Manipulation of chromatin to enhance CRISPR activity

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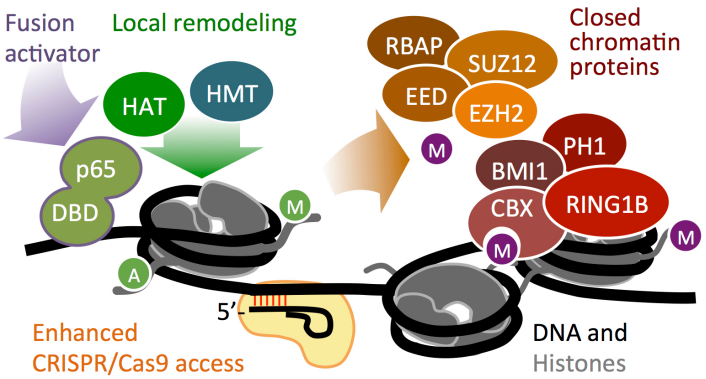

ABSTRACT

Recently, we demonstrated that closed, silenced chromatin impedes Cas9 editing by reducing the accessibility of DNA to Cas9/sgRNA binding at a chromosomal transgene. Here, we compared two methods for modifying chromatin to enhance Cas9 efficiency: a chromatin-disrupting inhibitor drug, UNC1999 and a transiently expressed targeted transcriptional activator, Gal4-p65. In both cases, we observed loss of the silencing mark histone 3 lysine 27 trimethylation (H3K27me3). Only Gal4-p65 treatment increased target gene expression. Our results confirm the previous observation that Gal4-p65-induced over-expression impedes Cas9 activity. We show that a recovery period of 9 days after Gal4-p65 expression generates a more Cas9-permissive state. Cas9 activity was restored following local modification of chromatin with transiently expressed Gal4-p65 while no enhancement was observed with UNC1999. These results implicate activator-mediated chromatin remodeling as an effective method to enhance Cas9 editing at closed chromatin.

INTRODUCTION

CRISPR/Cas9, a nucleic acid modifying system derived from prokaryotes, is a popular tool for rapid, high-throughput genome engineering.[1,2] However, recent work has shown that chromatin, the RNA-DNA-protein complex that controls chromosomal organization and gene expression in mammalian nuclei,[3–5] inhibits Cas9 binding and nuclease activity *in vitro*[6–8] and in mammalian cells.[9,10] While many Cas9 gene editing efforts have been successful, the complex structure of chromatin and its variation in different cells still pose a barrier to reliable and consistent use.[8–11]

A growing body of work has determined the strong influence of chromatin states on the accessibility of DNA to Cas9, guide RNA complexes (Cas9/gRNA), and Cas9 editing efficiency.[8–11] A series of *in vitro* studies used reconstituted nucleosomes complexed with synthetic DNA to demonstrate that Cas9-mediated binding and cleaving of nucleosome-bound DNA is completely blocked.[6–8] It is important to note here that the synthetic sequence associates more tightly with nucleosomes than natural sequences.[12,13] Utilizing transgenic cell lines, we and others showed that ectopic constitutive HP1-mediated[9] and facultative Polycomb Repressive Complex (PRC)-mediated[10] heterochromatin inhibit, and in some cases completely block, Cas9 binding and editing in HEK293 cell lines. Editing at three out of nine sites within PRC-repressed chromatin was not blocked, suggesting that inhibition of Cas9 can be discontinuous across closed chromatin regions.[10] Chromatin-mediated inhibition of Cas9 was also demonstrated *in vivo* (zebrafish).[14] Research so far suggests that disruption of closed chromatin might support a Cas9-permissive state. To achieve control over Cas9-mediated editing in heterochromatin, further investigation of the relationship between chromatin states, gene expression, and Cas9 editing in cells is needed.

To this end, we investigated methods for improving Cas9 editing by artificially opening chromatin previously inhibited by Polycomb-mediated heterochromatin (Fig. 1A). Polycomb group (PcG) proteins are critical for controlling gene expression during stem cell maintenance[19,20] and oncogenesis[21–24] by depositing and maintaining the silencing mark histone 3 lysine 27 trimethylation (H3K27me3). Polycomb-regulated genes are distributed over thousands of sites along chromosome arms in gene-rich regions. Therefore, PRC's represent a significant barrier to genome editing. In our previous work, we demonstrated that artificial conversion of closed chromatin via siRNA-mediated knockdown of the PcG protein Suz12 was accompanied by an increase in Cas9-mediated editing efficiency.[10] However, it is difficult to control the consistency and

magnitude of siRNA delivery. Therefore, we set out to explore alternative methods to induce an open, Cas9-accessible state at sites of previously silenced chromatin.

Here, we expanded our investigation to two methods for disrupting closed chromatin: a PcG-inhibiting drug (Fig. 1B) and site-directed chromatin modification with a targeted transcriptional activator fusion protein (Fig. 1C). Experiments with transcription-activating proteins in cells[18] and with nucleosome arrays *in vitro*[7,8] suggest that targeting silenced chromatin for remodeling could improve Cas9 editing in human cells. To test this hypothesis, we determined changes in transgene expression and histone marks following chromatin modification. We next determined the effect of global and targeted chromatin reopening approaches on Cas9 efficiency. We found that strong induction of transcription from a nearby promoter followed by culturing for several days to allow recovery of a generally permissive chromatin state restored full Cas9 activity at a target site downstream of the promoter. Our results inform a general approach for overcoming challenges associated with site-specific Cas9 inaccessibility.

RESULTS AND DISCUSSION

We observed that depletion of H3K27me3 via UNC1999 was not sufficient to generate an expression-permissive state. We hypothesized that treatment with with a broad acting, PcG-inhibiting drug would disrupt closed chromatin and allow for Cas9 to bind and edit a previously blocked target site (Fig. 1B). UNC1999 is a small molecule drug that disrupts heterochromatin in mammalian cells by binding to the active site of the methyltransferase Enhancer of zeste 2 (EZH2) to prevent the methylation at H3K27me3, a modification that supports PRC formation and a closed chromatin state.[17] We chose UNC1999 because it can be delivered to difficult-to-transfect cell lines via dilution in growth media, has been used for complete depletion of H3K27me3 in MCF7 and HEK293 cells, and is not toxic to mice.[17] We used Gal4EED/luc cells as a model system for PRC-mediated closed chromatin.[10,25] Upon treatment with doxycycline (dox), the cells express a Gal4-embryonic ectoderm development (EED) fusion protein which binds to a Gal4 enhancer sequence (UAS) upstream of a *luciferase* transgene. Gal4EED recruits PcG proteins, resulting in accumulation of the H3K27me3 silencing mark and *luciferase* silencing. We used the Luc14 cell line, which does not contain Gal4EED, as an open chromatin control. The absence of the silencing mark H3K27me3 was confirmed previously in Daer et al. 2017. The luciferase transgene was inserted via random chromosomal integration.[25] Based on a series of PCR's with overlapping amplicons that spanned the transgene, we estimated that the transgene-genome boundary was generated 2050 to 2457 bp upstream of the nucleation site (Gal4 UAS) (Fig. S1). We induced

repressive chromatin at *luciferase* with dox-supplemented media for 96 hours. At 120 hours, we added a sub-lethal dose of UNC1999 (10 μM) for 120 hours (see Table S1 for kill curve results). At 264 hours, we measured Luciferase activity and performed chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) to determine H3K27me3 occupancy.

While UNC1999 did not significantly increase Luciferase activity (Fig. 2A), we saw a significant decrease in H3K27me3 compared to the DMSO control ($p < 0.01$) (Fig. 2B). We conclude that at 10 μM, UNC1999 can effectively remove the silencing mark H3K27me3 from the silenced transgene but does not initiate transcriptional activation under the conditions tested here. This finding is consistent with previous work where removal of the H3K27me3 silencing mark alone did not activate gene expression in embryonic stem cells,[26] and DNA methylation and gene silencing persisted after depletion of EZH2 in cancer cells.[27]

Next, we investigated whether removal of H3K27me3 is sufficient to improve Cas9 editing. We discovered that UNC1999 treatment did not restore Cas9-mediated editing to levels observed in open chromatin. We compared Cas9 activity after the same treatments we used for the expression and ChIP-qPCR assays. Briefly, cells were silenced with dox and then treated with either vehicle control DMSO or UNC1999 (Fig. 3A). At 24 hours post DMSO or UNC1999 removal, we transfected with Cas9/sgRNA expressing plasmids with sgRNAs targeting sg046, 32, 25, and 48 (Fig. 3B). Sg046, 32, and 48 were chosen to represent sites where Cas9 activity is inhibited by closed chromatin.[10] These sites also represent varying distances from the heterochromatin nucleation site upstream of the transcription start site. Sg046 and 32 are proximal to the Gal4 UAS sequence (within 300 bp) while sg048 is distal (>1000 bp downstream of the TSS). Sg025 is a target site that showed no change in editing efficiency within closed chromatin[10] and was therefore included as a control. Their sequences are listed in Supplementary Table S3. At 72 hours post transfection, we collected the treated cells and assayed for Cas9 editing using deep sequencing to quantify the proportions of insertion and deletion (INDEL) variants generated by non-homologous end-joining (NHEJ) repair. We confirmed that mean editing was reduced at sg046, sg032, and sg048 in closed chromatin, vehicle-treated (DMSO) cells compared to the open chromatin control (sg046 $p < 0.001$, sg032 and sg046 $p < 0.005$) (Fig. 3B). UNC1999 treatment did not restore Cas9 editing at any of the four target sites tested. Furthermore, at two target sites, treatment with UNC1999 showed an even greater inhibition than in the vehicle control (sg046 $p < 0.001$ and sg025 $p < 0.01$). The position of the most frequently edited sites (the nucleotide immediately adjacent the predicted cut site) was the same under all conditions (Fig. 3C), suggesting that

treatment with UNC1999 did not alter the pattern of editing. We conclude that UNC1999 treatment under these conditions does not improve Cas9-mediated editing at sites in heterochromatin.

Next, we used a fusion activator protein to modify the closed state and found that Gal4-p65 changes both the transcription state (from silenced to active) and histone modifications. Our previous work demonstrated that a fusion transcriptional activator composed of the Gal4 DNA binding domain and the p65 protein (NF-kB) can activate gene expression at a PcG-silenced *luciferase* gene.[10] P65 is known to interact with several transcriptional activators, which potentially counteract PcG: histone acetyltransferases (CREBBP and EP300), H3K4 methyltransferases (EHMT1 and SETD7), and others (STRING database[28]). Here, we tested whether the transcription-activating fusion protein Gal4-p65 induces sustained transcriptional activation and chromatin remodeling at the PcG-silenced *luciferase* transgene. We measured the change in *luciferase* expression, the silencing-associated H3K27me3 mark, and the activation-associated H3K4me3 mark 9 days after treatment with the Gal4-p65 activator. We detected strong and sustained re-activation of *luciferase* at 3 days and 9 days following transfection with Gal4-p65-expressing plasmids (Fig. 4A), consistent with our previously reported results.[10]

Nine days after transfection with the Gal4-p65 expressing plasmid, we saw a significant reduction in the amount of H3K27me3 ($p < 0.005$) (Fig. 4B), comparable to UNC1999 treatment. To determine whether p65 localization results in addition of active chromatin modifications, we tested for the presence of H3K4me3. We observed some reduction of H3K4me3 in the silenced state compared to Luc14 ($p = 0.06$) (Fig. 4B) and a significant increase of H3K4me3 in the reopened state versus the silenced state ($p < 0.01$). Interestingly, our data show that the active and silenced states both have the H3K4me3 mark (Fig. 4B), which is associated with bivalent promoters and often seen together with H3K27me3.[29] This increase in H3K4me3 accompanies *luciferase* activation, suggesting that a greater proportion of H3K4me3 to H3K27me3 is important for expression.

Gal4-p65-induced activation of *luciferase* and the accompanying changes in chromatin structure (Fig. 4B) led us to hypothesize that local chromatin remodeling via Gal4-p65 would enhance Cas9-mediated editing at a site that was previously blocked by heterochromatin (Figure 1C). In previous work we tested this idea by co-transfecting *luciferase*-silenced HEK293 cells with a Gal4-p65-expressing and Cas9/gRNA-expressing plasmids.[10] *Luciferase* became activated, suggesting a transcriptionally permissive state, but editing efficiency was not significantly higher at the target site we tested, sg034. We had also observed a decrease in editing efficiency at sg034 in

the open chromatin state that was hyper-activated by Gal4-p65 suggesting competition between Gal4-p65 and Cas9/gRNA might contribute to reduction in Cas9 activity. To investigate further, here we carried out the same treatment (co-expression of Gal4-p65 with CRISPR plasmids) at sites located in the same 1.1 kb region as sg034, downstream of the PcG nucleation site. We detected editing efficiencies at or below closed chromatin levels for sg032, 25, and 48 or sg046, respectively, (Fig. 5B, Closed +Gal4-p65).[10] The most frequent INDELs showed no obvious change in position relative the predicted cut sites (Fig. 5C), suggesting no change in Cas9 specificity or NHEJ products.

To reconcile the opposing effects of Gal4-p65 targeting, induction of an active chromatin state versus inhibition of Cas9's access to DNA, we asked whether transient Gal4-p65 expression followed by a recovery period to allow for depletion of Gal4-p65 (referred to here as "pre-treatment") would generate a Cas9-accessible state. Having established that transcription and activation-associated histone modifications were sustained 9 days after treatment with Gal4-p65 (Fig. 4), we compared pre-treatment with Gal4-p65 would improve Cas9 editing compared to co-treatment with Gal4-p65 and untreated (closed chromatin control) (Fig. 5A). For pre-treatment, we transfected the Gal4-p65 plasmid alone and then the Cas9/gRNA plasmid 9 days later (2 passages) as described earlier. As with co-treatment, we did not observe any significant differences in editing efficiency at the target site sg025. At one proximal site (sg032) and the distal site (sg048), the mean editing efficiency approached full recovery (open chromatin levels). The most significant enhancement of editing was observed at the distal site sg048 ($p < 0.005$, Closed +Gal4-p65, 9 day recovery versus closed chromatin). The target site closest to the heterochromatin nucleation site and the TSS, sg046, showed no significant increase in editing compared to the closed chromatin control. Editing was significantly higher than in the Gal4-p65 co-treated cells ($p < 0.01$), suggesting that the site was initially blocked during Gal4-p65 over-expression, but did not take on a completely Cas9-permissive state after recovery. Overall, these results suggest that initial, transient overexpression results in Gal4-p65 accumulation and activator recruitment that inhibits Cas9 activity by blocking access of Cas9/gRNA to the target sites. This is followed by gradual remodeling of chromatin into a state that enables Cas9-mediated editing (Fig. 5) at specific sites.

In summary, we investigated methods for improving Cas9 editing at sites located in silenced chromatin. First we tested how artificial opening of closed chromatin changes gene expression and chromatin marks. Next we looked at how these changes affect Cas9 editing activity. We compared

two approaches: indirect activation via the disruption of closed chromatin (Fig. 3A) and fusion-mediated activation (Fig. 5A). We targeted Cas9 to four sites along a *luciferase* transgene, three of which show inhibition by heterochromatin (sg046, 32, and 48) and one control site that does not show inhibition (sg025). Removal of the silencing mark H3K27me3 by a global chromatin inhibitor drug was not sufficient to recover Cas9 editing. We confirmed our observation from previous work that strong transcriptional activation inhibits Cas9 activity.[10] We identified pretreatment with a transcriptional activator as a means to remodel heterochromatin to enhance Cas9 access to target DNA.

While UNC1999 effectively removed H3K27me3 from the *luciferase* transgene (Fig. 2B), it was not sufficient to induce gene activation (Fig. 2A) or enhance Cas9 editing at the sites tested (Fig. 3B and C). Our results support previous observations where removal of H3K27me3 did not result in gene activation[26] or remodeling of chromatin to an open state; gene silencing and other repressive modifications can persist after loss of PcG proteins.[27] We conclude that incompletely activated chromatin is not sufficient to improve DNA accessibility to Cas9-mediated editing (Fig. 3B and C). However, broad acting chromatin remodelers are worth further investigation. They provide several potential advantages over fusion protein-dependent approaches. Inhibitors enable manipulation of chromatin at many sites at once, allowing for multiplex targeting of difficult sites. Furthermore, there are target sites in which gene activation may not be desirable, such as master transcriptional regulators or oncogenes. Low molecular weight compounds do not require transfection and can affect sites for which DNA-binding modules cannot be designed (e.g., low complexity sequences). Future work should investigate additional chromatin inhibiting drugs and drug combinations such as histone deacetylase (HDAC) inhibitors and DNA methyltransferase (DNMT) inhibitors for their impact on Cas9 activity in heterochromatin.

We have determined specific conditions in which targeted hyper-activation can generate a Cas9-accessible state in the ectopic chromatin model system used here. Pre-treatment with the Gal4-p65 activator shows promising improvement in editing at a target site previously inhibited by PcG-mediated heterochromatin (Fig. 5B and C). We confirmed our previous observation that high-transcriptional activity can impede Cas9 editing possibly through steric hindrance and competition with the high occupancy of the Gal4 UAS with activator proteins and RNA PolII. Co-treatment with the Gal4-p65 activator showed no improvement in editing and, at one site, significant inhibition compared to the silenced state (Fig. 5B and C). In contrast, pre-treatment with the activator caused removal of the H3K27me3 silencing mark, addition of the H3K4me3 activating

mark, gene activation (Fig. 4), and enhanced Cas9 editing near open-chromatin levels at target sites previously inhibited by closed chromatin (Fig. 5B and C). This suggests that chromatin remodelers recruited by Gal4-p65 or transcriptional elongation remove the nucleosomes or the silencing marks. The key advantage of the activator Gal4-p65, or in general any DNA-binding domain with an effective transcriptional activator, is the ability to target a single genomic locus without disrupting the expression of other genes. We demonstrated that targeted remodeling using a strong activator (p65) enhances Cas9-mediated editing >1000 bp downstream from the Gal4 binding site (UAS). To stimulate distal remodeling in a similar manner at endogenous genes, the Gal4 binding domain could be replaced with other customizable DNA binding modules such as dCas9/sgRNA, zinc fingers (ZF) or transcription activator-like (TAL) effectors and be targeted to accessible DNA sites up- or down-stream of the desired editing target, making this approach widely applicable.[18] Weak transcriptional activators or pioneer factors that remodel chromatin without creating steric hindrance to Cas9/gRNA could provide options that do not require a recovery period (e.g. the 9-day post-transfection period used here). Cas9 editing efficiency has been enhanced by proximal binding (within 100 bp) of non-enzymatic dCas9 [30,31]. Barkal et al. showed that the underlying mechanism is local chromatin remodeling.[31] In conclusion, our study lays the foundation for a new fusion-activator based strategy to enable Cas9-mediated editing in heterochromatin.

METHODS

Cell Lines and Plasmids

Cas9 transfections were done using pU6-(BbsI)_CBh-Cas9-T2A-EGFP (DNASU UnSC00746685) built from pX330-U6-Chimeric_BB-CBh-hSpCas9 (a gift from Feng Zhang, Addgene plasmid #42230) (described in Daer et al. 2017). The pU6-(BbsI)_CBh-Cas9-T2A-EGFP expresses the human optimized spCas9 protein and EGFP from the same mRNA transcript. Guide RNAs sg025, sg032, sg046, and sg048 were cloned into pU6-(BbsI)_CBh-Cas9-T2A-EGFP (described in Daer et al. 2017). Gal4-p65 transfections were done with plasmid CMV-Gal4p65_MV1 (DNASU UnSC00746686) which expresses the Gal4-mCherry-p65 fusion protein from a CMV promoter. Sequences for each plasmid used in this study are available online with annotations at https://benchling.com/hayneslab/f_/V1mVw1Lp-chromatin-crispr-interference/.

Cell Culturing and Transfection

Cell culturing, luciferase silencing, and transfections of the Luc14 and GAL4EED HEK293 cell lines were performed as described in Daer et al. 2017. Briefly, cells were transfected in 12-well plates

with Lipid-DNA complexes (500 ng plasmid, 3 µl Lipofectamine LTX, 1 µl Plus Reagent (Life Technologies)) and cells collected for analyses 72 h post transfection for all but the p65 pre-treatment assays. EZH2 inhibition was carried out with UNC1999 (Cayman Chemical) dissolved in DMSO to 10 mM. Toxicity was determined using a dilution curve (Table S1). Gal4EED cells were treated for 96 hours with dox to induce silencing. After 24 hours without dox, cells were treated with 10 mM UNC1999 by diluting 1 µl of 10 mM UNC1999 per 1 ml media for 72 h. Control cells were treated with 1 µl of DMSO (vehicle) per 1 ml media. Cells were grown for 1 day in media without UNC1999 prior to Luciferase assays (described below), transfection with Cas9/sgRNA plasmids (described above) or ChIP-qPCR (Supporting Information).

Luciferase Assays

Transfection efficiencies for p65 transfected cells were determined by measuring the percent of cells that were mCherry positive using the BD Accuri C6 Flow Cytometer and accompanying software (BD Biosciences) (described previously in Daer et al). Cell counts to normalize Luciferase activity were determined by counting the number of live cells in 20 µl from each sample by flow cytometry. Luciferase assay was performed in triplicate with a Synergy H1 Multi-Mode Reader (Biotek) using the Steady-Luc Firefly HTS Assay Kit (Biotium) (described previously in Daer et al). Luciferase activity normalized per cell was calculated:

$$Luciferase \times cell^{-1} = (Luciferase\ signal - background\ signal) \div cell\ count\ in\ 20\ \mu l \times (100\ \mu l \div 20\ \mu l)$$

Cas9 Activity Assays

After 72 h of growth post-transfection with pU6-(BbsI)_CBh-Cas9-T2A-EGFP (DNASU UnSC00746685) with sgRNAs sg025, sg032, sg046, and sg048,[10] cells were collected. Transfection efficiency was determined as described above. Genomic DNA was extracted from the remaining 200 µl of cells using the QIAamp DNA Mini Kit (Qiagen) and eluted in 100 µl of nuclease-free water. PCR of gDNA was performed using GoTaq 2x Mastermix (Promega) using primers P198/198 (see Table S3 for sequences) [95°C for 10 min; 34x (95°C, 30 sec; 58°C, 30 sec; 72°C, 20 sec.)]. Nested PCR was performed by diluting 2 µl of PCR product into 500 µl of water and Phusion High-Fidelity DNA Polymerase (Thermo Scientific) (primers described in Table S2 and S3). PCR reactions were purified using the Genelute PCR Cleanup Kit (Sigma-Aldrich) and submitted to the Center for Computational and Integrative Biology (CCIB) Core Facility (Massachusetts General Hospital) according to their requirements for sequencing.

Quantitative PCR of ChIP DNA

Chromatin Immunoprecipitation is described in Supporting Information. Relative quantification of ChIP samples was performed using real-time quantitative PCR and SYBR Green I master mix (Roche) as previously described.[10] To adjust for input dilution, log2(20) was subtracted from input Cp values. Enrichment of H3K27me3 and H3K4me3 (%IP DNA bound of input) was calculated as 100 x 2^(Cp_{input} - Cp_{IP}).

Luciferase primer sequences:
P360 5'-CGGCGCCATTCTATCCTCTA-3' (forward);
P361 5'-ATTCCGCGTACGTGATGTTC-3' (reverse)

TBP primer sequences:
P363 5'-CAGGGGTTCAGTGAGGTCG-3' (forward);
P364 5'-CCCTGGGTCACTGCAAAGAT-3' (reverse)

STATISTICAL ANALYSES

The percentage of edited reads was normalized to transfection efficiency for each sample. Averages and standard deviations were calculated for each of n = 3 biological replicates. To analyze ChIP-qPCR assay data, averages and standard deviations were calculated for each of n = 3 replicate IPs from a single chromatin preparation. For each cell/treatment data set, %IP DNA for site 1 and TBP were normalized to %IP DNA for TBP. The differences of means to compare cell/ treatment data sets for both CRISPR editing and ChIP-qPCR experiments were calculated using the two sample, one-tailed Student's t test. For $p < 0.05$, 95% confidence with 2 degrees of freedom and a test statistic of $t_{(0.05,2)} = 2.920$. For $p < 0.01$, 99% confidence with 2 degrees of freedom and a test statistic of $t_{(0.01,2)} = 6.965$.

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COMPETING INTERESTS

Authors R. Daer and K. Haynes have filed a provisional patent application for methods included in this manuscript.

SUPPORTING INFORMATION. Figure S1. PCR analysis of the *pUAS-TK-luciferase* transgene; Table S1. UNC1999 kill curve; Table S2. List of primers for PCR of genomic DNA from Cas9/sgRNA treated samples; Table S3. Sequences of primers from Table S2.; Method: Crosslinked Chromatin Immunoprecipitation

ABBREVIATIONS. PRC = Polycomb Repressive Complex; PcG = Polycomb group

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FIGURES AND LEGENDS

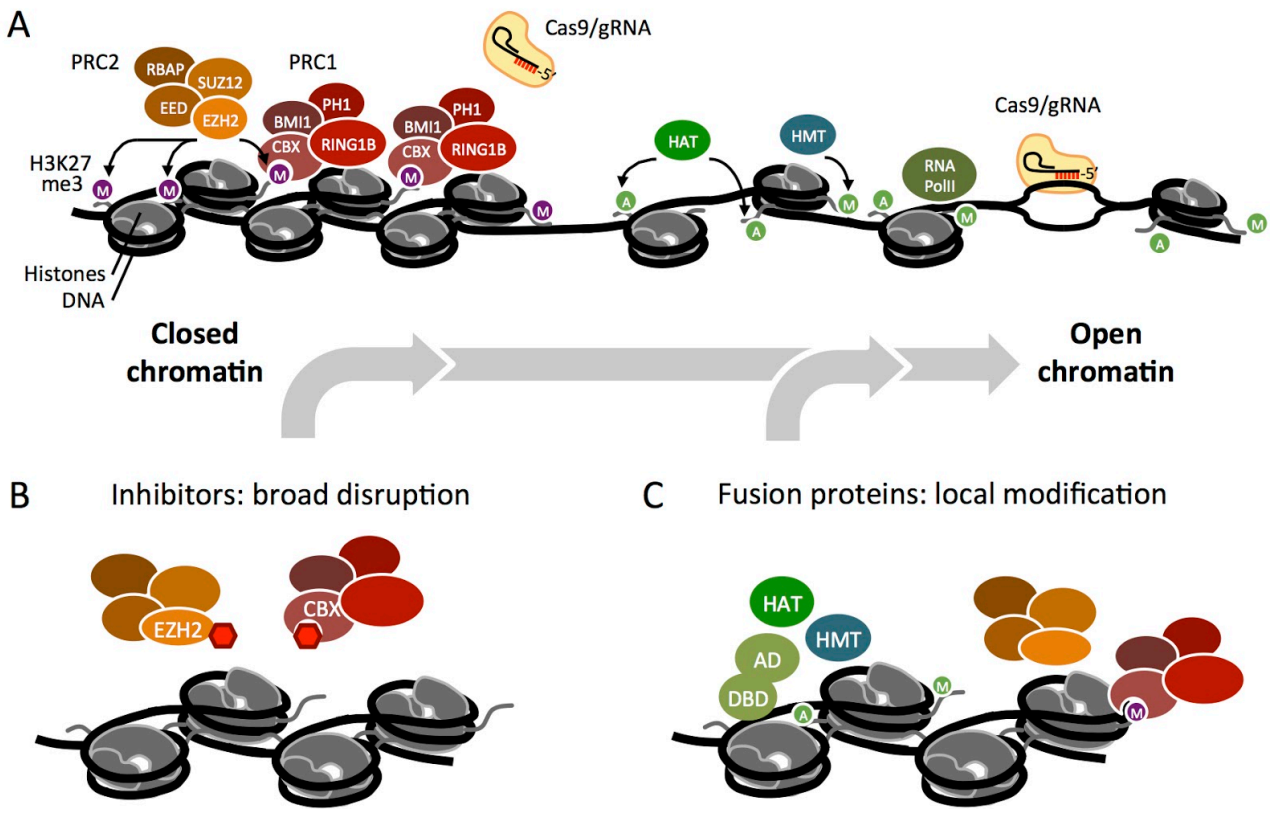

Figure 1. Research of Cas9 activity in chromatin suggests that facultative heterochromatin inhibits Cas9 editing while open chromatin is permissive to Cas9. (A) The PRC2 (Polycomb repressive complex 2) holoenzyme generates the silencing mark histone 3 lysine 27 trimethylation (H3K27me3) (purple M). PRC2 includes Suppressor of Zeste 12 (SUZ12), Embryonic ectoderm development (EED), Retinoblastoma-binding protein (RbAp), and Enhancer of zeste 2 (EZH2).[3,5] Polycomb repressive complex 1 (PRC1) includes Chromobox protein homolog (CBX), Ring finger protein 1b (RING1B), and Polycomb group RING finger protein 4 (BMI1).[3,5] Polycomb proteins support histone compaction and block access of DNA to RNA polymerase and Cas9.[9,10] Chromatin remodelers (not shown), histone acetyltransferases (HATs), and histone methyltransferases (HMTs), generate modifications that support open chromatin, accessible DNA, and a transcriptionally permissive state.[15,16] (B) Inhibitors (red hexagons) of chromatin-silencing proteins disrupt closed chromatin in a global, non-specific manner.[17] (C) Synthetic fusion proteins containing a DNA binding domain (DBD) and activating domain (AD) can be used to recruit chromatin-remodeling proteins to a specific locus.[18]

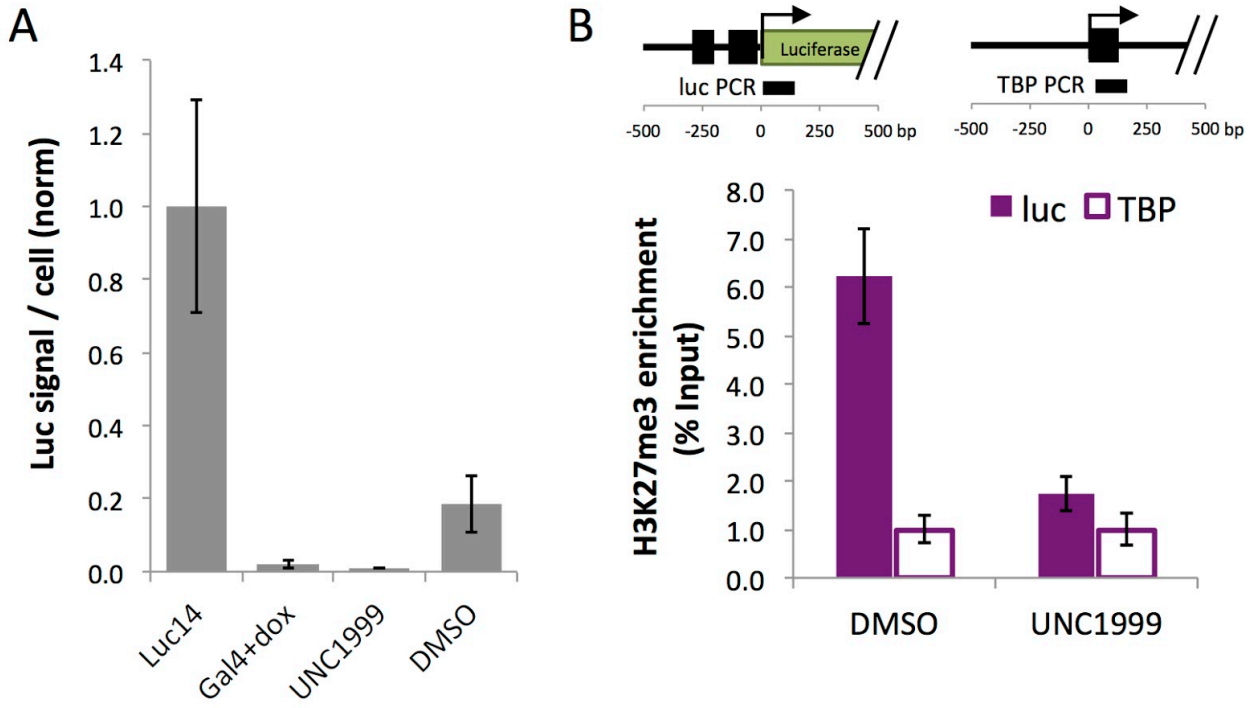

Figure 2. UNC1999 depletes histone 3 lysine 27 trimethylation (H3K27me3). *Luciferase* expression in UNC1999-treated cells was near or below luciferase levels in untreated cells (Gal4+dox) and cells treated with the vehicle control (DMSO). Error bars indicate standard deviation of $n = 3$ biological replicates. (B) Chromatin immunoprecipitation assay followed by quantitative PCR (ChIP-qPCR) showed significant reduction in H3K27me3 enrichment at *luciferase* after treatment with UNC1999 compared to the vehicle control ($p < 0.01$). A constitutively active control locus, TATA-binding protein (TBP), was used as a negative control for ChIP-enrichment of H3K27me3. Error bars indicate standard deviation of $n = 3$ replicate pulldowns from a single chromatin prep.

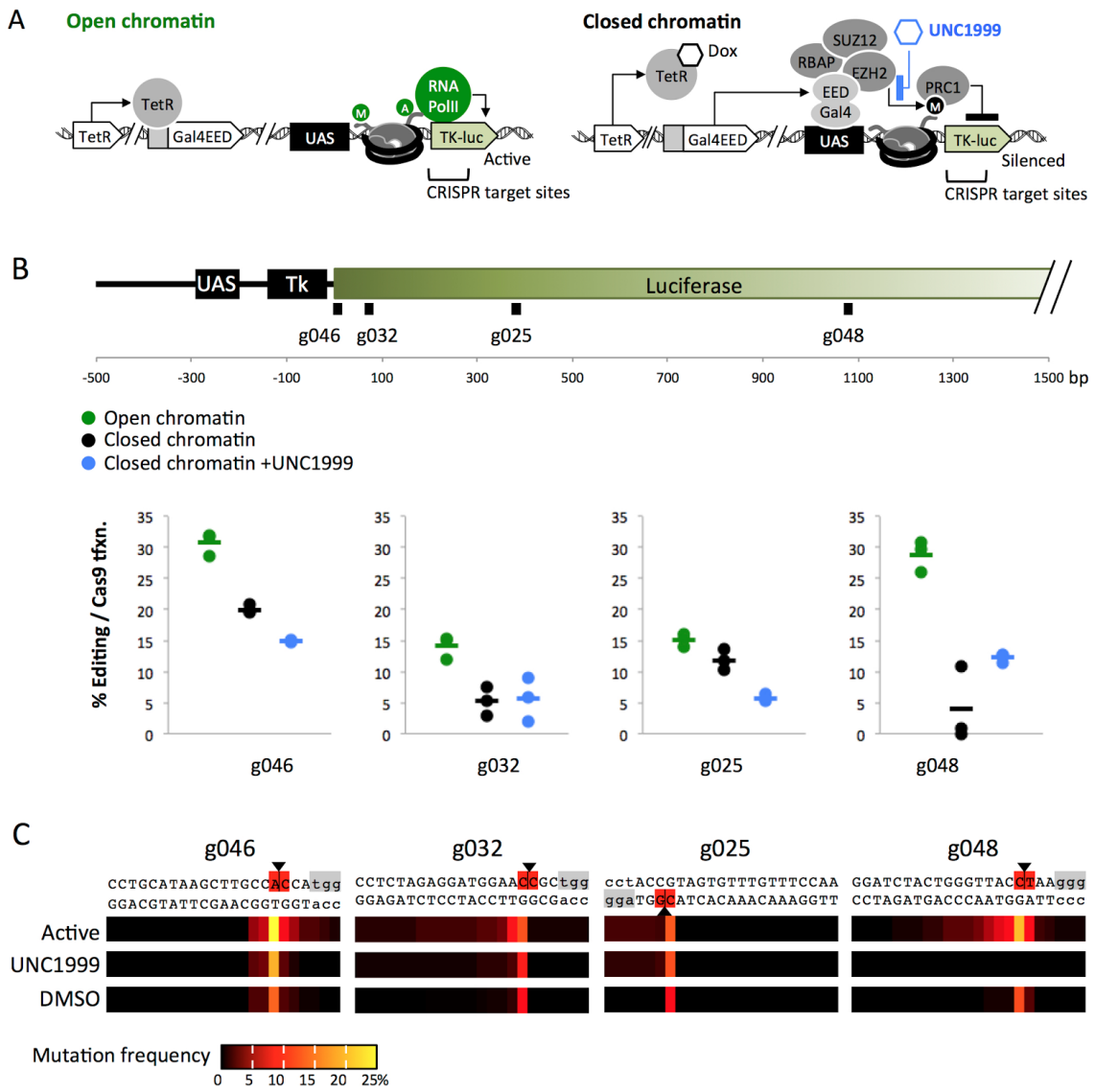

Figure 3. UNC1999 effect on Cas9 editing. (A) Overview of the experimental approach. Open chromatin is characterized by transcription of *luciferase* and absence of the silencing mark histone 3 lysine 27 trimethylation (H3K27me3).[10] Closed chromatin is induced upon addition of doxycycline (dox) and is characterized by reduced expression at *luciferase* (Fig. 2A) and accumulation of H3K27me3 (Fig. 2B and Daer et al. 2017). Treatment with UNC1999 (blue hexagon) inhibits Enhancer of zeste 2 (EZH2), resulting in the loss of H3K27me3 (Fig. 2B and Konze et al. 2013). (B) Map of the *luciferase* transgene and the sgRNA target sites. Charts show Cas9 editing efficiencies (deep sequencing data) at each target site for three different conditions. "Open chromatin" (green) represents untreated Luc14 cells transfected with Cas9/sgRNA expressing plasmids with sgRNAs targeting sg046, 32, 25, and 48. "Closed chromatin" (black) and "Closed chromatin +UNC1999" (blue) are Gal4EED/luc+dox silenced cells treated with either the vehicle (DMSO) or UNC1999, respectively. For all samples, editing efficiencies were measured 72 hours post transfection with Cas9/sgRNA plasmids. Bars indicate the mean of three biological replicates (dots). (C) Heat maps indicate the frequency at which each DNA base position was affected by an insertion or deletion (INDEL).

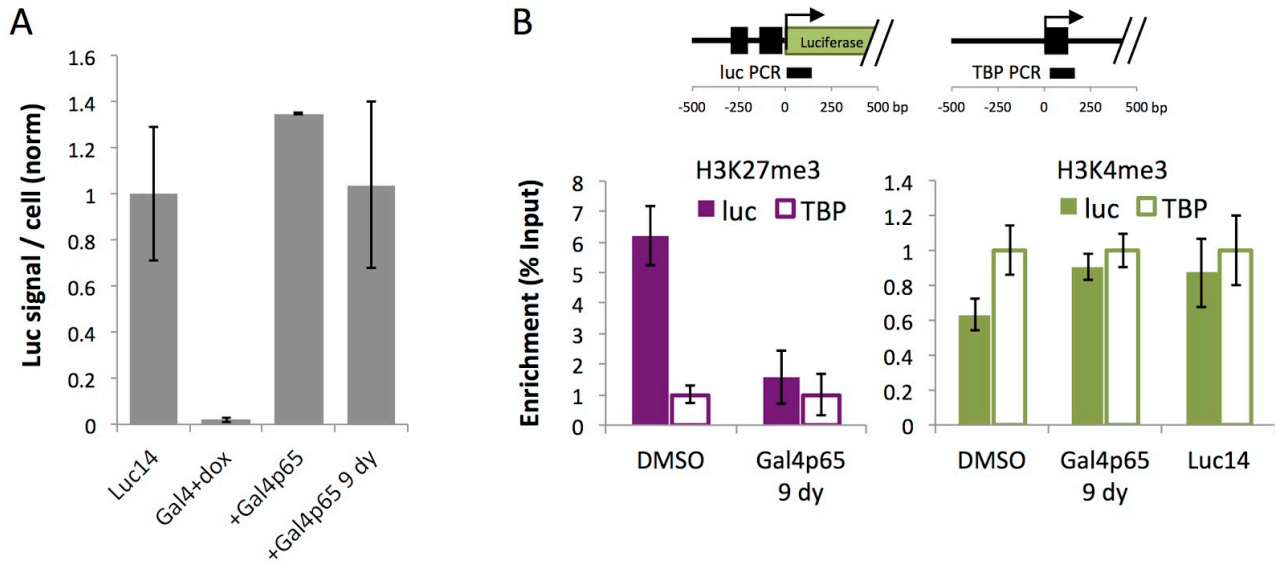

Figure 4. Targeted transcriptional activator Gal4-p65 increases *luciferase* expression and modifies chromatin. GAL4EED cells were treated with doxycycline (dox) for 96 hours and transfected with the Gal4-p65 expressing plasmid. (A) Gal4-p65 increases gene expression at the previously silenced *luciferase* transgene. Luciferase activity was measured after 72 hours or 216 hours (9 days). Error bars indicate standard deviation of *n* = 3 biological replicates. (B) Gal4-p65 reduces H3K27me3 at the *luciferase* transgene. H3K4me3 is present in each chromatin state but increased after treatment with Gal4-p65. Error bars indicate standard deviation of *n* = 3 replicate pulldowns from a single chromatin prep.

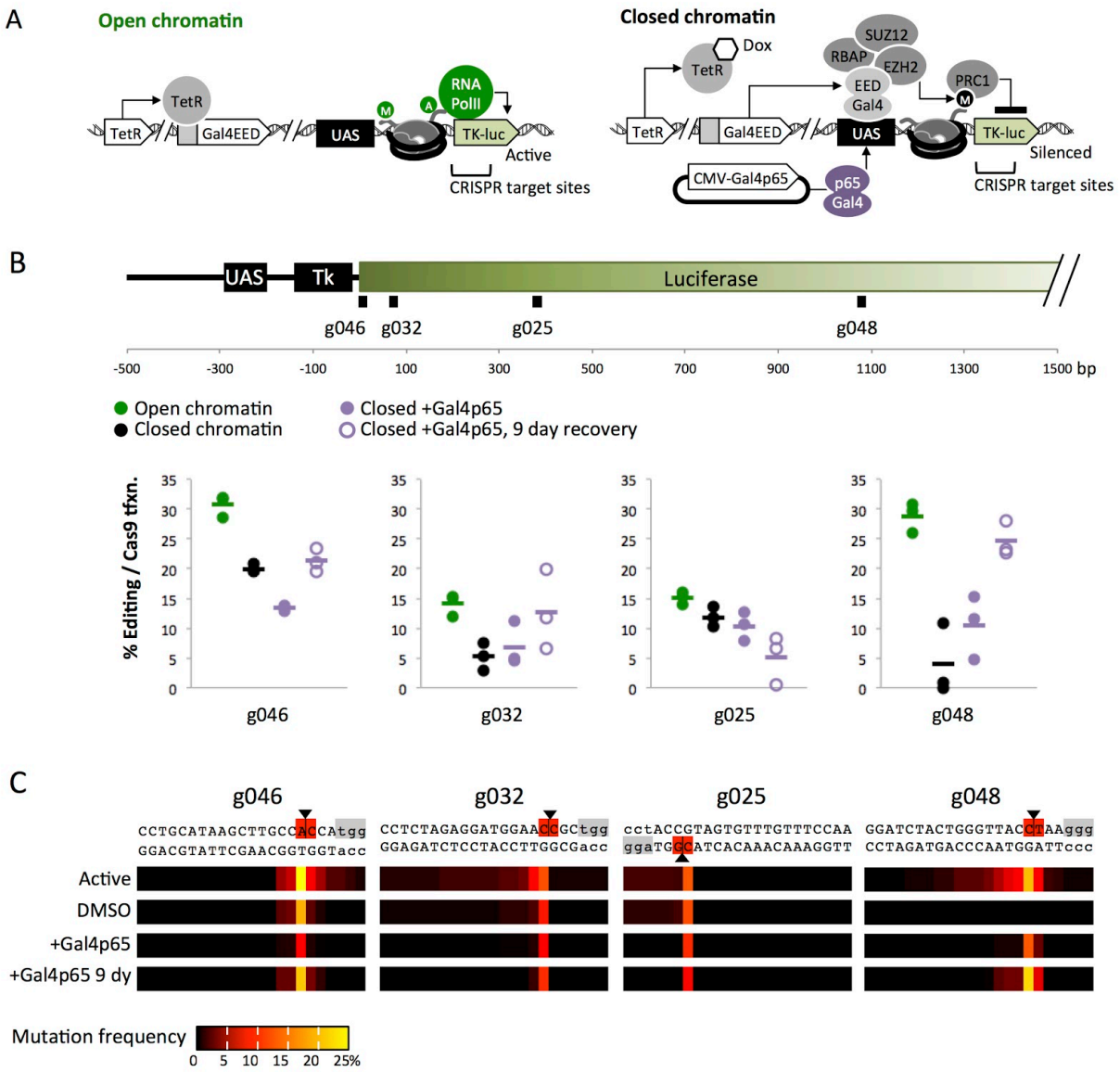

Figure 5. Pre-treatment with a transcriptional activator improves Cas9 editing at closed chromatin while co-treatment does not. (A) Summary of the experimental approach. Chromatin states are shown as described in Figure 3. Dox-treated Gal4EED/luc cells were transfected with the CMV-Gal4-p65 plasmid, which results in long-term activation of *luciferase* expression and local chromatin remodeling (Fig. 4B). (B) Comparison of Cas9 editing efficiencies at each sgRNA target site for different chromatin states. The luciferase map, open chromatin, and closed chromatin samples are described in Figure 3. "Closed +Gal4-p65" (purple filled) are Gal4EED/luc+dox silenced cells that were co-transfected with CMV-Gal4-p65 plasmid and Cas9/sgRNA expressing plasmids with sgRNAs targeting sg046, 32, 25, and 48 at 24 hours after dox removal. "Closed +Gal4-p65, 9 day recovery" (purple outlined) are Gal4+dox silenced cells transfected with only CMV-Gal4-p65 plasmid at 24 hours post dox removal, then cultured for 216 hours (9 days) and then transfected with Cas9/sgRNA plasmids. For all samples, editing efficiencies were measured after 72 hours of Cas9/sgRNA expression. Dots = individual replicates, bars = mean of three biological replicates. (C) Heat maps of the mutation frequency for a representative biological replicate. "Open chromatin" and "Closed chromatin" data are the same data presented in Figure 3.