for transfection, the distributions of both ZFa (proxied by TagBFP or mCherry) and output reporter gene (mGL) are unimodal. All units for single cell distributions of fluorescence intensity are arbitrary units (a.u.) Histograms and joint contour plots represent single-cell distributions sampled across bioreplicates (n=4).

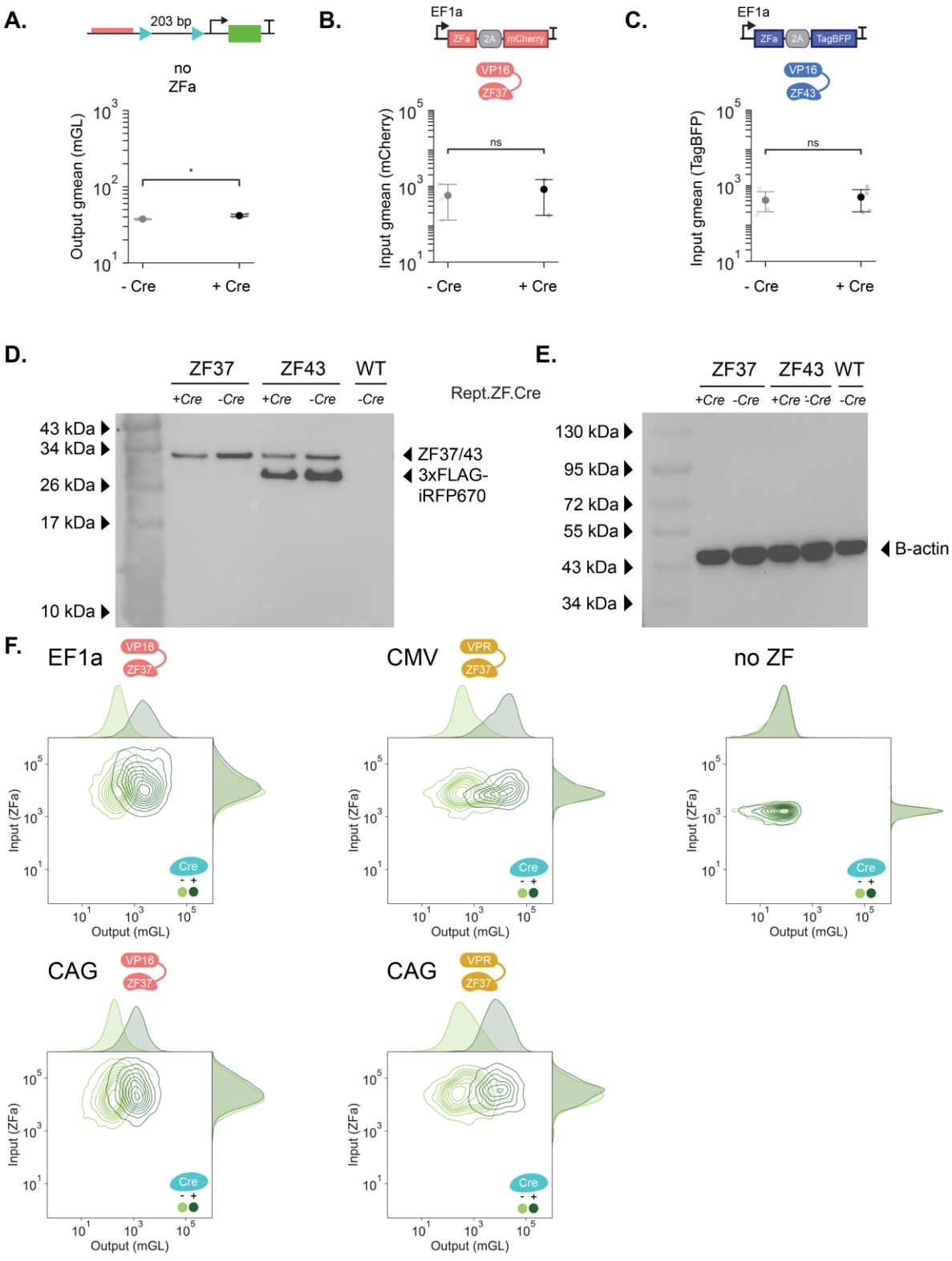


Figure S3. No output reporter expression without ZFa and ZFa levels are unaffected by Cre. | A. Gmean fluorescence intensity of mGL expressed from the 203-bp DIAL promoter transfected on plasmids into HEK293T cells without ZFa, and with or without Cre (n=3, p=0.02011). In the absence of ZFa, the output of the DIAL promoter is off and unaffected by the presence of Cre. **B.** Gmean fluorescence intensity of mCherry coexpressed with ZFa (VP16-ZF37) transfected on plasmids into HEK293T cells along with the 203-bp DIAL promoter and with or without Cre (n=8,

p=.3555). The presence of Cre does not affect the ZFa levels. **C.** Gmean fluorescence intensity of TagBFP coexpressed with ZFa (VP16-ZF43) transfected into HEK293T cells along with the 203-bp DIAL promoter and with or without Cre (n=8, p=.5343). The presence of Cre does not affect the ZFa levels**. D.** Western blot quantification of HEK293T cells transfected with the ZFa (FLAG-VP16-ZF37 or FLAG-VP16-ZF43), and GIB-inducible split Cre for constant total plasmid amount transfected. FLAG-VP16-ZF43 was co-transfected with a 3xFLAG-tagged iRFP670 for copy number normalization. Visualization done with Anti-FLAG Ab at 1:20k dilution and secondary Ab at 1:50k dilution. **E.** Western blot quantification of HEK293T cells transfected with the ZFa FLAG -VP16-ZF37 or FLAG -VP16-ZF43), and GIB-inducible split Cre for constant total plasmid amount transfected. Visualization done with Anti-Beta actin Ab at 1:50k dilution and secondary Ab at 1:50k dilution**. F.** Output single cell joint distribution of mGL regulated by 203-bp DIAL promoter and input ZFa transfected into HEK293Ts with and without Cre. Input ZFa are FLAG-VP16-ZF37 regulated by EF1a, FLAG-VP16-ZF37 regulated by CAG, FLAG-VPR-ZF37 regulated by CMV, or FLAG-VP16-ZF37 regulated by EF1a. ZFa input is quantified by immunofluorescent staining with FLAG Ab at 1:400 dilution and secondary AlexaFluor-555 Ab at 1:1000 dilution. **G.** All units for single cell distributions of fluorescence intensity are arbitrary units (a.u.). Histograms, joint contour plots, and western blots show representative replicates. All units for fluorescence intensity are arbitrary units (a.u.). Large markers represent the mean of biological replicates with span indicating standard error (n≥3). Histograms, joint contour plots, and western blots show representative replicates. Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05.

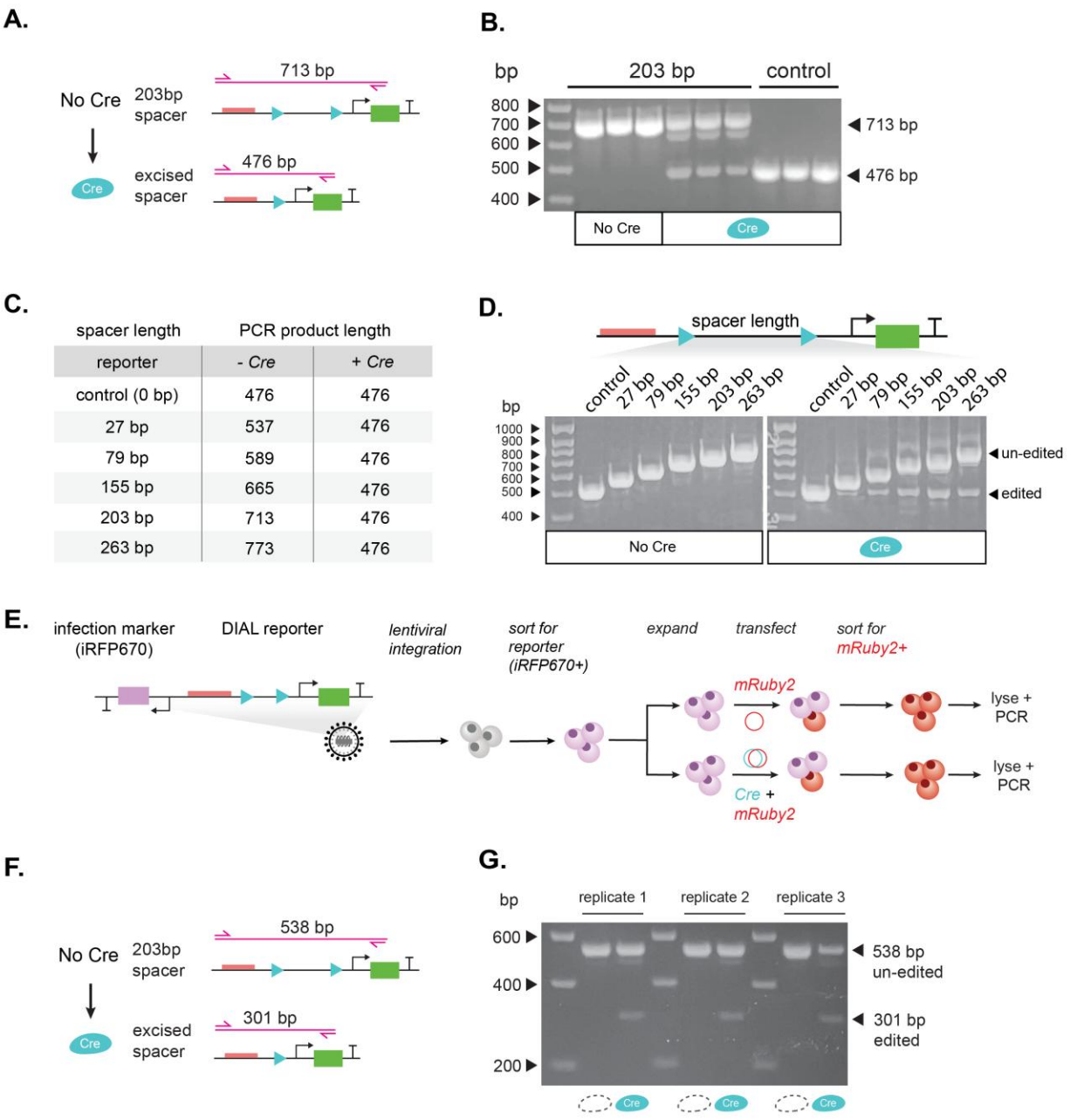
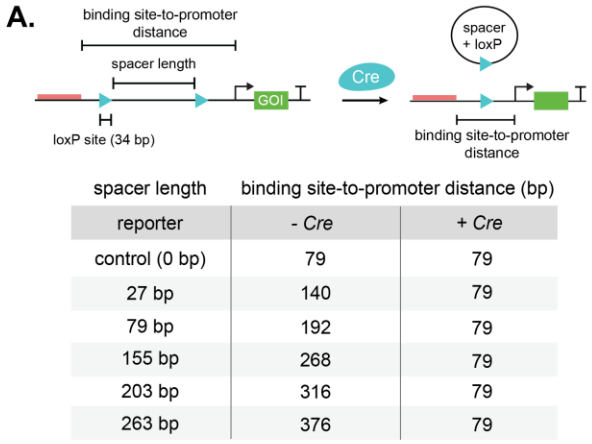


Figure S4. Cre-mediated DNA editing changes the state of the DIAL promoter. | A. Schematic of PCR used to visualize changes to the state of the DIAL promoter. The primers bind to sequences upstream of the ZF binding sites or the mGL reporter gene. In the presence of Cre, the spacer excision shortens the distance between binding sites and the minimal promoter, resulting in a shorter PCR product. **B.** Image of gel electrophoresis of PCR products using the primers shown in A. PCR was performed on cell lysis from HEK293T cells transfected with combinations of plasmids for Cre, the 203-bp spacer, post-excision control DIAL promoter, and VP16-ZF37. The first three lanes after the ladder are PCR products of conditions with 203-bp DIAL promoter and VP16-ZF37. The six following lanes are PCR products with control or 203-bp DIAL promoter, VP16-ZF37, and Cre. Samples are technical replicates (n=3). The condition with 203-bp DIAL promoter and Cre (middle 3 lanes) shows a shorter band at 476 bp which is the expected length of the PCR product from the post-excision promoter. **C.** Table of expected sizes of PCR product from post-excision control and different spacer length DIAL promoters, with and without Cre. According to our promoter design, DIAL promoters of all spacer lengths should have the same size PCR product for its post-excision state. **D.** Image of gel electrophoresis of PCR products using the primers shown in (A). PCR was performed on cell lysate from HEK293T cells transfected with combinations of plasmids for Cre, control or different spacer length DIAL promoters, and VP16-ZF37.

The left gel shows PCR products of conditions with control or different spacer length DIAL promoters regulating mGL with VP16-ZF37 and without Cre. The right gel shows PCR products of conditions with control or different spacer length DIAL promoters regulating mGL with both VP16-ZF37 and Cre. The PCR products in the right gel include a band around 476 bp, suggesting the emergence of a post-excision promoter state in the presence of Cre. **E.** HEK239T cells were lentivirally integrated with the 203-bp DIAL promoter regulating mGL and divergent iRFP670 gene at low MOI for low copy number. A polyclonal cell line was developed by sorting for iRFP670+ cells. At 0 dpt, cells were transfected with plasmids of Cre and mRuby2, or mRuby2 alone. At 3 dpt, cells were sorted for mRuby2+ and lysed. **F.** Schematic of PCR used to visualize changes to the state of the 203-bp DIAL promoter regulating mGL with divergent iRFP670. The primers bind to sequences upstream of the ZF binding sites or the mGL reporter gene. In the presence of Cre, the spacer excision shortens the distance between binding sites and the minimal promoter, resulting in a shorter PCR product. The table shows expected sizes of PCR products from the lentivirally integrated 203-bp DIAL promoter, with and without Cre. **G.** Image of gel electrophoresis of the PCR products using lysate and primers shown in E and F. The gel shows three different biological replicates with conditions with and without Cre. In the presence of Cre, a shorter band of 301 bp emerges indicating editing is taking place. However, the continued presence of the longer band at 538 bp indicates that spacer excision is not 100% efficient.



spacer length	binding site-to-promoter distance (bp)	
reporter	- *Cre*	+ *Cre*
control (0 bp)	79	79
27 bp	140	79
79 bp	192	79
155 bp	268	79
203 bp	316	79
263 bp	376	79

Figure S5. DIAL promoter system is tuned by spacer length and ZFa identity. A. Schematic of the pre- and post-excision molecular states of the DIAL promoter. The table summarizes the binding site-to-promoter distances for DIAL promoters with different spacer lengths, with and without Cre. **B.** Fold change and single cell distributions of the output reporter expressed from DIAL promoters with different spacer lengths transfected with ZFa (VP16-ZF43) on plasmids into HEK293T cells, with (dark green) or without (light green) of Cre. Fold change is the output mGL gmean fluorescence

intensity normalized to the condition without Cre within each spacer length. Histograms show decreasing pre-excision expression for increasing spacer length, which generates the larger fold change upon addition of Cre. **C.** Gmean fluorescence intensity of output mGL expressed from DIAL promoters and mCherry (co-expressed with ZFa VP16-ZF37) transfected on plasmids into HEK293T cells. Pre-excision expression decreases with increasing spacer length. In the presence of Cre, the output of the DIAL promoter converges to the output level of the control for all spacer lengths ($p>0.05$, ns). ZFa levels (proxied by mCherry gmean fluorescence intensity) are not affected by adding Cre nor increasing spacer length. **D.** Gmean fluorescence intensity of output mGL expressed from DIAL promoters and TagBFP (co-expressed with ZFa VP16-ZF43) transfected on plasmids into HEK293T cells. Pre-excision expression decreases with increasing spacer length. In the presence of Cre, the output of the DIAL promoter converges to the output level of the control for all spacer lengths ($p>0.05$, ns). ZFa levels (proxied by TagBFP gmean fluorescence intensity) are not affected by adding Cre nor increasing spacer length. **E.** Gmean fluorescence intensity of output mGL expressed from the 203-bp DIAL promoter transfected on plasmids into HEK293T cells with different zinc finger activators bearing different transactivation domains (ZF-TADs or ZFas), with or without Cre. Fold change is the output mGL gmean fluorescence intensity normalized to the condition without Cre within each ZF-TAD. Expression setpoints and fold changes increase slightly with stronger ZF-TAD. **F.** Gmean fluorescence intensity of output mGL expressed from the post-excision control DIAL promoter transfected on plasmids into HEK293T cells with different zinc finger activators bearing different transactivation domains (ZF-TADs), and with or without Cre. Expression setpoints increase slightly with stronger ZF-TAD. Presence of Cre decreases expression. All units for fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$.

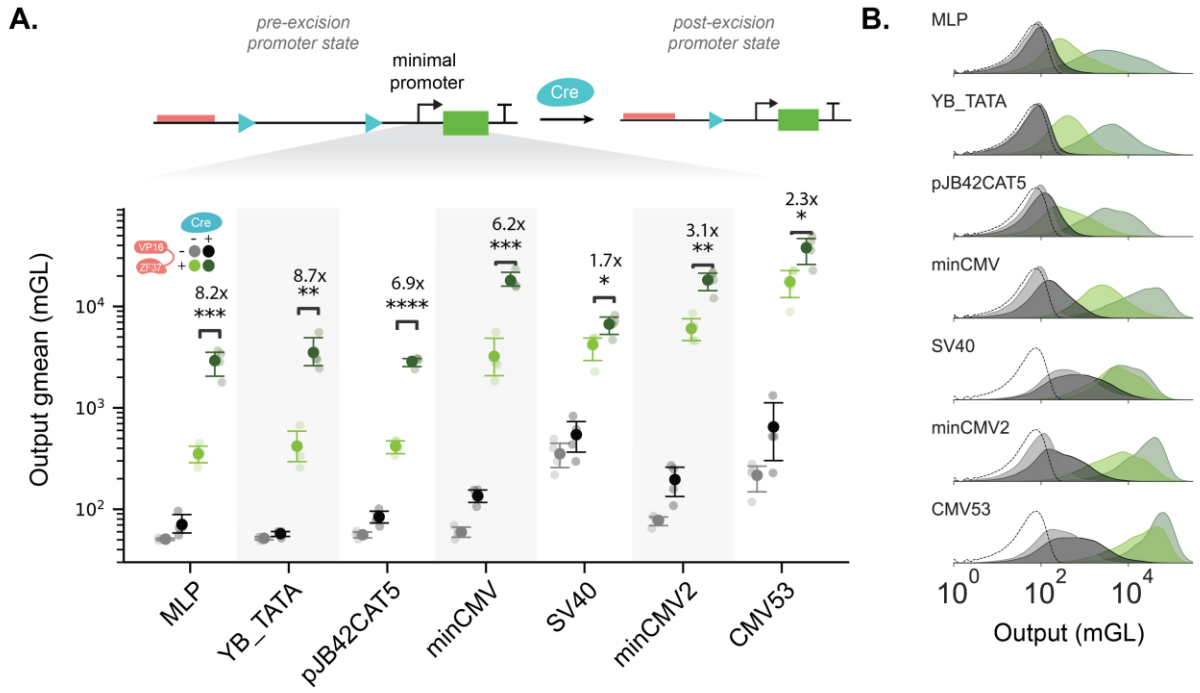


Figure S6. Minimal promoter choice tunes DIAL output expression. | A. Gmean fluorescence intensity of output mGL from 203-bp DIAL promoters with different minimal promoters. Constructs were co-transfected on plasmids into HEK293T cells with or without ZFa (VP16-ZF37) and Cre. Fold change annotated is the output mGL gmean fluorescence intensity normalized to the condition without Cre and with ZFa within each minimal promoter. The choice of minimal promoter influences the levels of pre- and post- excision expression, fold change, and basal activity. **B.** Single-cell distributions of output mGL expressed from the 203-bp DIAL promoters with different promoters as according to the legend in A. Dotted histogram represents untransfected cells. The choice of minimal promoter also influences the shape of the single cell distributions. All units forfluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=4). Histograms represent single-cell distributions sampled across bioreplicates (n=4). Statistical significance was calculated with two sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01; *** p<0.001.

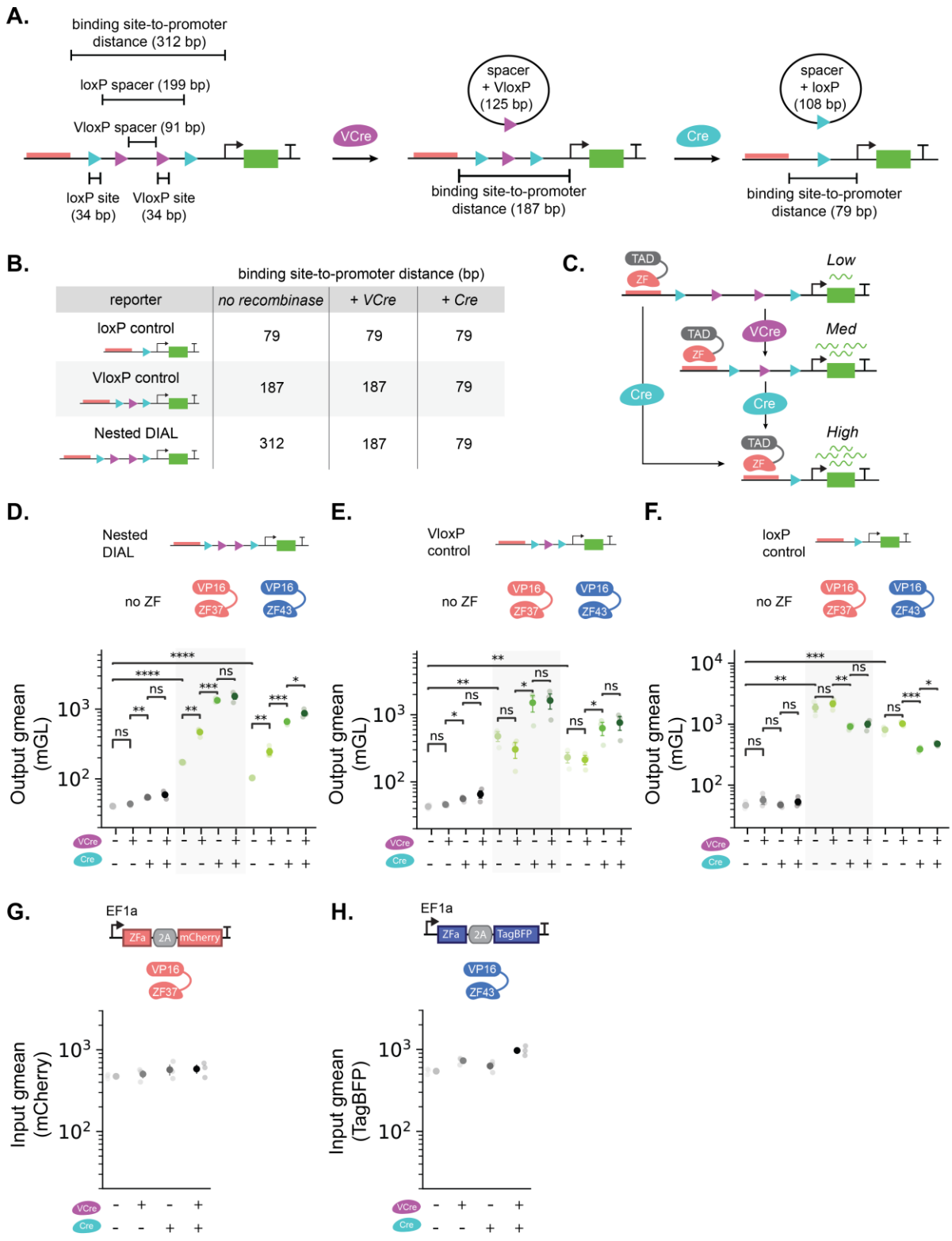


Figure S7. The nested DIAL promoter generates three promoter states and four states of expression. | A. Schematic of the pre- and post-excision states of the nested DIAL promoter. VloxP and loxP spacers are defined as the distance between the VloxP and loxP recognition sites, respectively. The binding site-to-promoter distance is defined as the total distance between the last binding site and the minimal promoter. VCre and Cre excise a VloxP and loxP site along with the spacer, respectively. The nested DIAL promoter has three possible promoter states. **B.** The table summarizes the binding site-to-promoter distances for the loxP control, VloxP control, and nested DIAL promoters,

with or without VCre and Cre. The VloxP and loxP control statically encodes the post-excision promoter state after adding VCre and Cre, respectively. Adding VCre to VloxP control and adding Cre to loxP control does not edit the promoter, not changing the binding site-to-promoter distance. **C.** Schematic of nested spacer achieving three different expression setpoints (excluding “off” state without ZFa). In the presence of ZFa, the nested spacer can go through all three promoter states and corresponding setpoints by sequentially adding VCre then Cre or skip directly to the “high” setpoint by adding Cre. (D-F) Gmean fluorescence intensity of output mGL expressed from the nested, VloxP control, and loxP control DIAL promoters transfected on plasmids into HEK293T cells with and without ZFa (VP16-ZF37 or VP16-ZF43) or Cre. In the absence of ZFa, the outputs of the DIAL promoter are negligible and not substantially different. **D.** In the presence of ZFa and VCre, the output of the nested DIAL promoter increases and converges to the output level of the VloxP control DIAL promoter shown in E. In the presence of ZFa and Cre, the output of the nested DIAL promoter increases converges to the output level of the loxP control DIAL promoter shown in F. **E.** In the presence of ZFa, adding VCre slightly reduces the output of the VloxP control DIAL promoter. Adding Cre increases the output, converging to the output level of the loxP control shown in F. **F.** In the presence of ZFa, adding Cre slightly reduces the output of the loxP control DIAL promoter, consistent with previous data. This decrease is not observed with the addition of VCre. **G.** Gmean fluorescence intensity of mCherry co-expressed with ZFa (VP16-ZF37) transfected on plasmids into HEK293T cells along with the nested DIAL promoter with or without Cre or VCre. The presence of recombinase does not substantially affect ZFa levels. **H.** Gmean fluorescence intensity of TagBFP co-expressed with ZFa (VP16-ZF43) transfected on plasmids into HEK293T cells along with the nested DIAL promoter with or without Cre or VCre. The presence of recombinase does not substantially affect ZFa levels. All units for geometric mean fluorescence intensity are arbitrary units (a.u.). Large markers represent the mean of biological replicates with span indicating standard error (n=3). Statistical significance was calculated with two-sided Student’s *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$; **** $p<0.0001$.

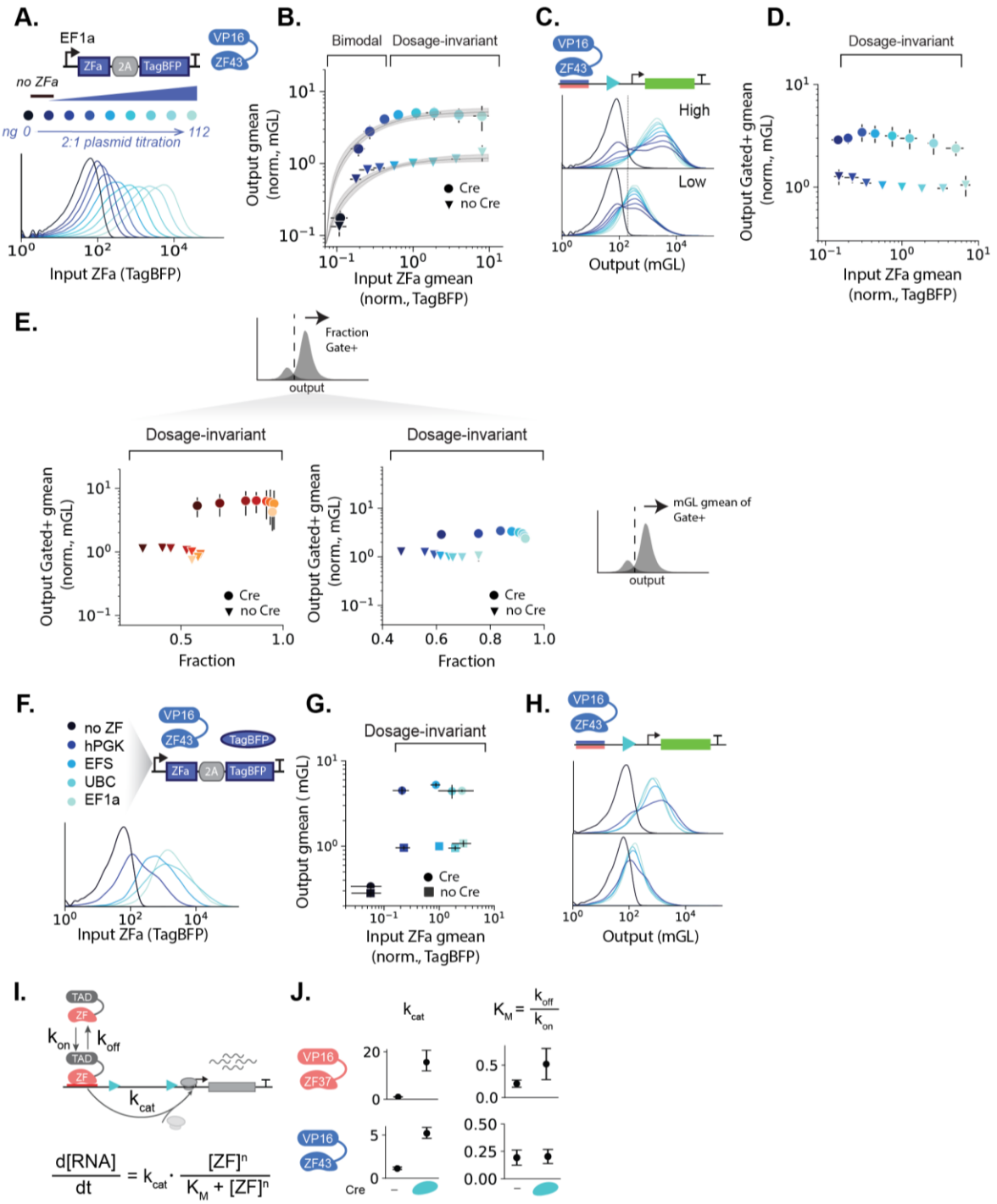


Figure S8. DIAL robustly generates expression setpoints with VP16-ZF43. | A. Single-cell distributions of TagBFP coexpressed with ZFa (VP16-ZF43) transfected on plasmids into HEK293T cells with 203-bp DIAL promoter regulating mGL. Conditions with and without Cre are combined. Schematic of plasmid titration of ZFa. **B**. Output reporter mGL gmean fluorescence intensity expressed from 203-bp DIAL promoter versus input ZFa (VP16-ZF43) gmean fluorescence intensity (proxied by co-expressed TagBFP) for ZFa titration shown in A with and without Cre. Values are normalized to the condition without Cre with 0.125x ZFa (14 ng of ZFa). Overlaid lines represent model fit with 95% confidence interval. The output initially increases at low ZFa levels, where the distribution is bimodal, followed by a dosage-invariant regime. **C.** Single cell distributions of output mGL expressed from the 203-bp DIAL promoter with titrated ZFa (VP16-ZF43) on plasmids transfected into HEK293T cells with and without Cre as described in A and B.

Gate drawn (mGL-A>200) to isolate populations with high output levels of the DIAL promoter. In the presence of Cre, the output mGL increases. At low levels of ZFa, output is bimodal. **D.** Output reporter mGL gmean fluorescence intensity (gated+ by the line in C) versus input ZFa gmean fluorescence intensity (proxied by co-expressed mCherry, as shown in A not gated+ for mGL) for ZFa titration shown in A and B with and without Cre. Values are normalized to the condition without Cre with 0.125x ZFa (14 ng of ZFa). Once gated, the reporter output is dosage-invariant throughout the ZFa plasmid titration. **E.** Output reporter mGL gmean fluorescence intensity of reporter expressing cells from the ZFa (left: VP16-ZF37 or right: VP16-ZF43) titration (gated by the line in C or Figure 3B) versus fraction of cells expressing mGL (above gate) transfected in HEK293T cells as described in A-C and Figure 3B-D. Colors represent ZFa titration amount (A or Figure 3B). The output gmean fluorescence intensity is dosage invariant to ZFa plasmid titration. The fraction above the mGL-gate correlates with ZFa level. The fraction has no effect on output level of the cells expressing mGL from the DIAL promoter. **F.** Input TagBFP (proxy for ZFa VP16-ZF43) single-cell distributions as encoded with promoters of varying strengths on plasmids in transfected HEK293T cells with 203-bp DIAL promoter regulating mGL. Conditions with and without Cre are combined. **G.** Output mGL gmean fluorescence intensity expressed from 203-bp DIAL promoter versus input ZFa (VP16-ZF43) gmean fluorescence intensity (proxied by co-expressed mCherry) expressed from different strength promoters transfected on plasmids into HEK293T cells with or without Cre. Values are normalized to the condition without Cre with EFS promoter. Colored according to legend in F. **H.** Single-cell distributions of output mGL expressed from 203-bp DIAL promoter when using different promoters to control ZFa transfected on plasmids into HEK293T cells, with and without Cre. Conditions correspond to G and are colored according to legend in F. **I.** Schematic of two-state transcriptional model of promoter activation. Binding of the ZFa and transcriptional activation is modeled as a single step. Model and full reactions for fitting ZFa plasmid titration data is described in the Methods. **J.** Model parameters after fitting ZFa plasmid titration data for ZFas VP16-ZF37 and VP16-ZF43, as shown in Supplementary Table 3 and 4. The binding affinity, K_M, of ZFa does not change upon editing whereas the putative rate of transcriptional activation, k_{cat}, increases upon editing. All units for fluorescence intensity are arbitrary units (a.u.). For experimental data (B, D, E, G), large markers represent the mean of biological replicates with span indicating standard deviation (n=5 for VP16-ZF43, n≥4 for VP16-ZF37). Histograms represent single-cell distributions sampled across bioreplicates.

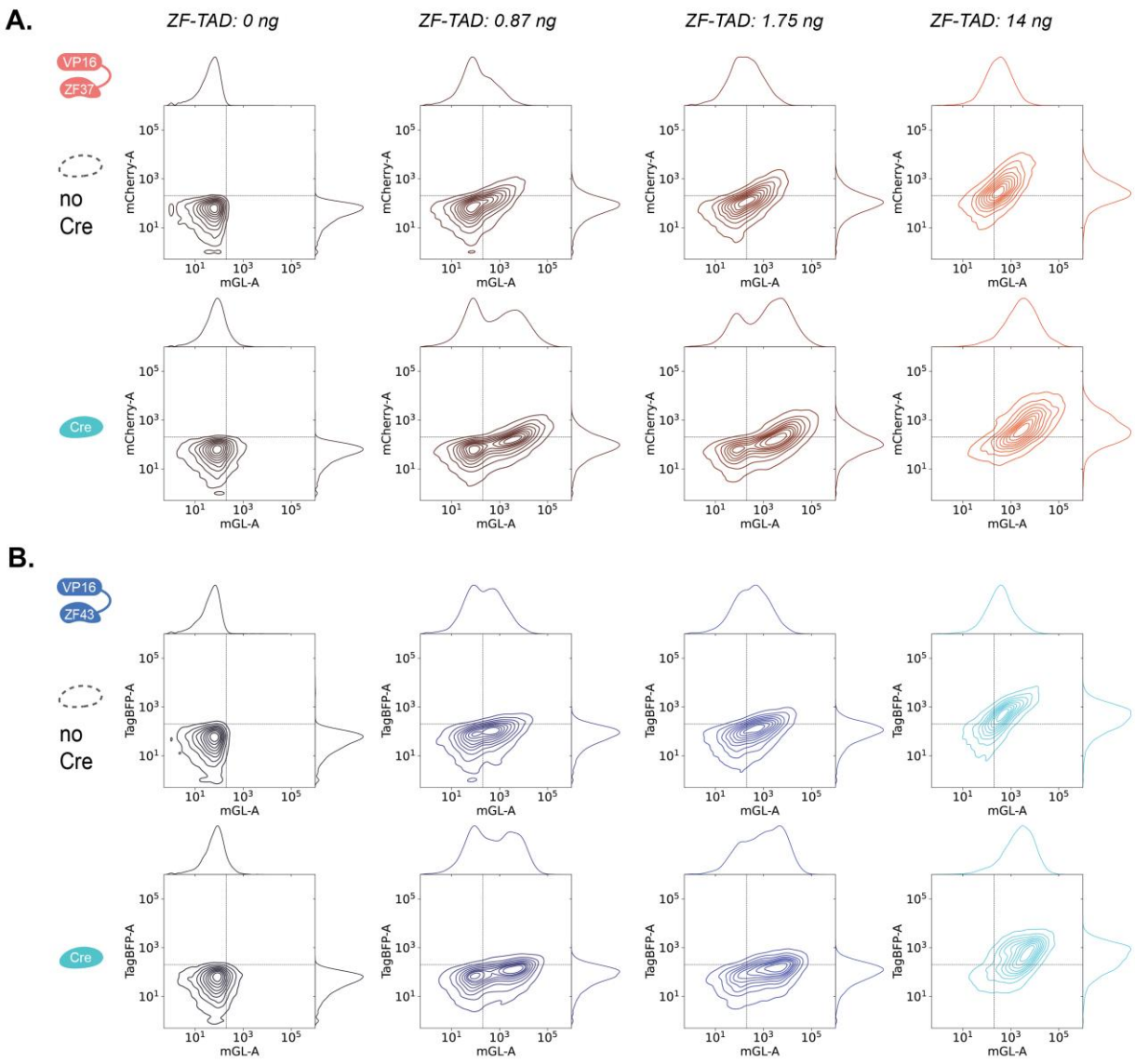


Figure S9. Single-cell joint distributions for titration of ZFa plasmid. | Single cell histograms and joint contour plots of output mGL expressed from the 203-bp DIAL promoter with titrated plasmid amounts of ZF-TAD (**A:** VP16-ZF37 or **B**: VP16-ZF43, proxied by co-expressed mCherry or TagBFP respectively) transfected on plasmids into HEK293T cells with or without Cre. Histograms and joint contour plots represent single-cell distributions sampled across bioreplicates (n≥4 for VP16-ZF37, and n=5 for VP16-ZF43). At low levels of the ZFa (ZF-TAD), output mGL is bimodal. In the bimodal distributions, the cells not expressing the mGL output reporter correspond to the lowest levels of mCherry or TagBFP similar to the condition with no ZFa, suggesting a threshold of ZFa is needed to induce expression from the DIAL promoter.

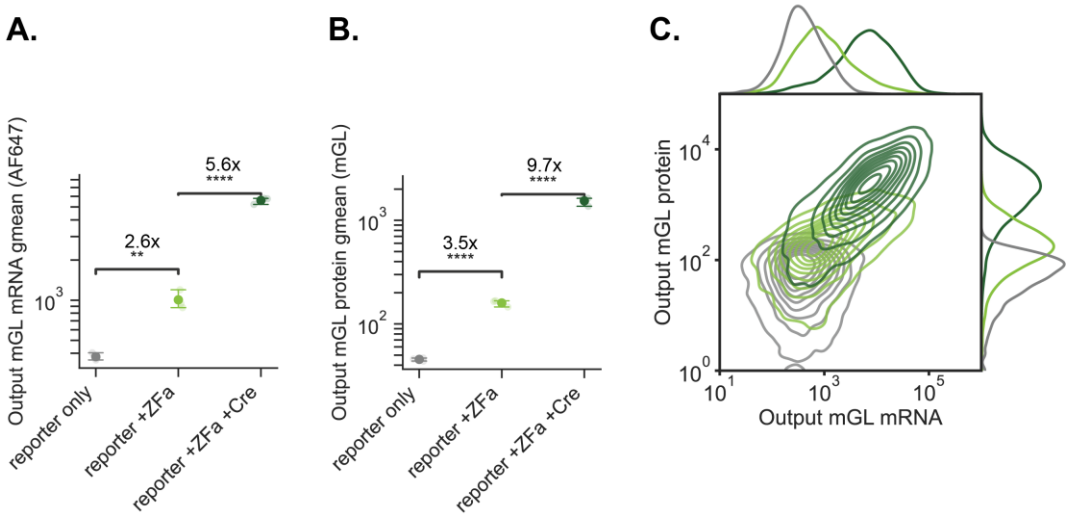


Figure S10. RNA FISH shows a shift in transcript levels with DNA editing. | A-B. Output mGL gmean fluorescence intensity of mRNA and protein from 203-bp DIAL promoter transfected into HEK293T cells with and without ZFa (VP16-ZF37) and Cre. Cells underwent hybridization chain reaction Flow-FISH to label mRNA using AF647. Fold change increase is labeled as normalized to the lower gmean between conditions**. C.** Single cell distributions and joint contour plots of output mGL mRNA and protein according to conditions and legend in A-B. All units for fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01; *** p<0.001; **** p<0.0001.

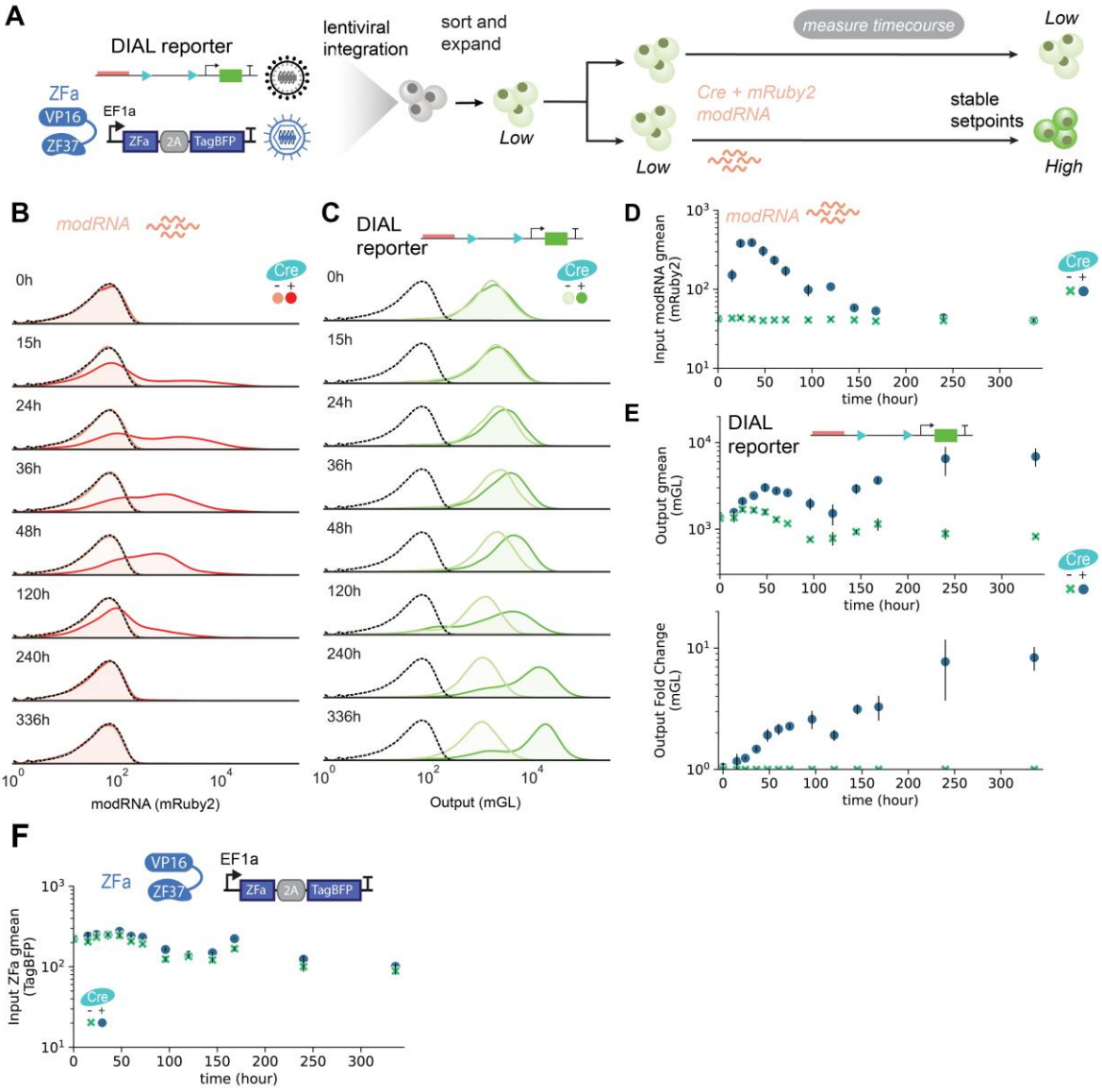


Figure S11. Transient expression of Cre stably shifts output setpoint from DIAL reporter. | A. Steps of establishing a polyclonal HEK293T line to demonstrate long term stability of DIAL setpoints from transient Cre expression. Following delivery of lentiviruses of ZFa (VP16-ZF37-2A-tagBFP) and 203-bp DIAL promoter regulating mGL, cells were expanded and sorted for TagBFP+ and mGL+. For the conditions with +Cre, Cre modRNA and mRuby2-2A-PuroR modRNA were co-delivered at 0 hours. Cells were not passaged. Measurements were taken at the various timepoints for conditions with and without Cre. **B.** Input mRuby2 (expressed via transfected modRNA, proxy for co-delivered Cre modRNA) single cell distributions over time for the polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL and VP16-ZF37-2A-TagBFP. Conditions are with and without modRNA as described in A. **C.** Output reporter mGL single cell distributions for the polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL and VP16-ZF37-2A-TagBFP, with conditions with and without modRNA as described in A. **D.** Input mRuby2 (expressed via transfected modRNA, proxy for co-delivered Cre modRNA) gmean fluorescence intensity over time for the polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL and VP16-ZF37-2A-TagBFP, with conditions with and without modRNA as described in A. **E.** Output reporter mGL gmean fluorescence intensity and fold change for the polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL and VP16-ZF37-2A-TagBFP, with conditions with and without modRNA as described in A. Fold change is the output mGL gmean fluorescence intensity normalized to the condition without modRNA Cre within each timepoint. **F.** Input ZFa (proxied by TagBFP) gmean fluorescence intensity from polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL and VP16-ZF37-2A-TagBFP, with conditions with and without modRNA as described in A. All units for fluorescence intensity are arbitrary

units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard deviation (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3).

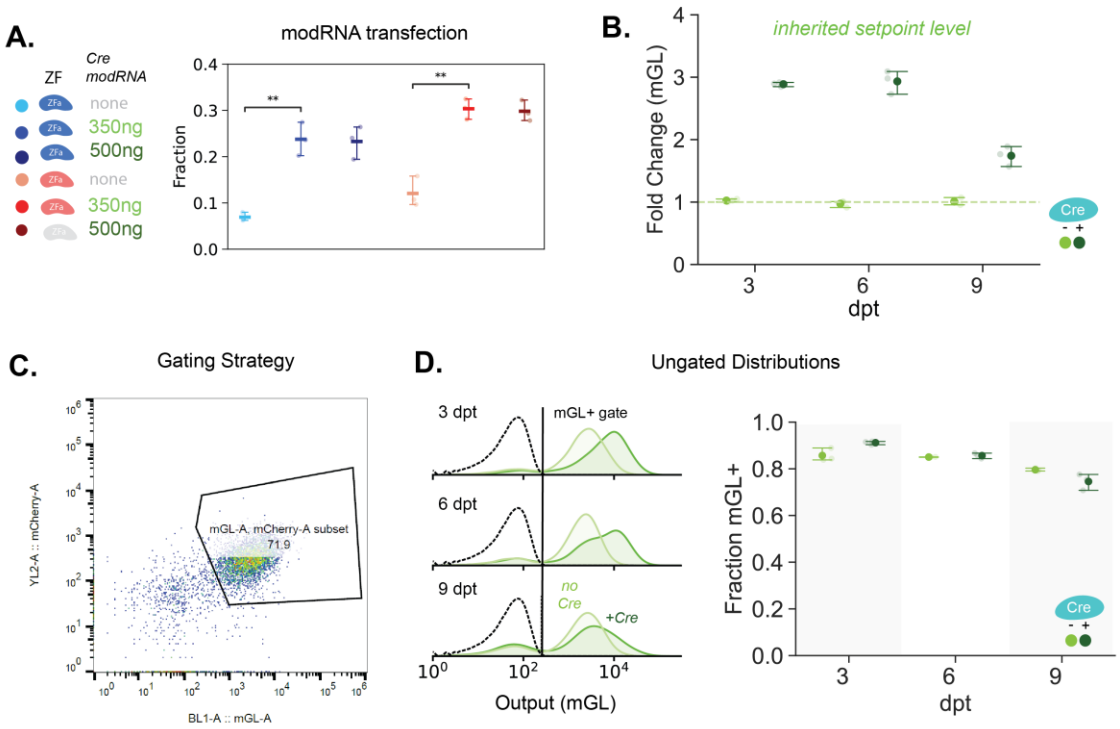


Figure S12. DIAL transmits transient inputs into heritable states. | A. Fraction of output mGL (above a gate based on the condition without Cre) from the 203-bp DIAL promoter with ZFa (blue: VP16-ZF43 or red: VP16-ZF37) transfected on plasmids into HEK293T cells. Cre modRNA (0, 350, or 500 ng) was transfected at 1 dpt. **B.** Fold change of the output mGL expressed from the 203-bp DIAL promoter integrated with ZFa into HEK293T cells, co-transfected with Cre modRNA and TagBFP modRNA on day 0, as in Fig 3D. Fold change is the output mGL gmean fluorescence intensity normalized to the condition without Cre at each time point. **C.** Representative gating strategy for mGL+ and mCherry+ of a polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL and VP16-ZF37-2A-mCherry, as shown in Fig 3B-E. **D.** Output reporter mGL ungated single cell distributions and fraction mGL+ over multiple passages measured on 3, 6, and 9 dpt from polyclonal line with 203-bp DIAL promoter regulating mGL and VP16-ZF37-2A-mCherry, as shown in Fig 3B-E. Conditions are with (dark green) and without (light green) Cre modRNA. Vertical line is the gate for mGL+. The dotted curve is a representative single-cell distribution of unstained HEK293T cells with no reporter or ZFa integrated.All units for output geometric mean fluorescence intensity, and single cell distributions are arbitrary units (a.u.). Fold change and fraction positive is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01.

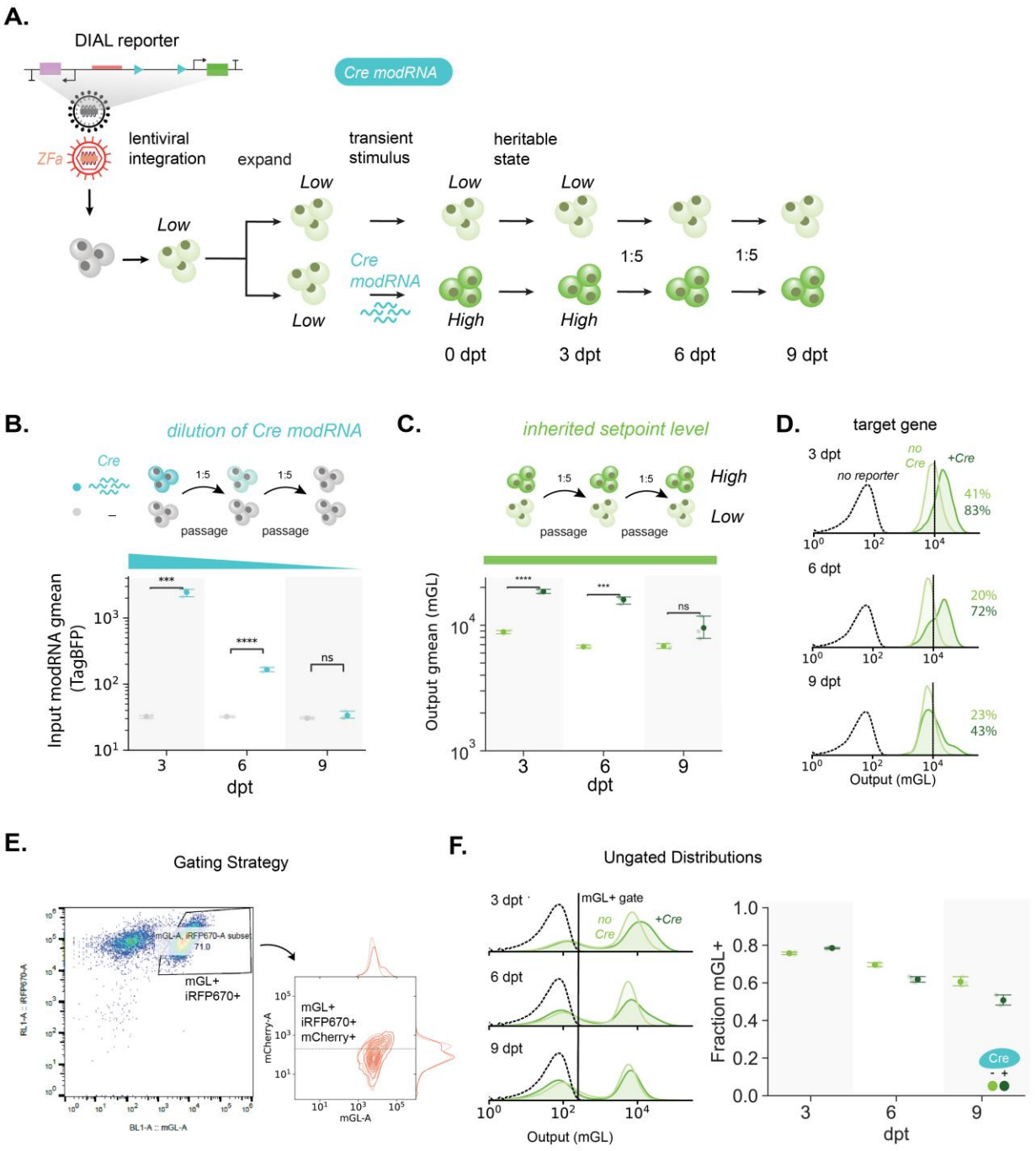


Figure S13. DIAL transmits inputs into heritable states when delivered via lentivirus encoded with a divergently expressed gene. | A. Steps of establishing a polyclonal HEK293T line to demonstrate heritability of DIAL setpoints. Following delivery of lentiviruses of ZFa (VP16-ZF37-2A-mCherry) and 203-bp DIAL promoter with divergent reporter iRFP670, sorting (iRFP670+ and mGL+), and expansion, transfection of modRNA Cre at 0 dpt sets heritable setpoints of expression level over multiple passages as indicated. **B-F**. Heritable setpoint results for a polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL with divergent EF1a-iRFP670-WPRE and lentivirally integrated VP16-ZF37-2A-mCherry. Prior to Cre modRNA delivery, cells were sorted for iRFP670+ and mGL+. Cre and TagBFP modRNA were delivered at 0 dpt. **B.** TagBFP (expressed via transfected modRNA, proxy for co-delivered Cre modRNA) gmean fluorescence intensity over multiple passages measured on 3, 6, and 9 dpt. Conditions are with and without co-transfected TagBFP modRNA and Cre modRNA added at 0 dpt. Results are for cells gated for iRFP670+, mGL+, and mCherry+. Protein expressed from the transfected modRNA dilutes or degrades

to background levels after multiple passages by 9 dpt. **C**. Output reporter mGL gmean fluorescence intensity from 203-bp DIAL promoter over multiple passages measured on 3, 6, and 9 dpt. Conditions are with and without co-transfected TagBFP modRNA and Cre modRNA added at 0 dpt. Cells are gated for iRFP670+, mGL+, and mCherry+. The difference in mGL setpoint level between conditions persists across multiple passages. **D**. Output reporter mGL single-cell distributions over multiple passages measured on 3, 6, and 9 dpt. Conditions are with (dark green) and without (light green) co-transfected TagBFP and Cre modRNA at 0 dpt. Cells are gated for iRFP670+, mGL+, and mCherry+. The dotted curve is a representative single-cell distribution of unstained HEK293T cells with no reporter or ZFa integrated. The vertical line represents a gate to isolate the lower peak and the higher peak. The percentages indicate the fraction of cells above the gate. **E.** Gating strategy for iRFP670+, mGL+, and mCherry+ for polyclonal lines with lentivirally integrated 203-bp DIAL promoter regulating mGL with divergent EF1a-iRFP670-WPRE and lentivirally integrated VP16-ZF37-2A-mCherry**. F.** Output reporter mGL ungated single cell distributions and fraction mGL+ from 203-bp DIAL promoter over multiple passages measured on 3, 6, and 9 dpt. Conditions are with (dark green) or without (light green) modRNA Cre. The dotted curve is a representative single-cell distribution of unstained HEK293T cells with no reporter or ZFa integrated. All units for geometric mean fluorescence intensity and single cell distributions of fluorescence intensity are arbitrary units (a.u.). Fraction of positive cells (e.g. mGL+) is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$.

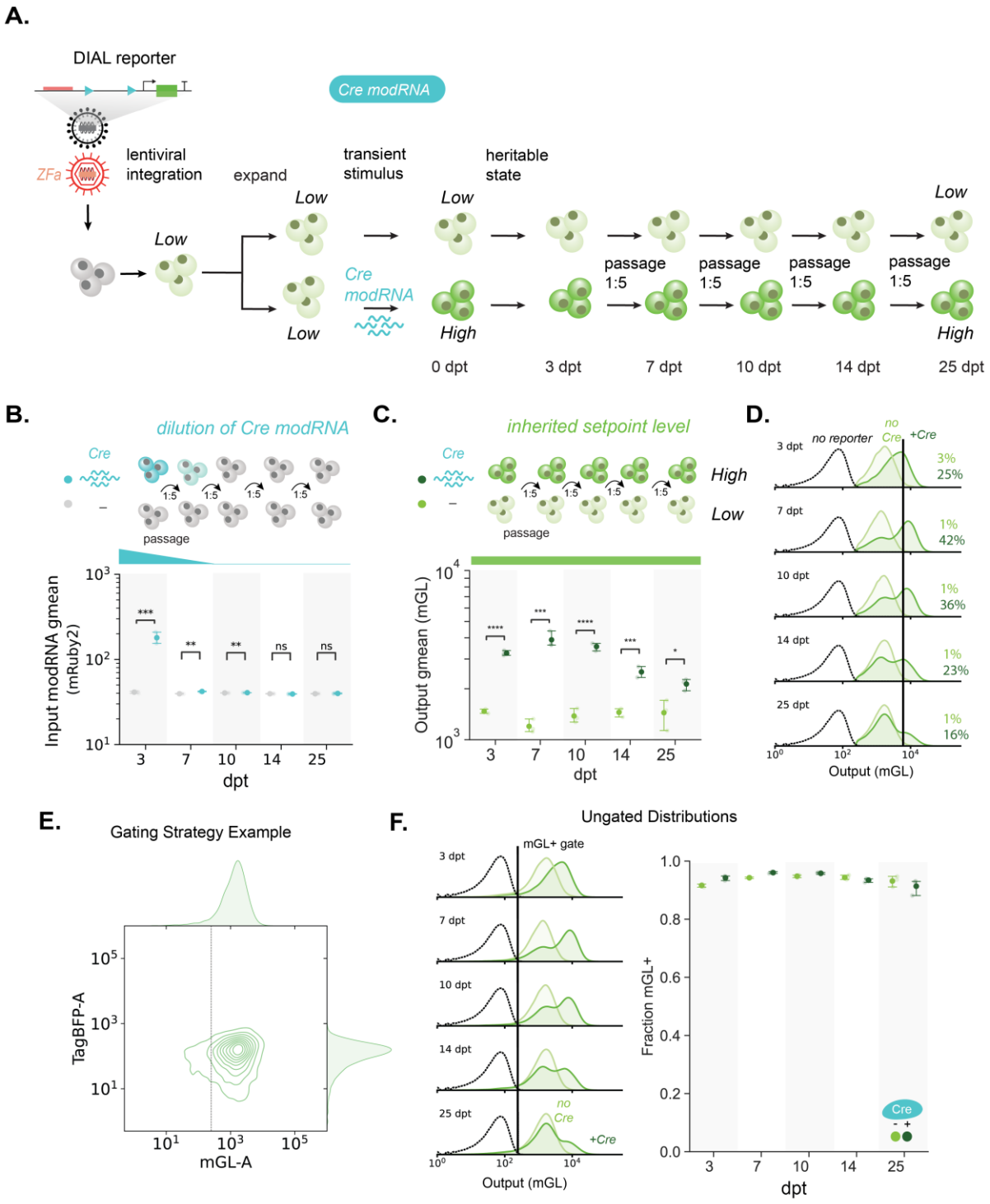


Figure S14: DIAL transmits inputs into heritable states of DIAL reporter over four passages. | A. Steps of establishing a polyclonal HEK293T line to demonstrate heritability of DIAL setpoints. Following delivery of lentiviruses of ZFa and 203-bp DIAL promoter and expansion, transfection of Cre modRNA sets heritable setpoints of expression level over multiple passages as indicated**. B-F.** Heritable setpoint results for a polyclonal HEK293T cell line with lentivirally integrated 203-bp DIAL promoter regulating mGL and lentivirally integrated VP16-ZF37-2A-TagBFP-BGH, following schematic in (A). Prior to Cre modRNA delivery, cells were sorted for TagBFP+ and mGL+. Cre and mRuby2-P2A-PuroR modRNA were delivered at 0 dpt. **B.** mRuby2 (expressed via transfected modRNA, proxy for co-delivered Cre modRNA) gmean fluorescence intensity over multiple passages measured on 3, 6, and 9 dpt Conditions are with

and without co-transfected mRuby2 modRNA and Cre modRNA added at 0 dpt. Results are for cells gated for mGL+. Protein expressed from the transfected modRNA dilutes or degrades to background levels after multiple passages by 7 dpt. **C.** Output reporter mGL gmean fluorescence intensity from 203-bp DIAL promoter over multiple passages measured on 3, 7, 10, 14, and 25 dpt. Conditions are with and without co-transfected mRuby2 modRNA and Cre modRNA added at 0 dpt. Cells are gated for mGL+. The difference in mGL setpoint level between conditions persists across multiple passages. **D.** Output reporter mGL single-cell distributions over multiple passages measured on 3, 7, 10, 14, and 25 dpt. Conditions are with (dark green) and without (light green) co-transfected mRuby2-P2A-PuroR and Cre modRNAs at 0 dpt. Cells are gated for mGL+. The dotted curve is a representative single-cell distribution of unstained HEK293T cells with no reporter or ZFa integrated. The vertical line represents a gate to isolate the lower peak and the higher peak. The percentages indicate the fraction of cells above the gate for the conditions with (dark green) and without (light green) Cre modRNA. **E.** Gating strategy for mGL+ for polyclonal lines with lentivirally integrated 203-bp DIAL promoter regulating mGL and lentivirally integrated VP16-ZF37-2A-TagBFP. **F.** Output reporter mGL ungated single cell distributions and fraction mGL+ from 203-bp DIAL promoter over multiple passages measured on 3, 7, 10, 14, and 25 dpt. Conditions are with (dark green) or without (light green) modRNA Cre. The dotted curve is a representative single-cell distribution of unstained HEK293T cells with no reporter or ZFa integrated. All units for fluorescence intensity are arbitrary units (a.u.). Fraction+ is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$.

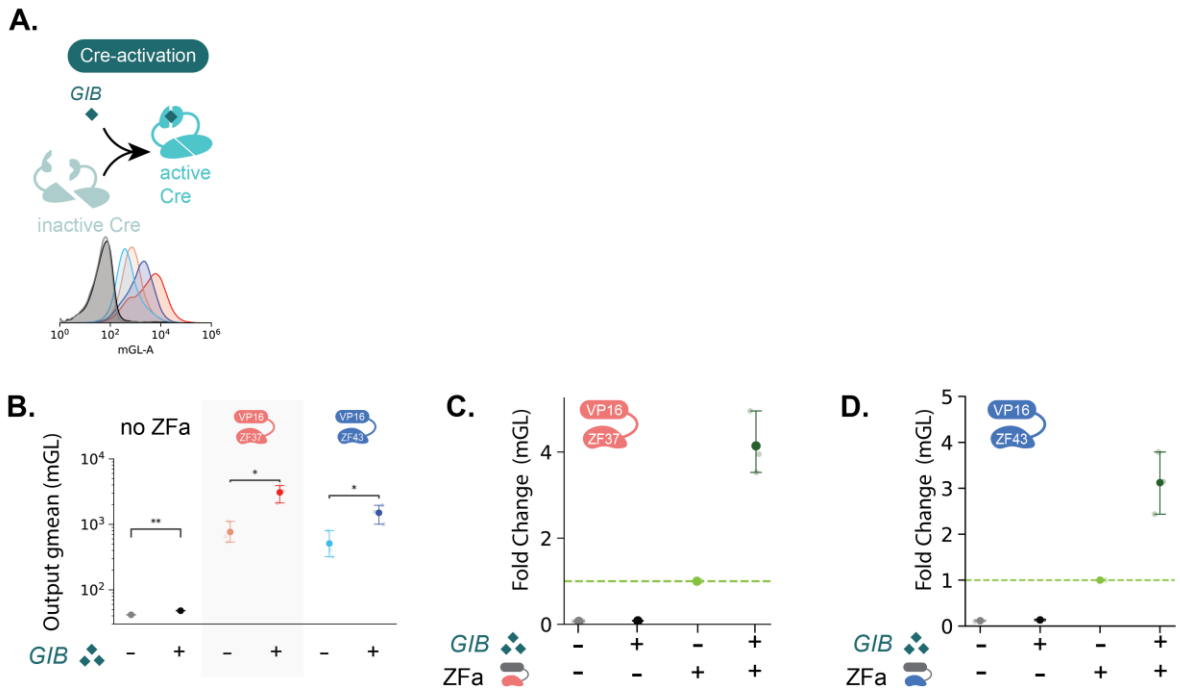


Figure S15: Small molecule inducible Cre can change setpoint level of DIAL reporter. | A. Schematic of gibberellin inducible split Cre. Output mGL single cell distributions from 203-bp DIAL promoter transfected with gibberellin inducible Cre on plasmids into HEK293T cells. Colors represent co-transfected ZFa and GIB according to legend in B. **B.** Output mGL gmean fluorescence intensity from the 203-bp DIAL promoter cotransfected with or without ZFa, and with gibberellin-inducible split Cre transfected on plasmids into HEK293T cells. Gibberellin (GIB) induces Cre activity. **C-D.** Output mGL fold change from the 203-bp DIAL promoter cotransfected with ZFa (C. VP16-ZF37; D. VP16-ZF43) and gibberellin-inducible split Cre transfected on plasmids into HEK293T cells. Fold change is gmean fluorescence intensity normalized to the conditions with ZFa and without GIB. All units for geometric mean fluorescence intensity and single cell distributions of fluorescence intensity are arbitrary units (a.u.). Fold change and fraction+ is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01.

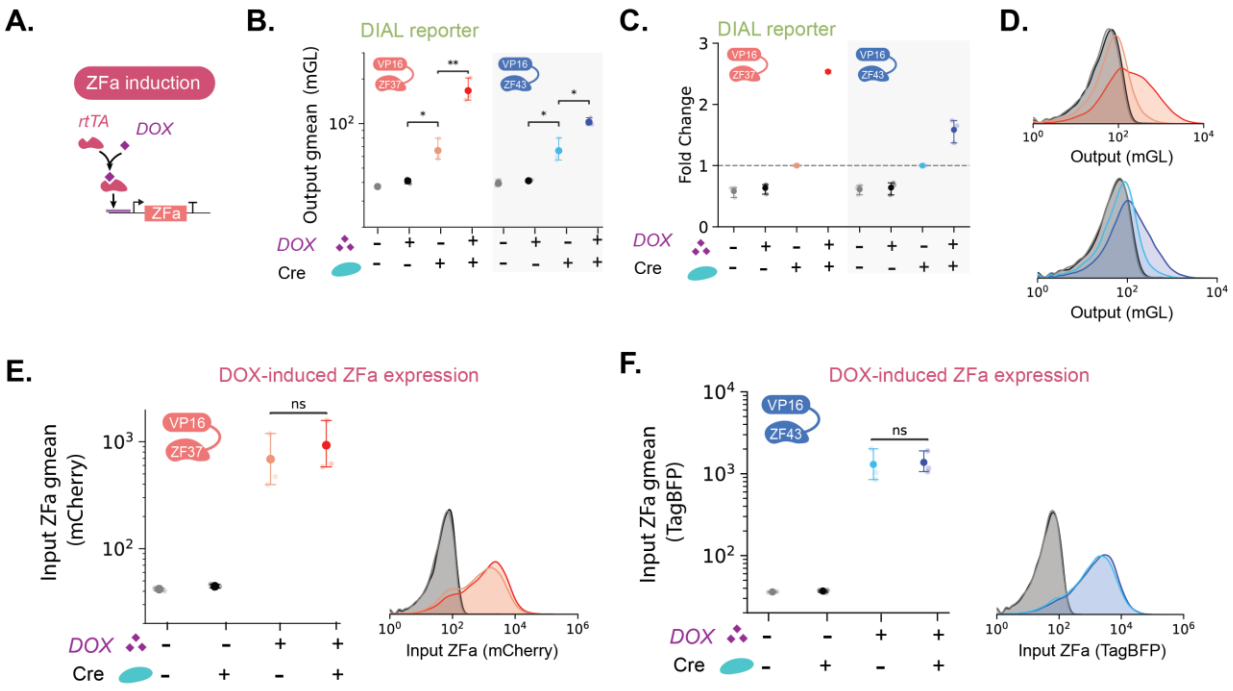


Figure S16. Small molecule inducible ZFa controls ON/OFF of DIAL reporter. | A. DOX-inducible ZFa is encoded downstream of a TET promoter. When rtTA is co-expressed, the presence of DOX will induce expression of the ZFa. The ZFa cassettes used were either VP16-ZF37-2A-mCherry or VP16-ZF43-2A-TagBFP. **B-D.** Output mGL gmean fluorescence intensity (B), fold change (C), and, single cell distributions (D) from 203-bp DIAL promoter transfected with DOX-inducible ZFa rtTA, and Cre (-/+) on plasmids into HEK293T cells. In the plots (left), the first four points are with VP16-ZF37, and the next four points are with VP16-ZF43. Distribution colors in D are according to the DOX and Cre combinations for each ZFa as shown in B-C. Fold change is gmean fluorescence intensity normalized to the +DOX, -Cre condition within each ZFa. **E-F.** Input ZFa gmean fluorescence intensity and single cell distributions as proxied by mCherry (VP16-ZF37) or TagBFP (VP16-ZF43) from DOX-inducible TET constructs transfected on plasmids with 203-bp DIAL promoter regulating mGL, rtTA, and Cre (-/+) into HEK239T cells. Distributions colors are according to the DOX and Cre combinations for each ZFa plot. All units for geometric mean fluorescence intensity and single cell distributions of fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01.

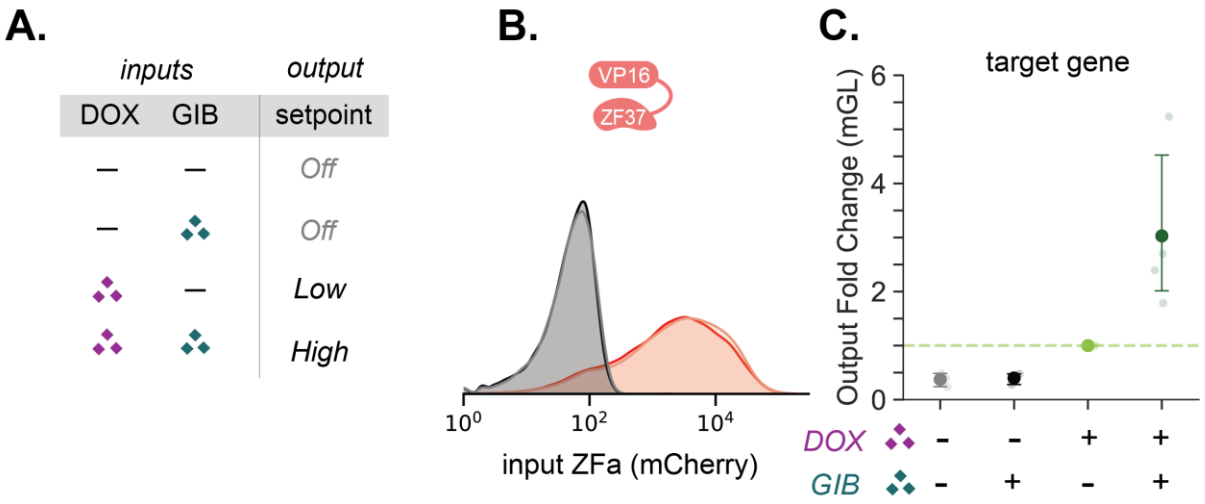


Figure S17: Supplemental data for dual control of ZFa and Cre with small molecules to control DIAL reporter output setpoint level.| A. Logic table for output expression from DIAL promoter with DOX-inducible ZFa and GIB-inducible Cre from a single spacer DIAL promoter via different combinations of DOX and GIB. **B.** Input ZFa single cell distributions as proxied by mCherry (VP16-ZF37) from DOX-inducible TET constructs transfected on plasmids with 203-bp DIAL promoter regulating mGL, rtTA, and GIB-inducible split Cre into HEK239T cells. Distribution colors are according to the DOX and GIB combinations, (-DOX/-GIB: gray, -DOX/+GIB: black, +DOX/-GIB: light red, +DOX/+GIB: dark red). **C.** Output mGL fold change from 203-bp DIAL promoter transfected with DOX-inducible ZFa rtTA, and GIB-inducible split Cre on plasmids into HEK293T cells with various combinations of GIB and DOX. Fold change is gmean fluorescence intensity normalized to the +DOX, -GIB condition. All units for geometric mean fluorescence intensity and single cell distributions of fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3).

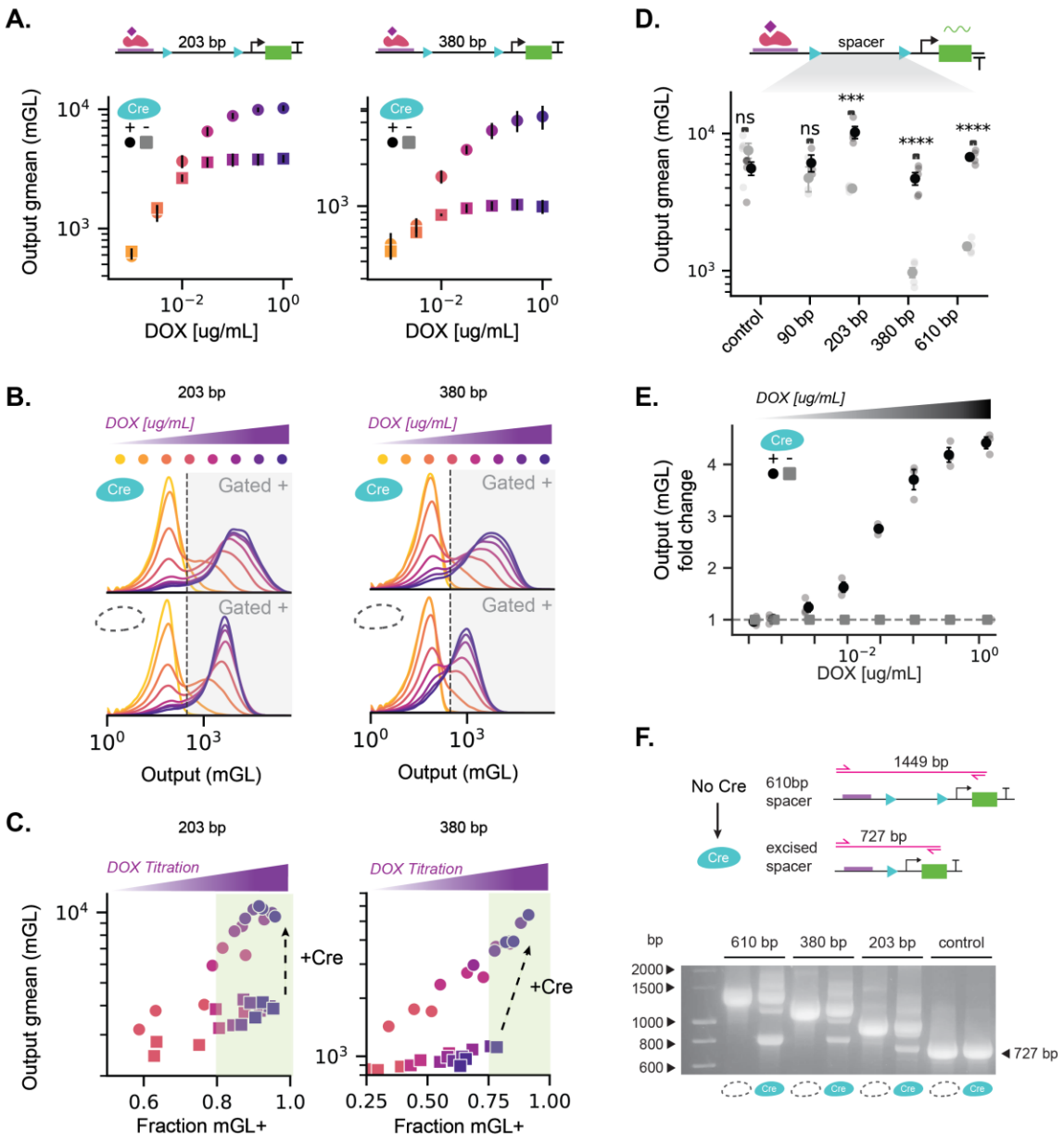


Figure S18. TET-DIAL enables small molecule control of defined setpoints for multiple spacer lengths. | **A.** Output reporter mGL gmean fluorescence intensity from 203-bp (n=3) or 380-bp (n=4) TET-DIAL promoter transfected with rtTA on plasmids into HEK293T cells with or without Cre versus input DOX concentration. DOX was induced at 1 dpt. **B.** Single-cell distributions of output mGL expressed from 203-bp (n=3) or 380-bp (n=4) TET-DIAL promoter with loxP sites on plasmids transfected with rtTA into HEK293T cells, with varying concentrations of DOX and with or without Cre. Colored according to A, with lightest yellow indicating no DOX added. Gates are drawn to isolate cells above the background (no DOX) condition. In the presence of Cre, the output mGL increases. At low concentrations of DOX, output is bimodal. **C.** Output reporter mGL gmean fluorescence intensity of reporter expressing cells (gated as shown in B) versus fraction of cells expressing mGL (above gate in B) from transfection of HEK293Ts cells as described in B. Colored according to A. Adding Cre induces a new setpoints of reporter expression that can be varied along with fraction of expressing cells by changing DOX concentrations. To set unimodal expression levels (off, low, and high) with high fraction positive in the "ON" state (green region), presence of DOX at high concentration can control whether expression is "OFF" or "ON", and presence of Cre can control the level of "ON" expression. Points represent individual bioreplicates (n=3 for 203-bp, n=4 for 380-bp)…**continued to next page.**

D. Gmean fluorescence intensity of mGL expressed from TET-DIAL promoters of varying spacer lengths transfected with rtTA on plasmids into HEK293T cells, with or without Cre and with DOX (1 µg/mL) (n=3 for 90-bp; n=4 for 203-bp and 610-bp; n=5 for 380-bp and control). 90 bp TET-DIAL promoter uses VloxP sites, and all others use loxP sites. **E.** Fold changes of output reporter mGL expression for the 610-bp TET-DIAL promoter versus DOX concentrations for DOX titration shown in Fig 4C. Fold changes are gmean fluorescence intensity normalized to the -Cre condition at each DOX concentration. (n=3)**. F.** PCR used to visualize changes to the molecular state of the TET-DIAL promoter, labeled with expected PCR product sizes for the 610-bp TET-DIAL promoter. The primers bind to sequences upstream of the TET binding sites or the mGL reporter gene. In the presence of Cre, the spacer excision shortens the distance between binding sites and the minimal promoter, resulting in a shorter PCR product. Image of gel electrophoresis of PCR products from TET-DIAL promoters of various spacer lengths in C. Conditions without Cre are PCR products from the plasmid. Conditions with Cre are PCR products from cell lysis of HEK293T cells transfected with plasmids for Cre, rtTA, and TET-DIAL promoter of corresponding spacer length. The conditions with Cre show a shorter band at 727 bp, the expected length of the PCR product from the post-excision promoter, suggesting the emergence of a post-excision promoter state in the presence of Cre. All units fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers in A, D, and E represent the mean of biological replicates with span indicating standard deviation (A, E) or standard error (D). Histograms represent single-cell distributions sampled across bioreplicates. Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$; **** $p<0.0001$.

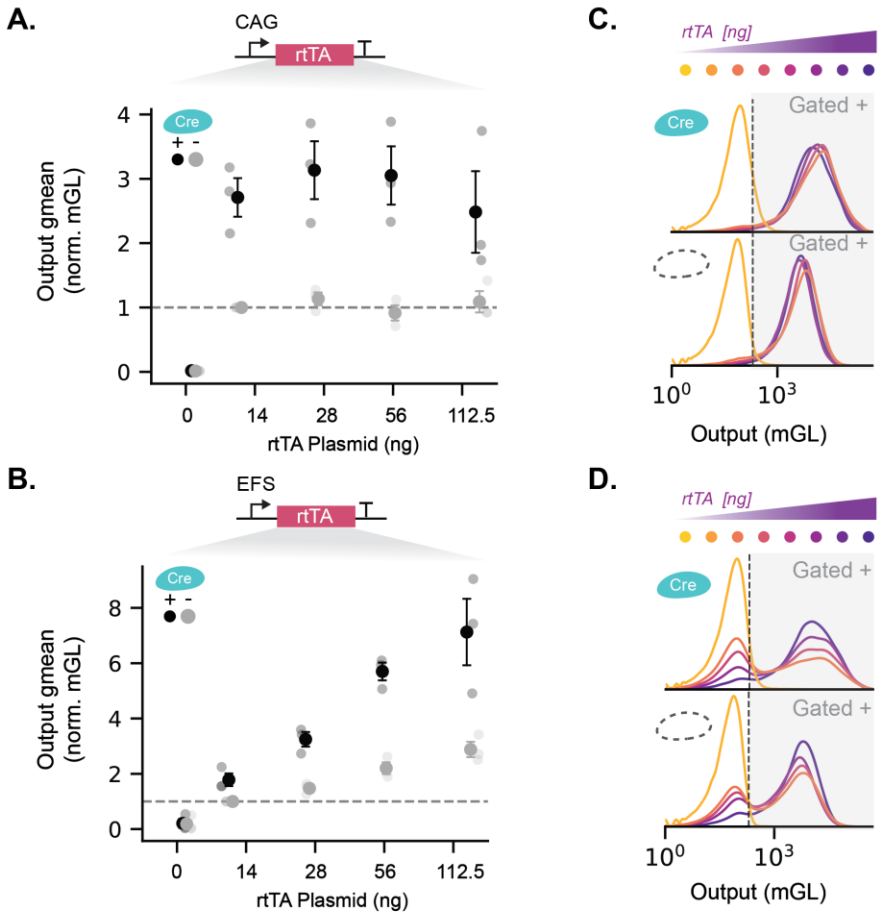


Figure S19. Expression of rtTA influences the bimodality of the TET-DIAL promoter. | A-B. Normalized gmean fluorescence intensity of mGL expressed from 203-bp TET-DIAL promoter with titrated levels of rtTA co-transfected on plasmids into HEK293T cells, with or without Cre. All conditions are at 1x DOX (1 ug/mL) and normalized to the 14 ng rtTA, -Cre condition within each bioreplicate. The transactivator rtTA was constitutively expressed from CAG (A) or EFS (B) promoter. **C-D.** Representative histograms for A and B. Single-cell distributions of output mGL expressed from the 203-bp TET-DIAL promoter titrated with CAG-rtTA (C) or EFS-rtTA (D) transfected on plasmids into HEK293T cells, with or without Cre. All units for fluorescence intensity are arbitrary units (a.u.). Large markers represent the mean of biological replicates with span indicating standard error (n=3). Histograms represent single-cell distributions sampled across bioreplicates (n=3).

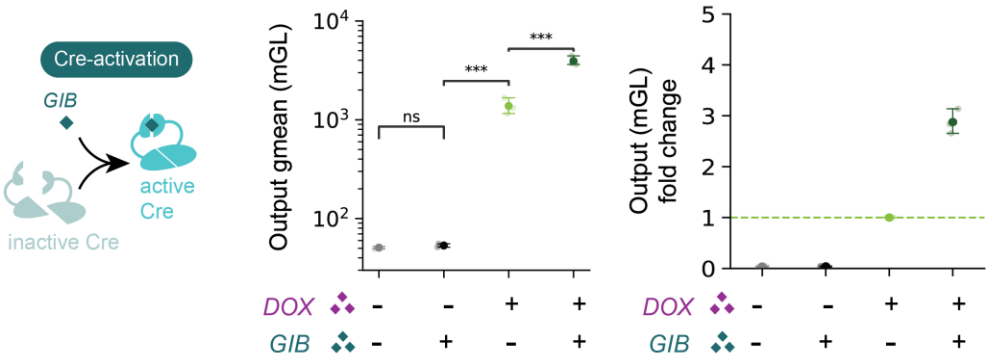


Figure S20. TET-DIAL and GIB-Cre enable dual small-molecule control. | Output reporter mGL gmean fluorescence intensity and fold change expressed from the 380-bp TET-DIAL promoter transfected with gibberellin (GIB) inducible split Cre, and rtTA, on plasmids into HEK293T cells. DOX (1 ug/mL) turns expression "OFF" or "ON", whereas presence of GIB (1 μM) determines levels of "ON" expression. All units for geometric mean fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$.

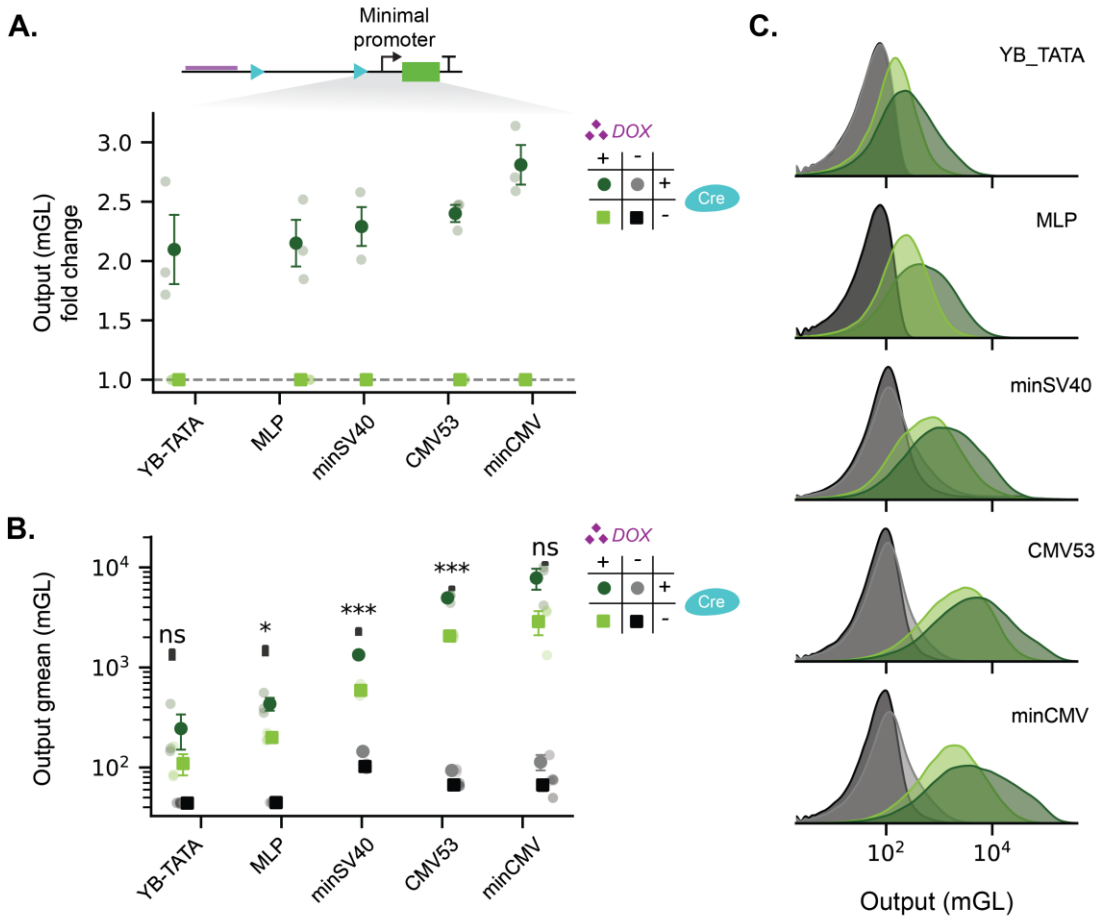


Figure S21. Minimal promoter choice tunes TET-DIAL output expression. | A. Fold change of output reporter mGL from 203-bp DIAL promoter with different minimal promoters. Constructs were co-transfected on plasmids into HEK293T cells with or without Cre in the presence of DOX (1 ug/mL). Fold change is the output mGL gmean fluorscence intensity normalized to the condition without Cre within each minimal promoter. **B.** Output reporter mGL gmean fluorescence intensity from 203-bp DIAL promoter with different minimal promoters. Constructs were co-transfected on plasmids into HEK293T cells with or without Cre, and DOX (1 µg/mL) as according to the legend in A. The choice of minimal promoter influences the levels of pre- and post- excision expression, and basal activity. **C.** Single-cell distributions of output mGL expressed from the 203-bp DIAL promoter with different minimal promoters as according to the legend in A. The choice of minimal promoter influences the shape of the single cell distributions. All units for geometric mean fluorescence intensity and single cell distributions of fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error (n=4 for YB-TATA, minSV40, minCMV, CMV53; n=3 for MLP). Histograms represent single-cell distributions sampled across bioreplicates. Statistical significance was calculated between the +DOX conditions with and without Cre using two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01; *** p<0.001.

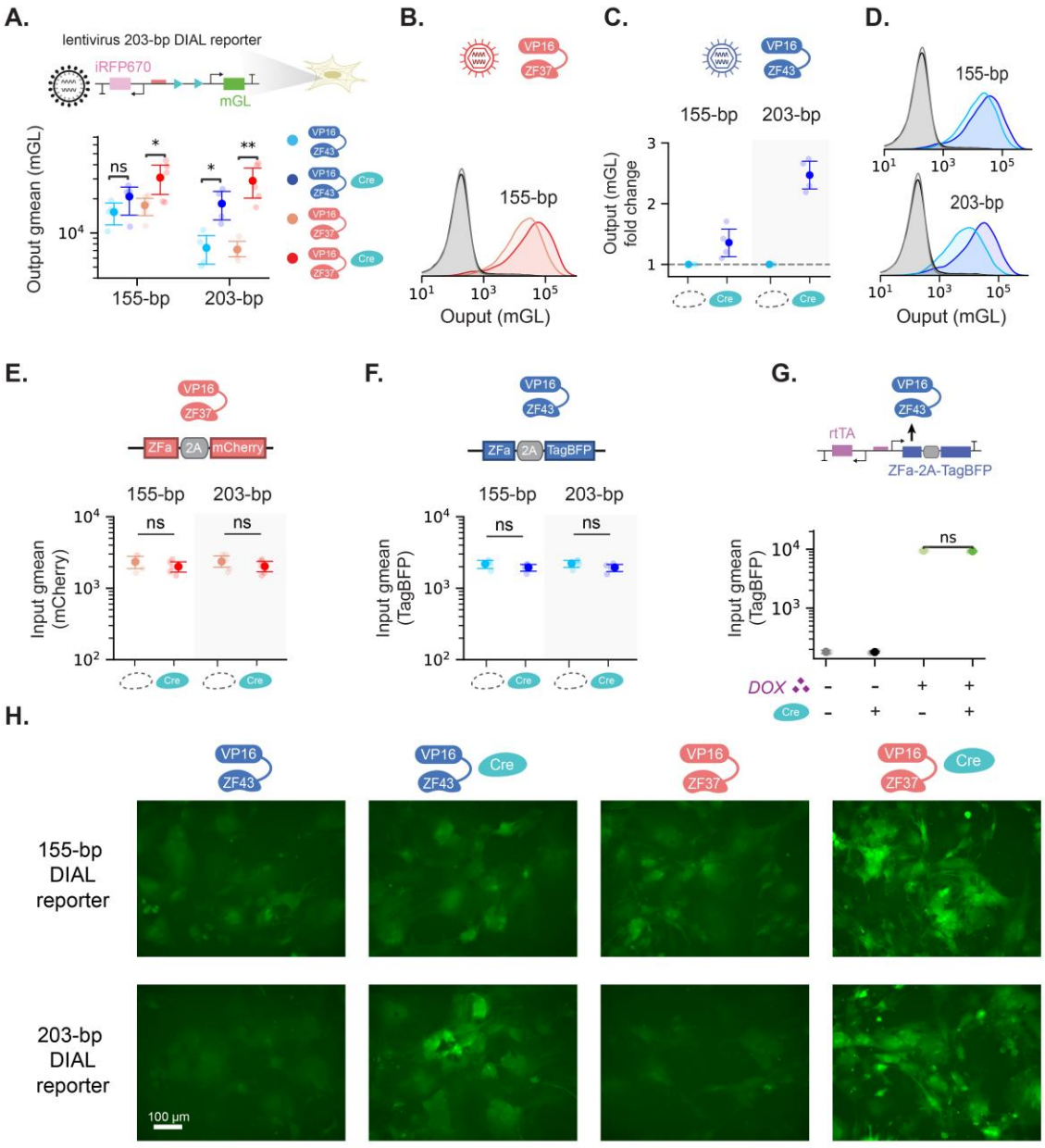


Figure S22. DIAL can be delivered to MEFs via lentivirus and Cre does not affect the level of ZFa expression.| (A-F, H) MEFs were infected and measured at 5 dpi as described in Figure 5A-C with n=4-5 biological replicates. **G.** MEFs were infected and measured at 3 dpi as described in Figure 5D with n=3 biological replicates. **A.** Gmean fluorescence intensity of output mGL expressed from 155-bp and 203-bp DIAL promoter, and **(B)** single-cell distributions of output mGL expressed from 155-bp DIAL promoter delivered via lentivirus co-infected with ZFa into mouse embryonic fibroblasts (MEFs), and with or without Cre (n=5 for VP16-ZF37, n=4 for VP16-ZF43). Cells were gated for divergent iRFP670 expression, co-expressed TagBFP for VP16-ZF43 expression if present, and co-expressed mCherry for VP16-ZF37 expression if present. Distribution conditions represent no ZFa and no Cre (gray), no ZFa and Cre (black), VP16-ZF37 and no Cre (light red), and VP16-ZF37 and Cre (dark red).**C.** Fold change of the output mGL reporter expressed from 155-bp and 203-bp DIAL promoters, co-infected with ZFa VP16-ZF43 into MEFs, with or without Cre (n=5 for VP16-ZF37, n=4 for VP16-ZF43). Fold change is the output gmean fluorescence intensity normalized to the condition without Cre within each spacer length. Cells were gated by expression of divergent iRFP670 and TagBFP co-expressed with VP16-ZF43. **D.** Single-cell distributions of output mGL expressed from 155-bp or 203-bp DIAL promoter, co-infected with or without ZFa VP16-ZF43 or Cre into MEFs (n=4). The conditions represent no ZFa and no Cre (gray), no ZFa and Cre (black), VP16-ZF43 and no Cre (light blue), and VP16-ZF43 and Cre (dark blue). Cells were gated by expression of divergent iRFP670. In the presence of ZFa, cells were also gated by TagBFP

co-expressed with VP16-ZF43. **E.** Gmean fluorescence intensity of mCherry co-expressed with ZFa VP16-ZF37 co-infected with 155-bp or 203-bp DIAL promoter into MEFs, with and without Cre (n=5). Cells were gated by expression of divergent iRFP670 and mCherry co-expressed with VP16-ZF37. **F.** Gmean fluorescence intensity of TagBFP co-expressed with ZFa VP16- co-infected with 155-bp or 203-bp DIAL promoter into MEFs, with and without Cre (n=4). Cells were gated by expression of divergent iRFP670 and TagBFP co-expressed with VP16-ZF43. **G.** Gmean fluorescence intensity of TagBFP co-expressed with DOX-inducible ZFa VP16-ZF43 in lentivirus with divergent rtTA into MEFs, co-infected with 203-bp DIAL promoter regulating mGL lentivirus, and with or without Cre retrovirus. Adding Cre does not affect DOX-inducible expression of ZFa. Cells were gated by expression of divergent iRFP670. In the presence of DOX, cells were also gated by expression of TagBFP co-expressed with VP16-ZF43. **H.** Representative fluorescence microscopy images of mGL expressed from 155-bp or 203-bp DIAL promoter infected via lentivirus into MEFs with ZFa retrovirus VP16-ZF37 or VP16-ZF43, and with or without Cre retrovirus. Images taken 3 days post-infection (dpi). (n=5 for VP16-ZF37, n=4 for VP16-ZF43 with same results). All mGL DIAL promoters contain a divergent EF1a promoter regulating iRFP670 encoded on lentivirus. All units for fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of biological replicates with span indicating standard error. Histograms represent single-cell distributions sampled across bioreplicates. Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$.

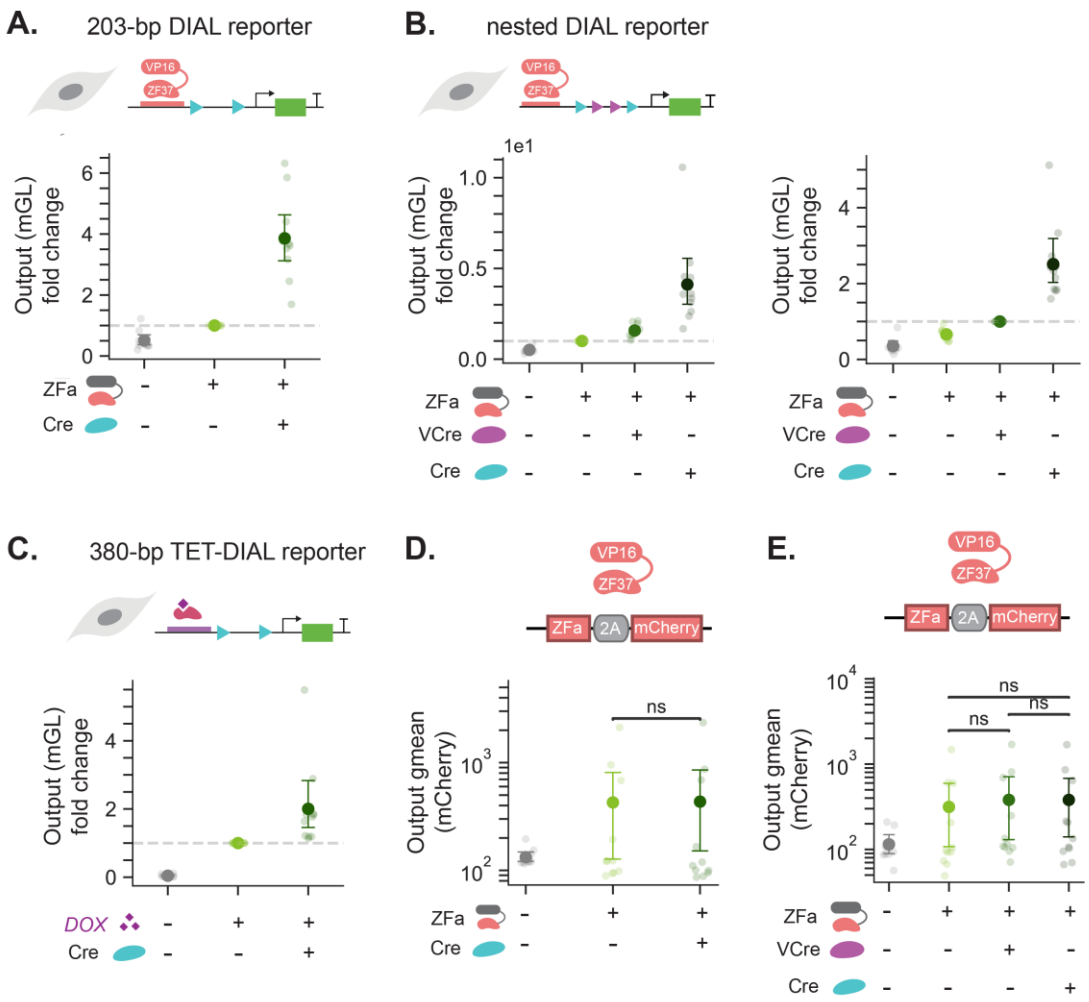


Figure S23. DIAL is portable to iPSCs and ZFa level is unaffected by recombinase expression.| A-C. Output mGL fold change expressed from 203-bp DIAL (n=11), nested DIAL (n=10), and 380-bp TET-DIAL (n=11) promoters transfected on plasmids into human induced pluripotent stem cells (iPSCs). 203-bp and nested DIAL were transfected with and without plasmids for ZFa (VP16-ZF37), VCre, and Cre. The TET-DIAL promoter was transfected with rtTA, and had conditions with and without DOX (1 μg/mL). Conditions with DOX are gated for output expressing cells. Fold change is the output mGL gmean fluorescence intensity normalized within each bioreplicate to the condition with +ZFa -Cre for 203-bp DIAL, +ZFa -VCre -Cre (left) or +ZFa +VCre -Cre (right) for nested DIAL, or +DOX -Cre for 380-bp TET-DIAL. **D-E.** Input gmean fluorescence intensity of mCherry co-expressed with ZFa VP16-ZF37 transfected on plasmids into iPSCs with 203-bp (n=11) (D) or nested (n=10) (E) DIAL promoter regulating mGL, with or without plasmids for ZFa, VCre, and Cre. The presence of recombinase does not affect levels of ZFa. All units for geometric mean fluorescence intensity are arbitrary units (a.u.). Fold change is unitless. Large markers represent the mean of individual bioreplicates with span indicating standard error. Statistical significance was calculated on individual bioreplicates with two-sided Student's *t*-test with ns p>0.05.

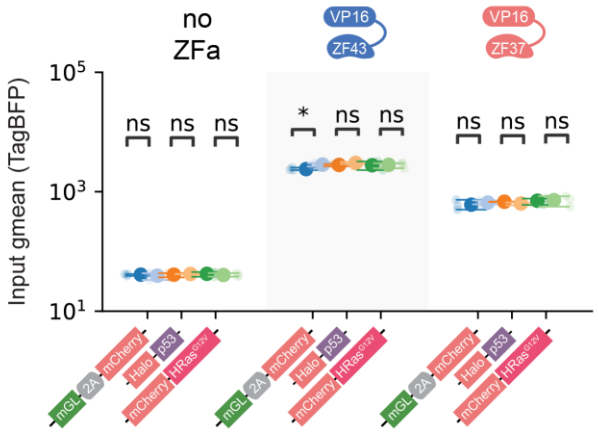


Figure S24. Cre does not affect ZFa levels when DIAL promoters regulate different target genes. | Gmean fluorescence intensity of TagBFP (proxy for ZFa VP16-ZF43 or VP16-ZF37) on plasmids transfected on plasmids into HEK293T cells. Conditions were co-transfected with the 203-bp DIAL promoter regulating different target genes, and with (light) or without (dark) Cre. Across diverse target genes like mGL-2A-mCherry (blue), Halo-p53 (orange), or mCherry-HRas^{G12V} (green), presence of Cre did not substantially affect ZFa expression levels. All units for gmean fluorescence intensity are arbitrary units (a.u.). Large markers represent the mean of biological replicates with span indicating standard error (n=3). Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$.

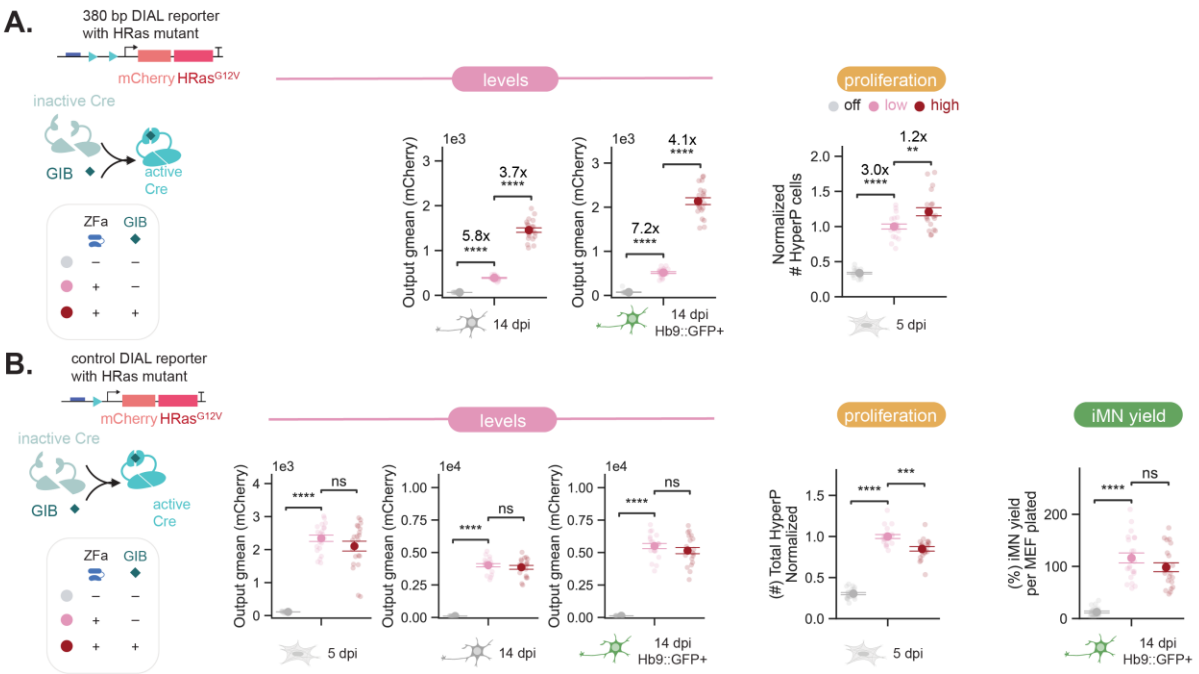


Figure S25. A single 380-bp DIAL promoter with gibberellin-inducible Cre generates multiple MAPK mutant levels to investigate dose dependent effects in fibroblast to motor neuron conversion. Control DIAL promoter does not generate different low and high setpoints. | A-B. MEFs were converted to iMNs following the workflow in Figure 6B. MEFs are infected with the conversion cocktail consisting of three motor neuron-specific transcription factors (3 TFs; Lhx3-Ngn2-Isl1) and a p53 mutant. The 380-bp or post-excision control DIAL promoter regulates the MAPK mutant HRas^{G12V} fused to mCherry. DIAL activity is induced by the presence of the ZFa (VP16-ZF37-2A-TagBFP) and promoter editing is controlled via the gibberellin-inducible split Cre. Phenotypes and expression levels are measured at 5 dpi (proliferation) and 14 dpi (iMN yield). Conversion to iMNs is measured via activation of the motor neuron Hb9::GFP reporter from the primary transgenic Hb9::GFP MEFs. Percent yield is defined as the number of Hb9::GFP+ cells at 14 dpi divided by the number of cells seeded. **A.** Output mCherry-HRas^{G12V} gmean fluorescence intensity regulated by the 380-bp DIAL promoter at 14 dpi and normalized number of hyperP cells at 5 dpi. mCherry-HRas^{G12V} levels were measured at 14 dpi for all cells, and at 14 dpi in Hb9::GFP+ iMNs. The OFF (gray), Low (pink), and High (red) setpoint are generated by combinations of ZFa and gibberellin. **B.** Output mCherry-HRas^{G12V} gmean fluorescence intensity regulated by the control DIAL promoter, normalized number of hyperP cells at 5 dpi, and percent iMN yield at 14 dpi. mCherry-HRas^{G12V} levels were measured at 5 dpi, at 14 dpi for all cells, and at 14 dpi in Hb9::GFP+ iMNs. All units for geometric mean fluorescence intensity are arbitrary units (a.u.). Fold change is unitless and annotated as the difference between means of conditions. For DIAL with GIB-Cre, conversion was conducted with four separate MEF batches with three to six replicates each (n=21). For number of hyperP cells normalization is performed within each MEF batch to control for batch-specific proliferation differences. Each replicate is divided by the mean number of hyperP cells across replicates for each batch. Large markers represent the mean of replicates with span indicating standard error (n=21). Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01; *** p<0.001; **** p<0.0001.

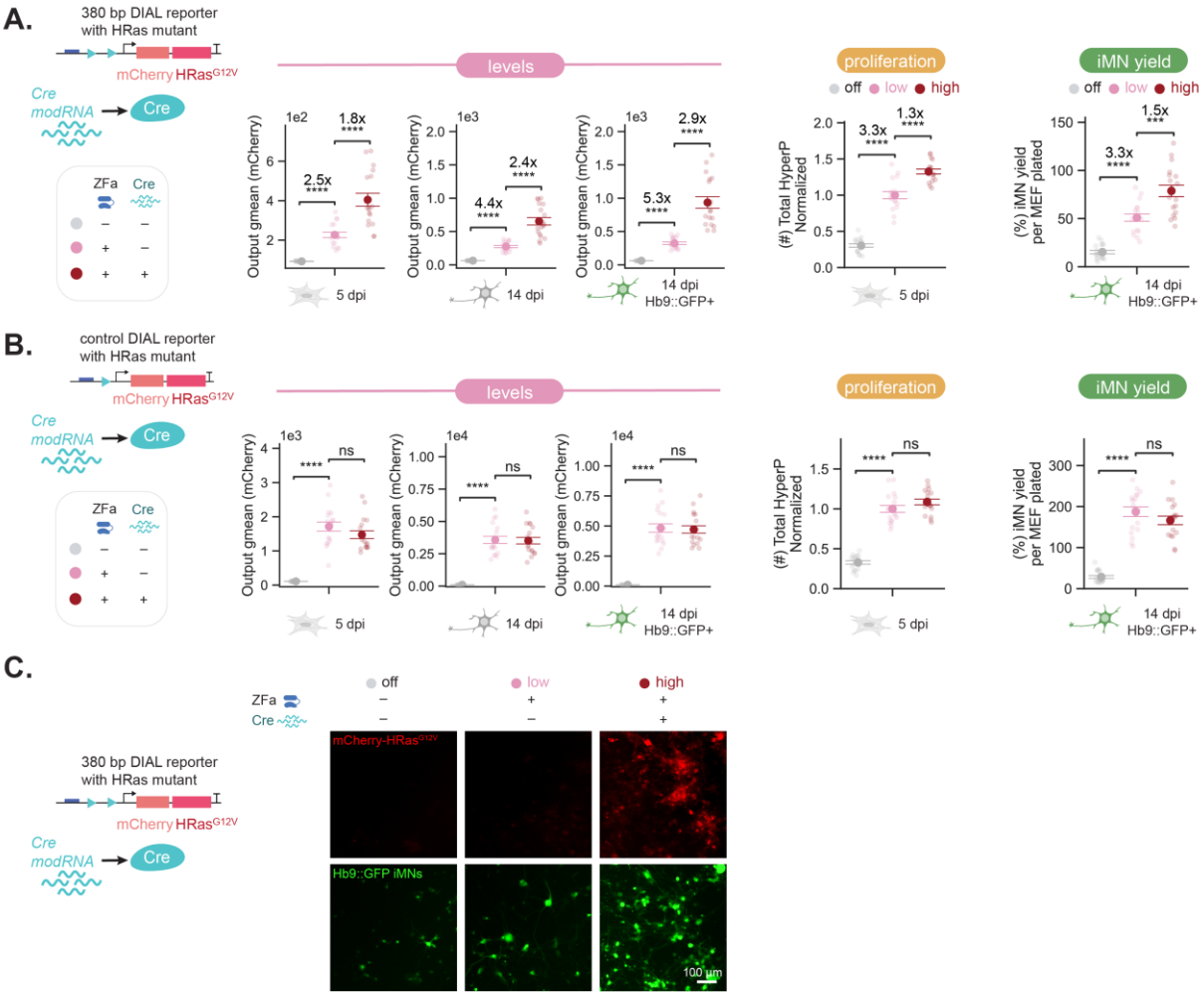


Figure S26. A single 380 bp DIAL promoter with modRNA Cre generates multiple MAPK mutant levels to investigate dose dependent effects in fibroblast to motor neuron conversion. Control DIAL promoter does not generate different low and high setpoints. | A-C. MEFs were converted to iMNs following the workflow in Figure 6B. MEFs are infected with the conversion cocktail consisting of three motor neuron-specific transcription factors (3 TFs; Lhx3-Ngn2-Isl1) and a p53 mutant. The 380-bp or post-excision control DIAL promoter regulates the MAPK mutant HRas^{G12V} fused to mCherry. DIAL activity is induced by the presence of the ZFa (VP16-ZF37-2A-TagBFP) and promoter editing is controlled via Cre modRNA. When Cre modRNA is used, control eeBxb1 modRNA is added to conditions +ZFa -Cre and retroviruses for GIB-Cre are not added. Phenotypes and expression levels are measured at 5 dpi (proliferation) and 14 dpi (iMN yield). Conversion to iMNs is measured via activation of the motor neuron Hb9::GFP reporter from the primary transgenic Hb9::GFP MEFs. Percent yield is defined as the number of Hb9::GFP+ cells at 14 dpi divided by the number of cells seeded. **A.** Output mCherry-HRas^{G12V} gmean fluorescence intensity regulated by the 380-bp DIAL promoter, normalized number of hyperP cells at 5 dpi, and percent iMN yield at 14 dpi. mCherry-HRas^{G12V} levels were measured at 5 dpi, at 14 dpi for all cells, and at 14 dpi in Hb9::GFP+ iMNs. The OFF (gray), Low (pink), and High (red) setpoints are generated by combinations of ZFa and Cre modRNA. **B.** Output mCherry-HRas^{G12V} gmean fluorescence intensity regulated by the control DIAL promoter, normalized number of hyperP cells at 5 dpi, and percent iMN yield at 14 dpi. mCherry-HRas^{G12V} levels were measured at 5 dpi, at 14 dpi for all cells, and at 14 dpi in Hb9::GFP+ iMNs. **C.** Images of mCherry-HRas^{G12V} and Hb9::GFP iMNs at 14 dpi. mCherry-HRas^{G12V} is regulated by the 380-bp DIAL promoter (n=18 replicates with same results). The mCherry OFF (gray), Low (pink), and High (red) setpoints are generated by combinations of ZFa and Cre modRNA. GFP is the output from the Hb9::GFP reporter for quantifying conversion to iMNs. All units for geometric mean fluorescence intensity are arbitrary units (a.u.). Fold change is unitless and annotated as the difference between means of conditions. For DIAL with modRNA Cre, conversion was conducted with three separate MEF batches with six replicates each (n=18). For number of hyperP cells normalization is performed within each MEF batch to control for batch-specific proliferation differences. Each replicate is divided by the mean number of hyperP cells across replicates for each batch. Large markers represent the mean of replicates with span

indicating standard error (n=18). Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$; **** $p<0.0001$.

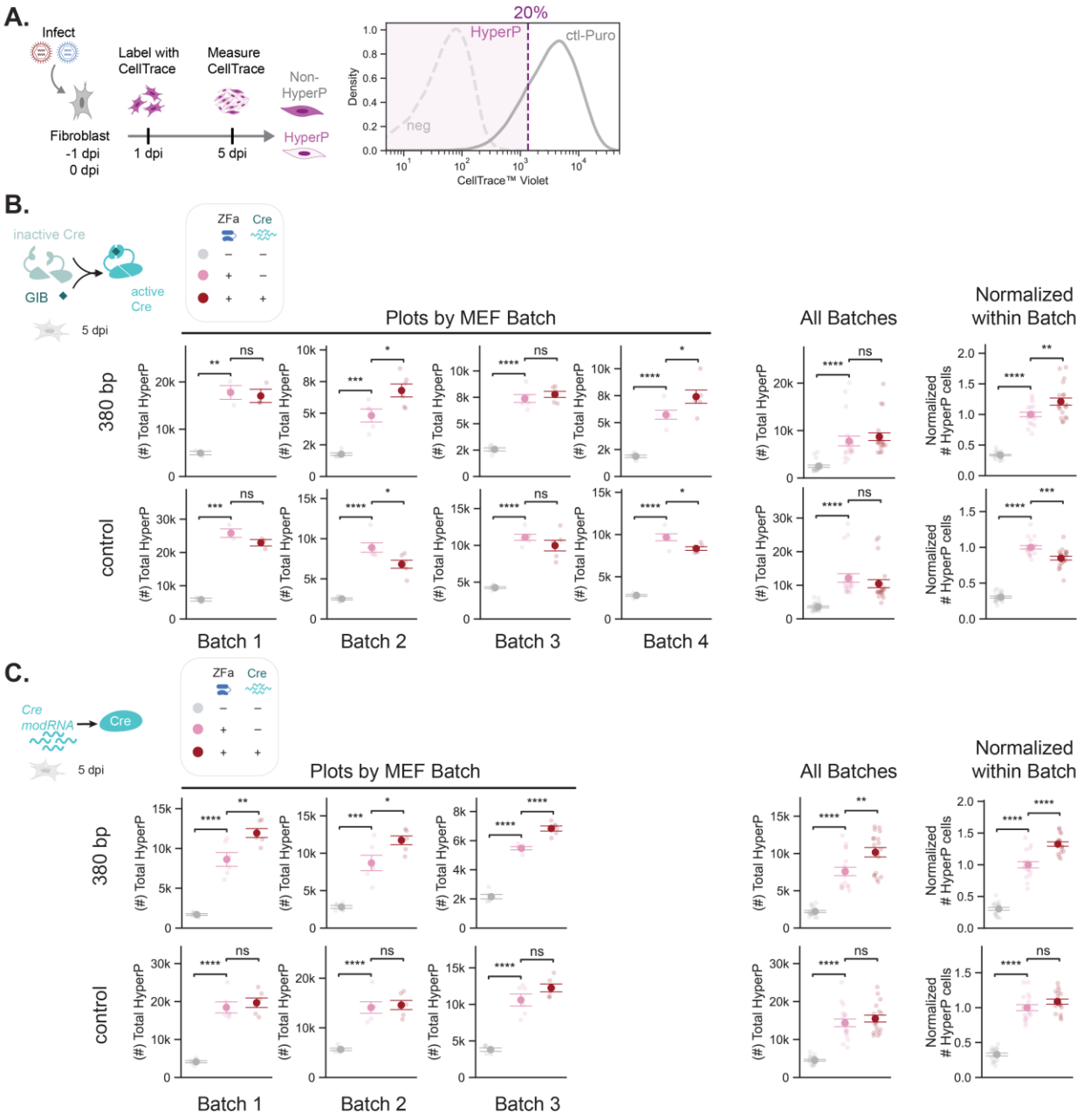


Figure S27. CellTrace assay for hyperproliferation. HRas^{G12V} expression increases hyperproliferation in a dose-dependent manner. | A. Schematic depicting CellTrace assay to measure the number of hyperP cells. For each batch of MEFs, cells are infected with the DIAL promoter regulating HRas^{G12V} and conversion cocktail at -1 dpi and 0 dpi, as well as a control condition with the puromycin resistance gene (ctl-Puro). CellTrace stain is added at 1 dpi. The stain dilutes as cells proliferate, where more CellTrace dilution indicates a history of more proliferation. At 5 dpi, cells are flowed for single cell data. For each MEF batch, the gate for hyperP cells is based on the lowest 20% of cells in the ctl-Puro condition. For the other conditions, we quantify the number of hyperP cells below this gate. **B-C.** Number of hyperP cells by MEF batch, combined MEF batches, and combined normalized within MEF batches at 5 dpi. MEFs were infected with the conversion cocktail in Figure 6B consisting of three motor neuron-specific transcription factors (3 TFs; Lhx3-Ngn2-Isl1) and a p53 mutant. Either a 380 bp or post-excision control DIAL promoter regulates mCherry-HRas^{G12V} as indicated. Gibberellin-inducible split Cre (GIB-Cre) or modRNA Cre was used to induce Cre editing as indicated. When modRNA Cre is used, control modRNA eeBxb1 is added to conditions +ZFa -Cre and retroviruses for GIB-Cre are not added. Normalization is performed within each MEF batch to control for batch-specific proliferation differences. Each replicate is divided by the mean number of hyperP cells across all replicates for each batch. For DIAL with GIB-Cre, conversion was conducted with four separate MEF batches with three or six replicates each (n=21). For

DIAL with modRNA Cre, conversion was conducted with three separate MEF batches with six replicates each (n=18). Large markers represent the mean of replicates with span indicating standard error (n=3 or n=6 for by MEF batch, n=21 or n=18 for combined batches). Statistical significance was calculated with two-sided Student's *t*-test with ns $p>0.05$; *$p<0.05$; **$p<0.01$; *** $p<0.001$, **** $p<0.0001$.

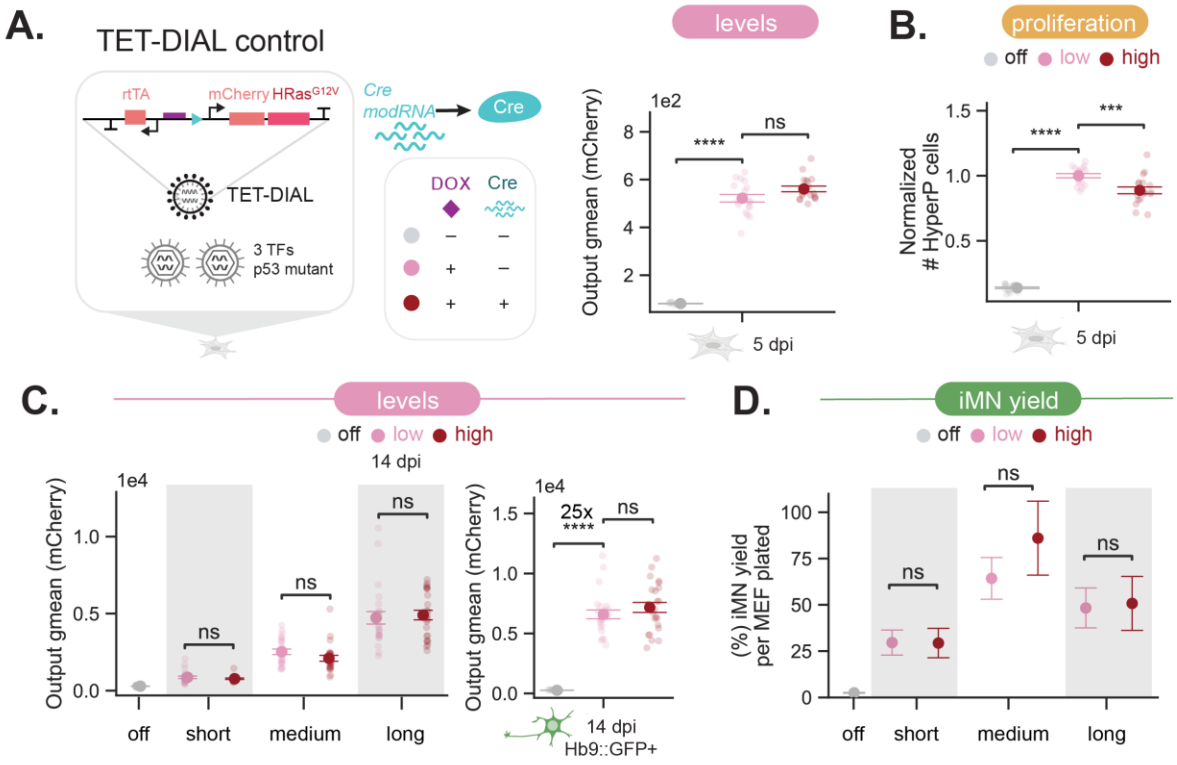


Figure S28. Control TET-DIAL promoter does not generate different low and high setpoints or titratable effects on proliferation and yield. | A-D. MEFs are infected with the conversion cocktail consisting of three motor neuron-specific transcription factors (3 TFs; Lhx3-Ngn2-Isl1) and a p53 mutant. The post-excision control TET-DIAL promoter regulates the MAPK mutant HRas^{G12V} fused to mCherry. DIAL activity is induced by the presence of DOX and promoter editing is controlled via Cre modRNA. A control modRNA eeBxb1 is added to conditions +DOX -Cre. Phenotypes and expression levels are measured at 5 dpi (proliferation) and 14 dpi (iMN yield). Conversion to iMNs is measured via activation of the motor neuron Hb9::GFP reporter from the primary transgenic Hb9::GFP MEFs. **A.** Output mCherry-HRas^{G12V} gmean fluorescence intensity regulated by the control TET-DIAL promoter at 5 dpi. **B.** Normalized number of hyperP cells at 5 dpi for the conditions and legend in G. Normalization is performed within each MEF batch. Each replicate is divided by the mean number of hyperP cells across all replicates for each batch. **C.** Output mCherry-HRas^{G12V} gmean fluorescence intensity regulated by the control TET-DIAL promoter at 14 dpi for off (no DOX), short, medium, and long pulses of DOX for all cells (left), as well as for off and long pulses in Hb9::GFP+ cells (right). **D.** Percent iMN yield for short, medium, and long pulses of DOX for off, low, and high mCherry-HRas^{G12V} levels. Percent yield is defined as the number of Hb9::GFP+ cells at 14 dpi divided by the number of cells seeded. All units for geometric mean fluorescence intensity are arbitrary units (a.u.). Fold change is unitless and annotated as the difference between means of conditions. For TET-DIAL and modRNA Cre, conversion experiments were conducted with three separate MEF batches with six replicates each (n=18). Large markers represent the mean of replicates with span indicating standard error (n=18). Statistical significance was calculated with two-sided Student's *t*-test with ns p>0.05; *p<0.05; **p<0.01; *** p<0.001; **** p<0.0001.

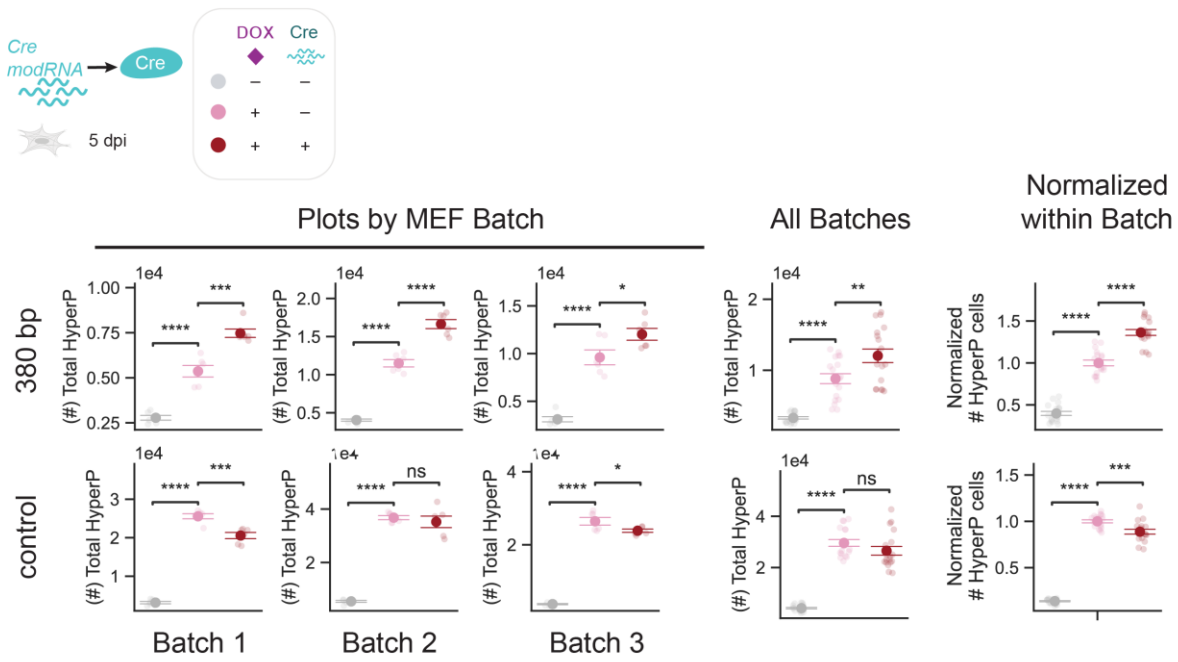


Figure S29. Hyperproliferation results for TET-DIAL regulating HRas^{G12V}. | Number of hyperP cells by MEF batch, combined MEF batches, and combined normalized within MEF batches at 5 dpi. MEFs are infected with the conversion cocktail consisting of three motor neuron-specific transcription factors (3 TFs; Lhx3-Ngn2-Isl1) and a p53 mutant. As indicated, the 380-bp or post-excision control TET-DIAL promoter regulates the MAPK mutant HRas^{G12V} fused to mCherry. DIAL activity is induced by the presence of DOX and promoter editing is controlled via modRNA Cre. A control eeBxb1 modRNA is added to conditions +DOX -Cre. Normalization of number of hyperP cells is performed within each MEF batch to control for batch-specific proliferation differences. Each replicate is divided by the mean number of hyperP cells across all replicates for each batch. For TET-DIAL and modRNA Cre, conversion experiments were conducted with three separate MEF batches with six replicates each (n=18). Large markers represent the mean of replicates with span indicating standard error (n=18). Statistical significance was calculated with two-sided Student's t-test with ns p>0.05; *p<0.05; **p<0.01; *** p<0.001; **** p<0.0001.

Supplementary Table 1: Plasmid Amounts for Transfection into HEK293T Cells or iPSCs

Figure	Description
Figure 1, S1, S2, S3A-C, S4A-D, S5 -S6, S7 DIAL development	HEK293Ts were transiently transfected with DIAL reporters containing mGL target gene (112.5ng), transfection marker (iRFP670, 112.5 ng), with or without ZFa (Fig 1C,1F, S1, S2, S3A-C, S4B, S5E-F, S6, S7: 112.5ng; Fig 1 1D,1E, 1H,1I, S4D, S5B-D:14 ng), and with or without Cre (11 ng). All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (349 ng). Experiments were at the 96-well scale.
Figure S4G Integrated line with Cre-mRuby2	Polyclonal HEK293Ts cells were integrated 203-bp DIAL reporter regulating mGL and divergent EF1a-iRFP670-WPRE. These HEK293T cells were transiently transfected with either mRuby2 (5565 ng) and filler plasmid (5565 ng), or mRuby2 (5565 ng) and Cre (5565 ng). Experiments were conducted at the 6-well scale.
Figure S3D-E Western blot	HEK293Ts were transiently transfected at a 6-well scale with either CAG-FLAG-VP16-ZF37-2A-mCherry-bGH (1800 ng) or CAG-FLAG-VP16-ZF43-2A-TagBFP-bGH (1800 ng), CAG-3xFLAG-iRFP670-bGH (1800 ng) and N- and C-terminal split GIB-Cre (900 ng each). Conditions that were +Cre received 1 uM GIB at 1 dpt, whereas –Cre conditions received fresh media. Lysates were collected at 3 dpt and protein concentrations were quantified via a Bradford Assay. Proteins were separated via electrophoresis on a bis-tris gel and transferred to a PVDF membrane. Membranes were blocked and incubated with primary and HRP secondary antibodies. HRP signal was detected using a commercial kit and visualized on a ChemiDoc MP Imaging system. Experiments were at the 6-well scale.
Figure S3F Immunofluorescent staining	HEK293Ts were transiently transfected with the 203-bp DIAL reporter with mGL target gene (866 ng), transfection marker (iRFP670, 866 ng), with or without Cre (86 ng), and ZFa as indicated (866 ng). All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (2684 ng). Experiments were at the 12-well scale.
Figure 2, S8-S9 ZFa titration	HEK293Ts were transiently transfected with the 203-bp DIAL reporter with mGL target gene (112.5 ng), transfection marker (iRFP670, 112.5 ng), with or without Cre (11 ng), and ZFa (2:1 plasmid titration: 0 to 112 ng, different promoters: 112 ng). All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (349 ng). Experiments were at the 96-well scale.
Figure S10 RNA FISH	HEK293Ts were transiently transfected with the 203-bp DIAL reporter with mGL target gene (2025 ng), transfection marker (CAG-mRuby2-SV40, 709nf ng), with or without Cre (203 ng), and ZFa (506 ng). All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (3443 ng). Experiments were at the 12-well scale.
Figure S15 Inducible Cre	HEK293Ts were transiently transfected with the 203-bp DIAL reporter with mGL target gene (112.5ng), transfection control (iRFP670, 112.5ng), each half of split GIB-inducible Cre (56ng each), and with or without ZFa or empty plasmid vector (112.5ng), for a total of 450ng DNA per condition. At 1 dpt GIB (1μM) was added. Experiments were at the 96-well scale.
Figure S16 Inducible ZFa	HEK293Ts were transiently transfected with the 203bp-spacer DIAL mGL reporter (112.5ng), transfection marker (iRFP670, 112.5ng), TET-VP16-ZF37-2A-mCherry-BGH or TET-VP16-ZF43-2A-TagBFP-BGH (14ng), rtTA (28ng), and with or without Cre (11ng). All conditions were filled with an empty plasmid vector to achieve same total DNA per condition (349 ng). At 1 dpt, DOX (1 ug/mL) was added. Experiments were at the 96-well scale.
Figure 3G-I, S17 Inducible Cre and Inducible ZFa	HEK293Ts were transiently transfected with the 203bp spacer DIAL mGL reporter (186 ng), transfection marker (iRFP670, 111 ng), TET-VP16-ZF37-2A-mCherry-BGH or TET-VP16-ZF43-2A-TagBFP-BGH (21 ng), rtTA (15 ng), and each half of split GIB-inducible Cre (50 ng each). All conditions had the same total DNA (432 ng). At 1 dpt, combinations of DOX (1 ug/mL) or GIB (1μM) were added. Experiments were at the 96-well scale.
Figure S12A modRNA test	HEK293Ts were transiently transfected with DIAL reporters containing mGL target gene (112.5ng), transfection marker (iRFP670, 112.5ng), and with or without ZFa

	(C, F: 112.5ng; D,E,H,I:14ng. All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (349 ng). ModRNA Cre was delivered at 1 dpt. Experiments were at the 96-well scale.
Figure 4 **Figure S18, S19, S21** **TET-DIAL development**	HEK293Ts were transiently transfected with the TET-DIAL promoter regulating the mGL target gene at varying spacer lengths (112.5 ng), transfection marker (iRFP670, 112.5ng), with or without Cre (11 ng), and EFS-rtTA-2A-mRuby2 (112.5 ng). For minimal promoter experiments, a CAG-rtTA-2A-TagBFP (112.5ng) was used in place of the EFS-rtTA-2A-mRuby2. All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (349 ng). Experiments were at the 96-well scale.
Figure S20 TET-DIAL with Inducible Cre	HEK293Ts were transiently transfected with the 380-bp TET-DIAL reporter regulating the mGL target gene (124 ng), transfection marker (iRFP670, 111 ng), each half of split GIB-inducible Cre (56 ng each), and CAG-rtTA-2A-TagBFP (137 ng). All conditions had the same total DNA (384 ng). Experiments were at the 96-well scale.
Figure S19 **rtTA titration**	HEK293Ts were transiently transfected with the 203-bp TET-DIAL promoter regulating the mGL target gene (112.5 ng), transfection marker (iRFP670, 112.5ng), with or without Cre (11 ng), and either EFS-rtTA-2A-mRuby2 or CAG-rtTA-2A-TagBFP (2:1 plasmid titration: 0 to 112.5 ng). All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (349 ng). Experiments were at the 96-well scale.
Figure 5E-H **Figure S23** **iPSC transfection**	iPSCs were transiently transfected with DIAL promoters regulating the mGL target gene (203 bp, nested, or TET-DIAL; 100 ng), transfected marker (TagBFP, 25 ng), with or without rtTA or ZFa (12.5 ng), and with or without Cre or VCre (25 ng). All conditions were filled with an empty plasmid vector to achieve the same amount of total DNA per condition (162.5 ng). Experiments were at the 96-well scale.
Figure 5I-K **Figure S4** **Additional genes**	HEK293Ts were transiently transfected with 203-bp DIAL reporter with various target genes (112 ng), ZFa (VP16-ZF37-2A-TagBFP, or VP16-ZF43-2A-TagBFP) (112.5 ng), transfection marker (iRFP670, 112.5ng), with or without Cre (11 ng), and ZFa (112 ng). Experiments were at the 96-well scale.

Supplementary Table 2: Instrument specifications of analytical flow cytometry

Experiments	Fluorescent Protein	Excitation Laser (nm)	Laser Setting (V)	Emission Filter (nm)
Figure 1-3 **Figure 5I-K** **Figure S1, S2, S3A-C, S5-S9, S11-S17, S20, S24**		FSC	60	
		SSC	360	
	mGreenLantern	Blue – 488 nm	220	BL1 (510/10)
	Halo (Janelia Fluor 549)	Yellow – 561 nm	390	YL1 (585/16)
	mCherry	Yellow – 561 nm	295	YL2 (615/25)
	TagBFP	Violet – 405 nm	190	VL1 (440/50)
	iRFP670	Red – 637 nm	280	RL1 (670/14)
Figure S3F		FSC	60	
		SSC	360	
	mGreenLantern	Blue – 488 nm	220	BL1 (510/10)
	Alexa Fluor™ 555	Yellow – 561 nm	400	YL1 (585/16)
	TagBFP	Violet – 405 nm	190	VL1 (440/50)
	iRFP670	Red – 637 nm	280	RL1 (670/14)
Figure S10		FSC	60	
		SSC	360	
	mGreenLantern	Blue – 488 nm	220	BL1 (510/10)
	mRuby2	Yellow – 561 nm	260	YL2 (615/25)
	TagBFP	Violet – 405 nm	190	VL1 (440/50)
	Alexa Fluor™ 555	Red – 637 nm	220	RL1 (670/14)
Figure 4 **Figure S18-S19**		FSC	60	
		SSC	360	
	mGreenLantern	Blue – 488 nm	220	BL1 (510/10)
	mCherry	Yellow – 561 nm	295	YL2 (615/25)
	TagBFP	Violet – 405 nm	190	VL1 (440/50)
	iRFP670	Red – 637 nm	280	RL1 (670/14)
Figure S21		FSC	60	
		SSC	360	
	mGreenLantern	Blue – 488 nm	180	BL1 (510/10)
	mCherry	Yellow – 561 nm	295	YL2 (615/25)
	TagBFP	Violet – 405 nm	190	VL1 (440/50)
	iRFP670	Red – 637 nm	280	RL1 (670/14)
Figure 5E-H **Figure S23**		FSC	60	
		SSC	340	
	mGreenLantern	Blue – 488 nm	380	BL1 (510/10)
	mCherry	Yellow – 561 nm	440	YL2 (615/25)
	TagBFP	Violet – 405 nm	300	VL1 (440/50)
	iRFP670	Red – 637 nm	460	RL1 (670/14)

Figure 5B-D **Figure S22A-F**		FSC	60	
		SSC	345	
	mGreenLantern	Blue – 488 nm	260	BL1 (510/10)
	mCherry	Yellow – 561 nm	300	YL2 (615/25)
	TagBFP	Violet – 405 nm	210	VL1 (440/50)
	iRFP670	Red – 637 nm	270	RL1 (670/14)
Figure 5E **Figure S22G**		FSC	60	
		SSC	325	
	mGreenLantern	Blue – 488 nm	250	BL1 (510/10)
	mCherry	Yellow – 561 nm	320	YL2 (615/25)
	TagBFP	Violet – 405 nm	220	VL1 (440/50)
	iRFP670	Red – 637 nm	250	RL1 (670/14)
Figure 6C-E **Figure S25, S26**		FSC	60	
		SSC	345	
	Hb9::GFP	Blue – 488 nm	240	BL1 (510/10)
	mCherry-HRas^{G12V}	Yellow – 561 nm	300	YL2 (615/25)
	TagBFP	Violet – 405 nm	210	VL1 (440/50)
	CellTrace™ Far Red	Red – 637 nm	260	RL1 (670/14)
Figure 6G-J **Figure S27, S28**		FSC	60	
		SSC	345	
	Hb9::GFP	Blue – 488 nm	240	BL1 (510/10)
	mCherry-HRas^{G12V}	Yellow – 561 nm	400	YL2 (615/25)
	TagBFP	Violet – 405 nm	240	VL1 (440/50)
	CellTrace™ Far Red	Red – 637 nm	260	RL1 (670/14)

Supplementary Table 3: VP16-ZF37 Plasmid Titration Fitted Model Parameters

Parameters	No Cre	Cre
k'_{cat}	0.99	15.63
K_M	0.21	0.52
α'	0.14	0.22

Supplementary Table 4: VP16-ZF43 Plasmid Titration Fitted Model Parameters

Parameters	No Cre	Cre
k'_{cat}	1.13	5.21
K_M	0.19	0.20
α'	0.08	0.08

Supplementary Information References

(1) Shapiro, H. M. *Practical Flow Cytometry*; John Wiley & Sons, 2005.
(2) Rico, L. G.; Bardina, J.; Bistué-Rovira, À.; Salvia, R.; Ward, M. D.; Bradford, J. A.; Petriz, J. Accurate Identification of Cell Doublet Profiles: Comparison of Light Scattering with Fluorescence Measurement Techniques. *Cytom. Part J. Int. Soc. Anal. Cytol.* 2023, *103* (5), 447–454. https://doi.org/10.1002/cyto.a.24690.
(3) *Antibodies 101: Introduction to Gating in Flow Cytometry*. https://blog.addgene.org/antibodies-101-introduction-to-gating-in-flow-cytometry (accessed 2025-08-19).
(4) *Flow Cytometry Gating for Beginners*. https://www.ptglab.com/news/blog/flow-cytometry-gating-for-beginners/ (accessed 2025-08-19).

Unedited gels and blots

Figure S3D (Figure shows relevant lanes for experiment)

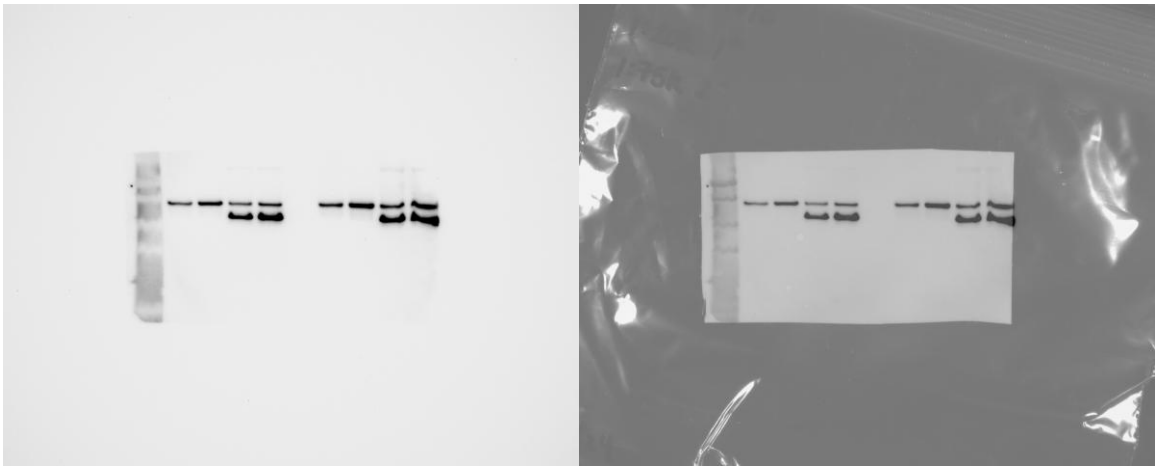


Figure S3E

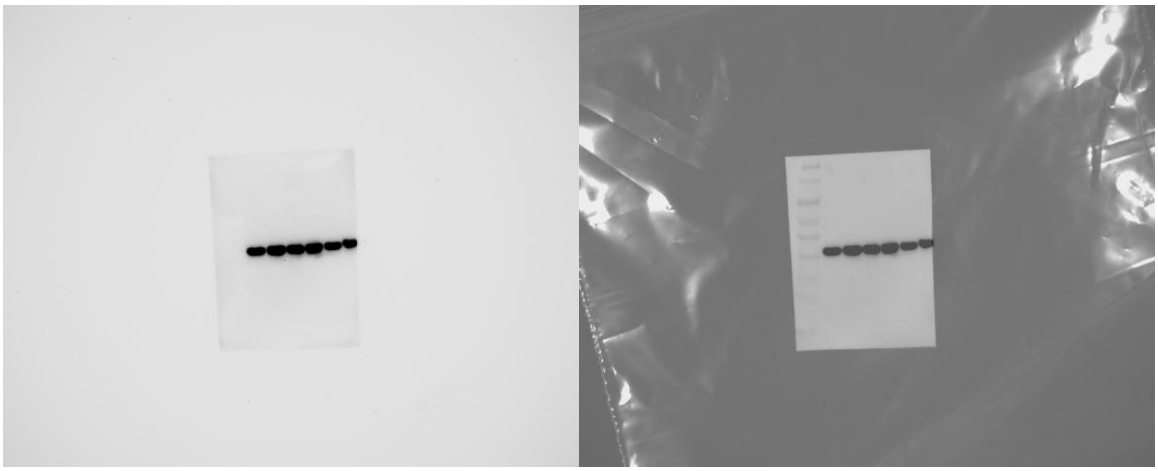


Figure S4B (Figure shows relevant lanes for experiment)

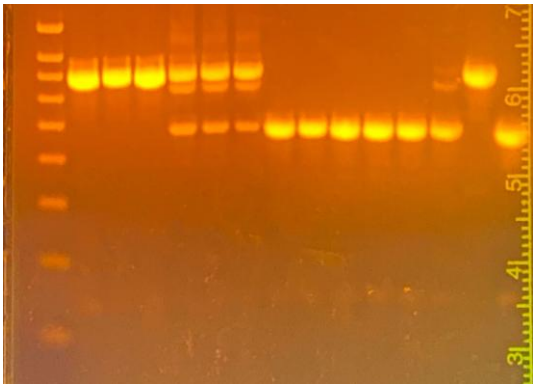


Figure S4D

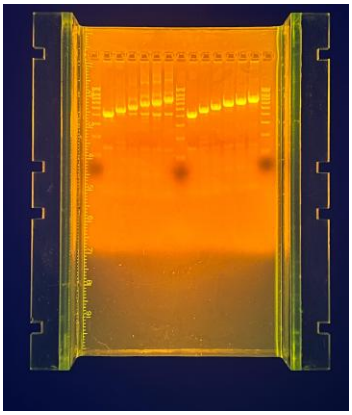


Figure S4G (Figure shows relevant lanes for experiment)

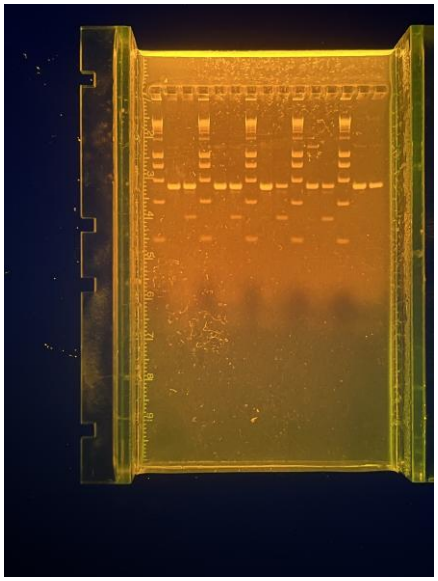


Figure S18F (Figure shows relevant lanes for experiment)