Novel CRISPR-Guided Epigenome Modifiers Provide Insights Into Dynamics of Gene Regulation

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Keywords CRISPR, histone tail modifications, chromatin, epigenetics, epigenome editing, gene regulation, KRAB, HDAC8, SIRT6, SETDB2

ABSTRACT

Distinct epigenetic signatures have been associated with gene expression states. Yet questions remain about a causal link between epigenetic modifications and transcriptional activity, and the contribution of various individual marks in gene regulation. Here, we generated and characterized an array of CRISPR-guided epigenome modifiers (CRISPR-GEMs), HDAC8, SIRT6 and SETDB2, which are associated with deposition of repressive marks, alongside the commonly used KRAB repressor domain. By concomitantly measuring changes in gene expression and chromatin marks at three target genes across various time points, we found that our novel CRISPR-GEMs and KRAB mediate gene repression through multiple different epigenetic pathways. In addition, HDAC8 and SIRT6 possess unique kinetics, which allow for a faster and more transient gene repression than KRAB. Interestingly, while early time point experiments showed only a mild correlation between changes in acetylation at H3K27 or H4K16 and gene expression, we observed a strong correlation between changes in acetyl or methyl marks on H3K9 and gene expression, suggesting that these epigenetic modifications may precede gene expression changes. These results provide further evidence regarding epigenetic regulation and the relationship between transcription and chromatin marks. Furthermore, our novel CRISPR-GEMs introduce a variety of kinetics and histone tail targets to the currently available, yet modest, toolkit of epigenome editors.

INTRODUCTION

Regulation of genes is a complex and hierarchical process that is determined by various factors, often divided into genetic and epigenetic elements. Since most somatic cells share the

same genomic sequence, it has been hypothesized that the expression of a gene is determined by an epigenetic code that can also be described through a host of processes which were shown correlate with gene expression, such as DNA methylation, histone tail modifications, chromatin accessibility and DNA architecture (1-9). Among these components of the epigenetic landscape, previous studies demonstrated histone tail modifications to be main predictors of gene expression, specifically highlighting residues on histone H3 lysine 4 (H3K4), lysine 9 (H3K9), lysine 27 (H3K27), and lysine 36 (H3K36) and histone H4 lysine 20 (H4K20) to be more essential for an accurate prediction (1-3,10,11). However, there also have been multiple observations which show gene expression that deviates from the "canonical" epigenetic code (12). For example, one study showed a host of actively transcribed genes during the fly development that lack any canonical active marks, such as mono- or tri-methyl marks on H3K4, or acetyl marks on H3K9 and H3K27 (13), and another study showed that H3K27 acetylation is dispensable for enhancer activity in mouse embryonic stem cells (14). It has also been shown that H3K4 mono-methylation is not required for promoters to transcribe (15), and that in yeast genes can be transcribed from heterochromatic regions despite the absence of active chromatin marks (16). In spite of these studies, and although only a mild correlation has been observed between individual chromatin marks and gene expression levels (3), the clustering of several epigenetic marks would sometimes yield a stronger correlation (10,11,17-21), suggesting that while some chromatin marks may be causative, others may be an outcome of parallel processes. Moreover, studies that attempt to draw whole-genome correlations inherently possess several shortcomings, such as coarse resolution and relying on cells that have reached a steady state, thus possibly overlooking outliers and transient effects that may be in disagreement with the current

paradigm. For example, a study demonstrated how actively perturbing the expression of a gene may lead in itself to epigenetic changes, such as accumulation of trimethyl marks at H3K27 (22), while other studies demonstrated how changes in gene expression are subsequent to changes in chromatin (23,24). These findings, altogether, raise the possibility that the "epigenetic code" is more intricate than previously thought, and is likely to involve additional processes of feedback and crosstalk between its various components.

Thus, to better understand epigenetic regulation, it is necessary to design and develop new targeted technologies that would possess the ability to edit individual epigenetic marks. In recent years, this endeavor has been facilitated by the discovery and application of multiple gene-targeting platforms such as zinc-fingers (ZFs), transcription-activator-like effectors (TALEs) and CRISPR/Cas9 (clustered, regularly interspaced, short, palindromic repeat / CRISPR-associated protein 9). While each of these platforms was successfully utilized for targeted gene activation or repression when fused to a catalytic domain (25-28), CRISPR/Cas9 has become the leading platform for this purpose due to high specificity (29), simplicity of scaling up to target thousands of genomic regions at once (30-33), and the relative ease of adding functional modules to this platform (34-36). Prominently, the utility of the dCas9, or mutant Cas9 that is lacking the nuclease activity, for gene repression or gene activation engendered two main sub-technologies: CRISPRi (CRISPR interference) and CRISPRa (CRISPR activation). The CRISPRi and CRISPRa platforms have been both optimized and are considered a powerful tool in turning on and off transcription from gene promoters and enhancers (26,30-34,37-41). The main effector used for CRISPRi is the Krüppel-associated-box (KRAB) domain, a potent repressor domain that functions within >400 zinc finger transcription factors (PMID: 14519192),

which is derived from the ZFP10 protein (26,29). It serves as a recruitment scaffold for other proteins, including KAP-1, SETDB1, HP1 as well as multiple members from the HDAC family (74-76). In addition to its robust gene repression capability, dCas9-KRAB was shown to drive the deposition of trimethyl marks on H3K9 (H3K9me3) and H3K27 (H3K27me3) as well as the removal of acetyl marks from H3K27 (H3K27ac) (29,51). Moreover, certain studies have demonstrated that stronger or longer gene repression may be achieved when dCas9-KRAB is supplemented with MeCP2 (42), HP1 (43), and the DNA methyltransferase domains DNMT3A and DNMT3L (44-47). CRISPRa, on the other hand, has been utilized with multiple different activator domains, such as VP16 (or VP64, its tetramer) (40), P300 (37) and P65 (41). These domains and others are sometimes combined to elicit stronger gene activation (32,48). Of particular interest to this study, there is a class of CRISPR-based tools where dCas9 is tethered to an enzyme that is known to directly catalyze changes in histones tail modifications and DNA methylation, which hereon will be referred to as CRISPR-Guided Epigenome Modifiers or CRISPR-GEMs. In the past few years, only about a dozen CRISPR-GEMs, consisting of various catalytic domains, were developed and shown to effectively manipulate chromatin marks and gene expression (37,44,49-54). These studies showed that induction of gene expression was dependent on DNA methylation (44,50) and could be associated with methylation of H3K4 and H3K79 (50,53,54) or acetylation of H3K27 (37). In contrast, attempts to repress gene expression by introducing repressive marks proved to be more challenging and yielded a few unexpected results. For example, deacetylation of H3K27 showed only marginal decrease in gene expression levels (49), and enrichment of methyl marks at H3K9 or H3K27 did not always lead to reduced gene expression levels (PMID: 28973434 or 52 in the Ref. section). Interestingly, there were

cases in which gene repression was observed in the absence of changes to chromatin marks (49,52), suggesting additional modes of repression, either by steric hindrance or by recruitment of additional endogenous factors that carry an unknown effect. Overall, despite major technological advancements that allow complete epigenetic characterization of a plethora of cell types from multiple origins, it is still a fundamental challenge to disentangle cause from effect in the crosstalk between gene expression and its epigenetic landscape. One of the leading paths to tackle this problem is the creation of reliable epigenome editors that could easily and efficiently target loci of interest.

In this study, we used the CRISPR/Cas9 platform to develop novel targeted epigenome editors to catalyze specific histone tail modifications which are associated with gene repression. We focused our efforts on four chromatin marks that strongly correlate with gene expression in the promoter region and have been linked to gene dysregulation in multiple diseases: H3K9ac (55,56), H3K27ac (56,57), H3K9me3 (58-61) and the relatively underexplored H4K16ac (55,62-68).We used the catalytic domains or full-length proteins of HDAC8 (69), SIRT6 (70,71) and SETDB2 (72) to create three novel dCas9 epigenome editor fusions for the deposition or removal of the intended epigenetic marks. To account for confounding effects that may result from delivery of dCas9 or any of the CRISPR-GEMs, for each experiment we elected to test the entire array of CRISPR-GEMs and their catalytically mutated versions in addition to dCas9. An important aspect of this study was the attempt to better characterize differences in activity and kinetics between the novel CRISPR-GEMs we developed, therefore we carried out the experiments over multiple time points and at three different loci. By performing this extensive characterization, we were able to uncover meaningful differences in the kinetics of the various

novel CRISPR-GEMs, allowing future users to decide which technology would best fit their needs. For example, we show that the effect of dCas9-HDAC8 on transcription was faster in comparison to the other fusions, but also lasted for shorter periods of time. We show that the kinetics of dCas9-SIRT6 were moderately slower than dCas9-HDAC8, but also, its repressive effect among all CRISPR-GEMs was the most potent at day 4 post-delivery. In addition, we demonstrate that although dCas9-KRAB leads to significant enrichment of H3K9me3 marks within 2 days of delivery, it requires substantially longer duration to reach its fullest potential of repression. Finally, we show that changes to H3K9 marks (both acetylation and methylation) strongly correlate with later changes in gene expression, supporting multiple other papers that showed that epigenetic changes precede changes in gene expression (23,24,53, PMID: 33770473) or observed no changes in gene expression at early time points, despite enrichment of repressive marks (52).

MATERIALS AND METHODS

Plasmids

CRISPR-GEMs and their corresponding mutant versions were constructed by assembly of epigenome modifiers in place of P300core in the dCas9-P300core plasmid backbone (Addgene #83889) using Gibson assembly (NEB, #E2611L). The epigenome modifiers sequences (Supp. Table 1) were obtained either by amplification of HEK-293T cDNA with custom designed primers (HDAC8, SETDB2), by ordering a gene block (SIRT6, IDT-DNA) or by amplification from a verified plasmid (KRAB, Addgene #71236). Mutant versions of the epigenome modifiers (Supp. Table 1) were obtained by overlap extension PCR, where mutated sequences were

introduced with custom primers. Individual guide RNAs (gRNAs) sequences were ordered as oligonucleotides (IDT-DNA), phosphorylated, hybridized, and cloned via Gibson assembly into BsmBI cloning sites of a lentiviral eGFP-P2A-BlastR backbone. This plasmid originated from the optimized SpCas9-sgRNA plasmid (Addgene #51024) where Puro-mCherry was replaced with eGFP-P2A-BlastR from Addgene # 83925. To generate the HDAC8 knock-out cell line, gRNAs were cloned into a Cas9-2A-GFP backbone (Addgene #48138). gRNAs were designed using an in silico tool to predict gRNAs (PMID: 29762716). gRNA sequences are listed in (Suppl. Table 1). All plasmids were validated by Sanger sequencing and will be deposited in Addgene.

Mammalian Cell Cultures and Transfection

HEK-293T cells (ATCC, Manassas VA, obtained through the Duke University Cell Culture Facility)were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained at 37°C and 5% CO2. Depending on harvest day and type of assays, transfections were either performed in 24-well or 6-well plates using a total amount of 1000 ng or 5000 ng plasmid DNA, respectively, mixed with Lipofectamine 3000 (Life Technologies, #L3000008) as per manufacturer's instruction. To generate the HDAC8 knock-out cell line, both Cas9-gRNA plasmids were introduced to the cells in a 1:1 ratio. For gene expression and ChIP assays, CRISPR-GEMs, gRNA-1 and gRNA-2 plasmids were transfected in a 2:1:1 mass ratio, respectively. Cells were propagated until the day of harvest as described for each experiment.

Generation of HDAC8 Knock-Out (KO) Cell line

200,000 HEK-293T cells were seeded in a 24 well-plate, and transfected the next day with two all-in-one Cas9-2A-GFP plasmids (1:1 ratio) that express Cas9 and two gRNAs flanking the first exon of HDAC8. After 24 hours, the cells were trypsinized, resuspended in FACS buffer (1X PBS, 1% BSA, 2mM EDTA), and sorted (SH800 cell-sorter, Sony Biotechnology) based on positive GFP expression (top 2%) into single-cell colonies in a 96-well plate with fresh DMEM medium. Medium was replaced every 4-5 days until formed colonies were visible under a microscope (2 weeks), and then were split and expanded into 48-well plate. DNA from colonies was extracted using QuickExtract (Lucigen, #QE09050) and was amplified with HDAC8 specific primers overlapping its first exon and Q5 hot start polymerase (NEB, #M0493L), products were visualized on a 1.2% agarose gel alongside a wildtype control to assess which colonies showed the deletion band. Gel bands at the correct size were excised, extracted with QIAquick Gel Extraction Kit (Qiagen, #28706), and sequenced using commercial Sanger sequencing services. Positive colonies were propagated and used for further validation by Western blot and gene-expression assays.

Western Blot

Equal amounts (20 μg) of cell proteins were separated on a SDS PAGE and transferred onto a nitrocellulose membrane for Western blots. The membrane was blocked for 1 h at room temperature in Tris‐buffered saline (50 mM Tris‐HCl, 150 mM NaCl, pH 7.4) containing 0.1% Tween 20 and 5% non‐fat powdered milk (TBST + 5% milk), followed by overnight incubation at 4°C with a mouse α-HDAC8 antibody (Biolegend, #685503, 1ug/ml dilution in TBST + 5% milk) or an HRP-conjugated primary α-GAPDH antibody (Sigma, #G9295, 1:5000 dilution in

TBST + 5% milk). After washing and, for the α-HDAC8 blot, incubation with a horseradish peroxidase-conjugated secondary goat α-mouse antibody (Sigma, #A8924, 1:5000 dilution in TBST + 5% milk), blots were developed using a chemiluminescence kit (Clarity™ Western ECL Substrate, Bio-Rad, #170-5060) and visualized on a ChemiDoc Imager (ChemiDoc XRS+, Biorad).

RNA-seq and Selection of Target Genes

RNA-seq was performed on both wildtype and HDAC8 knock-out HEK-293T cell lines. RNA was isolated from 1-2 million cells using the RNeasy Plus mini kit (Qiagen, #74136) and 500 ng of purified mRNA was used for the preparation of both libraries with Universal Plus mRNA-Seq (Nugen, #508203) as per manufacturer's instructions. Indexed libraries were validated for quality and size distribution using the Tapestation 2200 (Agilent) and quantified by Qubit 2.0 (Invitrogen) prior to multiplex pooling and sequencing at the Duke University Genome Sequencing Shared Resource facility. Libraries were pooled and 75 bp single end reads were sequenced on a NextSeq 500 (Illumina), de-multiplexed and then aligned to the HG19 transcriptome using HISAT2. Transcript abundance was calculated using htseq-count on the Galaxy platform (73).

To identify genes that may be more amenable to editing by CRISPR-GEMs, we filtered RNA-seq results only for protein-coding genes of TPM>0.5 which also showed enrichment above 2-fold in the HDAC8 knock-out cell line compared to the wildtype HEK-293T. We then used a publicly available dataset (ENCODE project, biosample ENCBS486AAA) and identified

H3K27Ac peaks that are in close proximity to candidate genes that were previously found verified by RT-qPCR.

RNA Extraction and Reverse-transcription Quantitative PCR (RT-qPCR)

RNA was isolated using the RNeasy Plus RNA isolation kit (Qiagen, #74136), and 500-1000 ng of purified RNA were used to synthesize cDNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen, #11754250). Real-time PCR was performed by either using PerfeCTa SYBR GreenFastMix (Quanta Biosciences, #95072-012) or PerfeCTa FastMix II for probe-based assays (Quanta Biosciences, #95118-012) with the CFX96 RealTime PCR Detection System (Bio-Rad). The results are expressed as fold change expression of the gene of interest normalized to RPS29 expression using the ΔΔCt method.

Chromatin Immunoprecipitation (ChIP) and ChIP-qPCR

ChIP was performed using the EpiQuik ChIP Kit (EpiGentek, #P-2002-3) according to manufacturer's instructions with 0.5 million cells and 2 µg antibody per IP. Soluble chromatin was immunoprecipitated overnight at 4°C with antibodies against H3K9ac (Abcam, #ab4441), H3K27ac (Abcam, #ab4729), H4K16ac (Sigma-Millipore, #07-329) and H3K9me3 (Abcam, #ab8898) and genomic DNA (gDNA) was purified for qPCR analysis. All sequences for ChIP-qPCR primers can be found in Supp.Table 3 qPCR was performed using PerfeCTa SYBR GreenFastMix (Quanta Biosciences, #95072-012) and the data are presented as fold-change gDNA relative to negative control (gRNA only) and normalized to a region of the GAPDH locus for H3K9ac, H3K27ac, H4K16ac or to a region of the MYOD1 locus for H3K9me3.

Production of Lentivirus and Transduction

Approximately 4.5 million HEK-293T cells were plated per 10-cm tissue-culture polystyrene dish. Twenty-four hours later, the cells were transfected using the calcium phosphate precipitation method with pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) second-generation envelope and packaging plasmids as well as gRNA/CRISPR-GEM that was intended to be packaged in the lentivirus. The medium was exchanged 12-16 h post transfection, and the viral supernatant was harvested 24 h and 48 h after this medium change. The viral supernatant was passed through a 0.45-μm filter and concentrated to 66X using a Lenti-X Concentrator (Clontech, #631280) in accordance with the manufacturer's protocol. For long-term gene expression and ChIP experiments, transduction of target HEK293T cells was performed first for CRISPR-GEM expressing virus at 20X in the presence of 4 μg/mL polybrene (Santa Cruz, #sc-134220), which was followed by 48 h recovery and then 3 d selection with puromycin (Sigma, # P8833). Subsequently, CRISPR-GEM expressing cells were transduced with gRNA expressing virus at 5X in the presence of 4 μg/mL polybrene and were later validated for >98% expression of fluorescent marker under a fluorescent microscope.

Statistical Analysis and Fitting of a Kinetic Model

All statistical tests in this study were done using Prism GraphPad. To calculate statistical significance between different biological samples shown in this study, we used Welch's test. Similarly, we used Spearman's correlation analysis to quantify the correlation coefficient between ChIP-pPCR and RT-qPCR data. Lastly, fitting gene expression data to a kinetic model

was done using a one-phase exponential decay equation, where the Y-intercept was limited to be below 2 and the plateau was set to be above 0.5 R^2 for all fitted models was above 0.5.

RESULTS

Design and development of CRISPR-GEMs.

To expand the epigenome editing toolkit and better dissect some of the individual effects on gene repression that dCas9-KRAB carries, we designed three novel CRISPR-guided epigenome modifiers (CRISPR-GEMs) consisting of SIRT6, HDAC8 and SETDB2 (core) fused to the C-terminus of *Streptococcus pyogenes* dCas9 (**Fig. 1A**). The first two proteins are members of the HDAC protein family that catalyze the removal of acetyl marks from histones H3 and H4 (69-71,77), while SETDB2 is a methyltransferase that specifically catalyzes H3K9 trimethylation (72), an epigenetic mark associated with silenced genes (1). Due to the large size of SETDB2 (719 amino acids [aa]) and the size limitations of lentivirus-based delivery, we used the SETDB2 core domain (consisting of the pre-SET and SET catalytic domains) in our experiments. To better assess the catalytic activity of the CRISPR-GEMs and account for indirect effects that may be due to recruitment of endogenous factors and/or steric hindrance, we generated catalytic mutant versions for each novel CRISPR-GEM in addition to the use of the dCas9 only control. For HDAC8 and SIRT6, we used previously described mutations that were shown to ablate their deacetylase activity: HDAC8$^{D101A/Y306F}$(69) and SIRT6^{H113Y}(70), while for SETDB2 we created a new variant, SETDB2$^{C293L/C295P}$, which relies on its shared homology with SETDB1 (**Fig. S1A**) and previously described mutations in SETDB1 that impaired its methyltransferase activity (75).

CRISPR-GEMs repress selected target genes

To uncover candidate genes that may be responsive to our newly developed CRISPR-GEMs, we aimed to create HEK293T knock-out cell lines for our epigenome modifiers, and identify differentially expressed genes by RNA-seq. We first generated an homozygous deletion of HDAC8 (HDAC8-KO clone #3, hereafter referred to as HDAC8-KO) using the CRISPR/Cas9 nuclease system with gRNAs flanking the first exon of the target gene (**Fig. S2A-D**). We then performed RNA-seq with RNA isolated from the HDAC8-KO cell line and WT HEK293T, and found 188 protein-coding transcripts that were differentially upregulated (transcripts with at least 0.5 transcripts per million [TPM]; TPM levels in HDAC8-KO compared to TPM levels in WT > 2) (**Supp. Table 4**). We further narrowed down these 188 transcripts by using publicly available H3K27ac ChIP-seq tracks from the ENCODE project (biosample ENCBS486AAA)and identified 13 genes with H3K27ac peaks in the vicinity of their promoter (±500 bp from TSS) (**Supp. Table 5**). Using RT-qPCR, we confirmed that 10 out of the 13 candidate genes were up-regulated in the HDAC8-KO compared to WT (expression ratio > 1.5; **New Fig. S2E**). We picked 3 genes (DKK1, IGFBPL1 and MLNR), whose levels were not affected by dCas9 in the HDCA8-KO cells, and assessed the capacity of CRISPR-GEMs to repress these target genes in the HDAC8-KO cell line three days post-transfection using RT-qPCR (**Fig. 1F**). Both DKK1 and IGFBPL1 showed significant repression with all three dCas9-GEMs when compared to the dCas9-only treatment while we observed significant repression of MLNR only by dCas9-SIRT6. Importantly, the level of repression obtained with all three dCas9-GEMs was similar to the one obtained using dCas9-KRAB. Since the effect of all three dCas9-GEMs could be examined on the same target genes, we decided to use those genes in subsequent experiments.

Chromatin analysis at day 2 post-transfection confirms catalytic activity of CRISPR-GEMs but only partially explains changes in gene expression.

To begin characterizing our newly developed CRISPR-GEMs and examine their potential applicability, we used the genetically unperturbed WT HEK293T cell line. Previously published CRISPR-guided epigenome modifiers were able to show desired changes to histone marks under transient expression within several hours for gene activation (53) and within 2-4 days for gene repression (42,52). Thus, we chose day 2 past co-transfection of CRISPR-GEMs and gRNAs as the first benchmark to assess their impact on epigenetic marks and gene expression (**Fig. 2A**). For our novel HDAC-based CRISPR-GEMs, we tested their ability to remove acetyl marks that strongly correlate with promoter activity (H3K9ac, H3K27ac, and H4K16ac) (3,68). For the dCas9-SETDB2 fusion, we tested its ability to deposit H3K9me3. In parallel, we also measured the effects of dCas9-KRAB that has been previously shown to repress transcriptional activity, alongside enrichment of H3K9me3 (29) and depletion of H3K9ac and H3K27ac marks (51). The effects of CRISPR-GEMs on epigenetic marks were quantitatively measured at all three target loci using ChIP-qPCR and evaluated relative to a no effector control as well as a dCas9 that did not contain an effector domain. For H3K27ac levels, dCas9-HDAC8 was the only CRISPR-GEM that showed a significant depletion at both DKK1 and IGFBPL1 (0.78- and 0.7-fold, respectively; $P<0.05$ compared to no effector). These changes were significant relative to dCas9 at IGFBPL1 but were not significantly different from dCas9-HDAC8-Mut at both loci (**Fig. 2B**). For H3K9ac levels, we observed a significant depletion compared to both controls at IGFBPL1 with all 3 CRISPR-GEMs (KRAB, HDAC8, SIRT6), while their corresponding

mutants did not have an effect on H3K9ac levels. At MLNR, we observed a significant depletion of H3K9ac by dCas9-HDAC8 compared to its catalytic mutant (0.53- vs. 0.9-fold and 0.51- vs. 1.28-fold, respectively, $P<0.05$) (**Fig. 2C**). For H4K16ac levels, we observed a significant decrease relative to dCas9 with both dCas9-KRAB and dCas9-SIRT6 at DKK1, and dCas9-KRAB and dCas9-HDAC8 at IGFBPL1. We note that at DKK1, dCas9 alone led to a global depletion of H3K9ac (0.47- to 0.56-fold) and H4K16ac (0.45- to 0.73-fold) levels, suggesting that the targeting of dCas9 to this locus may drive epigenetic changes, possibly by steric hindrance or by interacting with other endogenous factors (**Fig. 2C, D**). Next, we measured H3K9 trimethyl levels at the target loci (**Fig. 2E**). We found that targeting dCas9-KRAB led to enriched levels of H3K9me3 at all three tested loci (DKK1: 3.52-fold, $P<0.05$; IGFBPL1: 1.19-fold, $P<0.05$; and MLNR: 2.12-fold, $P=0.06$). H3K9me3 enrichment by dCas9-SETDB2, however, could only be observed at the DKK1 locus (2.19-fold compared to no effector; $P<0.05$). Together, these data show that within 2 days of expression in cells, both HDAC-based fusions, HDAC8 and SIRT6, are effective at removing certain acetyl marks at their target genes, and that dCas9-SETDB2 is able to deposit H3K9me3 at a target locus.

HDAC-based CRISPR-GEMs repress target genes within 2 days post-transfection.

We then examined the effect of CRISPR-GEMs on target gene expression by RT-qPCR (**Fig. 2F**). We found that dCas9-HDAC8 led to significant repression of DKK1 and IGFBPL1 (0.55- and 0.76-fold, respectively; compared to no effector and to dCas9 only; $P<0.05$) (**Fig. 2F**). In addition, we observed that dCas9-SIRT6 and dCas9-SETDB2 led to a significant repression of IGFBPL1 (0.76- and 0.86-fold; compared to no effector and to dCas9 only; $P<0.05$). The

repression of DKK1 and IGFBPL1 by dCas9-SIRT6 was found to be significant upon comparison to its catalytic mutant ($P<0.05$), and similarly, repression of DKK1 by dCas9-HDAC8 was more potent than its catalytic mutant ($P<0.05$). Notably, we saw a global reduction of MLNR mRNA levels at day 2 post-transfection by all CRISPR-GEMs (between 0.53- to 0.7-fold reduction compared to no effector), which suggested a possible confounding steric hindrance effect, thus diminishing our ability to observe significant differences between tested CRISPR-GEMs and their mutated counterparts. Remarkably, however, comparing across all three target genes, HDAC8 consistently showed the most potent repression at this time point. Interestingly, while we observed robust H3K9 trimethylation by dCas9-KRAB and dCas9-SETDB2 at the DKK1 locus, it did not lead to marked decline of its transcription levels at day 2 post-transfection. These results are in agreement with previous studies which showed that enrichment of H3K9me3 marks does not necessarily result in gene repression (52), possibly hinting that a longer expression of these CRISPR-GEMs is necessary for a potent gene repression.

Chromatin analysis at day 4 post-transfection reveals slower kinetics by SIRT6 and SETDB2 in comparison to HDAC8 and KRAB.

Various epigenome editing domains were previously shown to possess different kinetics that can result in significant changes to gene expression within days or even hours (50,53,78). Thus, we decided to measure changes to chromatin marks and gene expression of target genes at a later time-point, 4 days after transfection (**Fig. 3A**), and compare it to the data obtained at day 2 post-transfection. For H3K27ac levels, targeting dCas9-SIRT6 led to significant reduction at

DKK1 (0.75-fold; $P<0.05$) in comparison to no effector, dCas9 only and its catalytic mutant while it led to an increase of H3K27ac marks at IGFBPL1 and MLNR (**Fig. 3B**). Compared to day 2 post-transfection, dCas9-HDAC8 did not have an effect on H3K27ac levels 4 days post-transfection (**Fig. 3B**). For H3K9ac levels (**Fig. 3C**), targeting dCas9-SIRT6 resulted in a depletion at all 3 tested loci: DKK1, IGFBPL1 and MLNR (0.68-, 0.54- and 0.74-fold, respectively; compared to no effector, dCas9 only or catalytic mutant; $P<0.05$). For dCas9-HDAC8, however, we observed depletion of H3K9ac levels only in the MLNR locus when compared to no effector ($P<0.05$). For H4K16ac marks, targeting dCas9-HDAC8 led to significant depletion at DKK1 (0.82-fold; compared to no effector, dCas9 only and catalytic mutant; $P<0.05$), similar to the one observed with dCas9-KRAB (**Fig. 3D**). Interestingly, both dCas9-SIRT6 and its catalytic mutant resulted in a similar depletion of H4K16ac marks at the DKK1 and IGFBPL1 loci when compared to no effector ($P<0.05$) or dCas9 only (only at the DKK1 locus; $P<0.05$). While we observed multiple occurrences at day 2 post-transfection in which HDAC8 or KRAB led to significant deacetylation, the data we obtained at day 4 post-transfection indicated that most of these changes did not persist. Moreover, this pattern could also be observed for H3K9me3 marks, with a diminished effect of dCas9-KRAB at all 3 tested loci, and only a modest enrichment of H3K9me3 observed at the DKK1 locus (1.32-fold; $P<0.05$) (**Fig. 3E**). dCas9-SETDB2, however, showed a moderate enrichment of H3K9me3 at both IGFBPL1 and MLNR loci 4 days post-transfection (2.02- and 1.68-fold, respectively ($P<0.05$; compared to no effector and dCas9 only). Surprisingly, dCas9-SETDB2 also led to depletion of H3K9me3 marks at the DKK1 locus (0.43-fold; $P<0.05$). However, at all tested loci, SETDB2 and its catalytic mutant had similar effects on H3K9me3 levels, suggesting that the

mutations we introduced to SETDB2 did not completely abrogate its catalytic activity, or alternatively, both fusions recruit similar endogenous factors that indirectly alter H3K9me3 levels at target loci.

Measuring expression of target genes 4 days post-transfection reveals different CRISPR-GEMs kinetics.

Since we used a transient delivery method, we hypothesized that there may be some processes that could interfere with our endeavor to target epigenetic changes and gene repression, including a decline in CRISPR-GEMs transcripts, or endogenous feedback loops of target genes. We reasoned that measuring mRNA levels of repressed genes at a later time-point would be advantageous in highlighting epigenetic repression that is less likely to be due to steric hindrance, assuming that epigenetic changes that affect gene expression may last for prolonged periods of time. Therefore, we measured the relative mRNA levels of the target genes by RT-qPCR on day 4 post-transfection (**Fig. 3F**). Interestingly, we saw some changes in the trends of gene repression by various CRISPR-GEMs. First, dCas9-SIRT6 significantly repressed all target genes: DKK1, IGFBPL1 and MLNR (0.61-, 0.78- and 0.81-fold; compared to no effector; $P<0.05$). These changes were also significant when compared to dCas9-SIRT6 Mut. for the DKK1 and MLNR genes ($P<0.05$). Second, repression by dCas9-HDAC8 was still significant at DKK1 (0.63-fold; compared to no effector and dCas9-HDAC8-Mut.; $P<0.01$). Third, dCas9-SETDB2 showed significant repression of IGFBPL1 and MLNR (0.86- and 0.83-fold; compared to no effector, respectively; $P<0.05$), however these results were not significant when compared to its catalytic mutant. Fourth, dCas9-KRAB significantly repressed DKK1 and

MLNR (0.7- and 0.83-fold; compared to no effector, respectively). In summary, at day 4 post-transfection, dCas9-SIRT6 was the most potent in repressing every gene, followed by dCas9-KRAB in 2 out of the 3 genes we tested. Overall, a comparison of these results to repression data at day 2 (**Fig. 2F**) supports the hypothesis that different CRISPR-GEMs possess varying kinetics of gene repression.

Long-term effects on chromatin marks by CRISPR-GEMs are different from short-term effects.

Previous CRISPRi screens have mostly utilized dCas9-KRAB over a prolonged period of time (at least 10 days), often stably expressed by a lentiviral delivery system (30,31,33,42). Other studies that sought to achieve long term gene repression used transient or inducible delivery systems that resulted in eventual loss of the repressive machinery, and showed that a DNMT3A-L component is necessary for sustainable gene silencing (44,52 and PMID: 33838111). To interrogate the effects of novel CRISPR-GEMs over a prolonged period of time, we used a lentiviral delivery platform and constitutively expressed the gRNAs and the dCas9 fusions for 10 days prior to analysis of chromatin and RNA levels at target genes (**Fig. 4A**). We observed significant depletion of H3K27ac marks by dCas9-SIRT6 at all 3 loci: DKK1, IGFBPL1 and MLNR (0.7-, 0.27- and 0.44-fold, respectively; compared to no effector; $P<0.05$) (**Fig. 4B**). This effect was also significant when compared to dCas9 (DKK1 and IGFBPL1; $P<0.05$), or when compared to dCas9-SIRT6 Mut. (DKK1 and MLNR; $P<0.05$). For H3K9ac marks, we saw that dCas9-SIRT6 led to significant deacetylation in the MLNR locus when compared to no effector, dCas9 only, or dCas9-SIRT6 Mut. (0.62-fold compared to no effector;

P<0.05) (**Fig. 4C**). However, both dCas9-HDAC8 and dCas9-HDAC8 Mut. resulted in significant depletion of H3K9ac marks in the MLNR locus, compared to dCas9 only (0.72- and 0.77-fold compared to no effector; P<0.05). For H4K16ac levels, dCas9-HDAC8 successfully removed acetyl marks at the IGFBPL1 locus when compared to no effector or dCas9 only (0.27-fold; P<0.05) (**Fig. 4D**). Similarly, dCas9-SIRT6 significantly reduced H4K16ac marks when compared to no effector or its catalytic mutant (0.62-fold; P<0.05). Lastly, we measured H3K9me3 levels and found that dCas9-KRAB was able to enrich for this mark in all tested loci (DKK1: 3.31-, IGFBPL1: 2.69- and MLNR: 7.47-fold, respectively; P=0.06, P<0.05 and P<0.001, respectively) (**Fig. 4E**). Additionally, our results showed that dCas9-SETDB2 led to a modest deposition of H3K9me3 marks at the IGFBPL1 and MLNR loci when compared to dCas9 only or its catalytic mutant (1,13- and 2.58-fold; P<0.05). Altogether, these changes to chromatin at day 10 depict a vastly different epigenetic landscape than previously observed at early time points, suggesting that the continuous interactions of CRISPR-GEMs with endogenous factors may lead to new regulatory and transcriptional equilibria that are distinct from those that we observed at earlier time points under transient expression system.

KRAB demonstrates stronger gene repression over long-term constitutive expression.

Next, we measured the repression of all 3 target genes by RT-qPCR 10 days post-transduction. dCas9-KRAB showed a potent repression of all target genes (DKK1: 0.17-, IGFBPL1: 0.03- and MLNR: 0.23-fold; P<0.05) (**Fig. 4F**), while dCas9-SIRT6 showed a moderate repression of all target genes (DKK1: 0.84-, IGFBPL1: 0.33- and MLNR: 0.55-fold; P<0.05). Interestingly, the repression by dCas9-KRAB was substantially stronger than any other CRISPR-GEM at this time

point, suggesting that longer expression time and/or enrichment of H3K9me3 are required for its efficient repression. Bintu et al. previously proposed a three-state repression model for KRAB (78), which included a fast transition to a "reversibly repressed" state, followed by a slow transition to an "irreversible repressed" state. Our observations of a potentiating repression by KRAB are in agreement with this model.

Analysis of gene expression over multiple timepoints demonstrates gene- and effector-dependent kinetics.

Previous attempts to characterize gene repression by recruitment of epigenetic factors raised the possibility of a three-state model, which proposed a fast and reversible transition between native state and repressed state, and an additional slower phase, transitioning into an irreversibly repressed state (78). In order to characterize the kinetics and the efficiency of the repressive CRISPR-GEMs in this study, we proposed a simple first-order kinetic model to analyze the decline of target gene mRNA levels upon expression of CRISPR-GEMs during the reversible state (**Fig. 5A**). Briefly, assuming the effector is in excess, this model allows us to refer to the expression of and the decline in target mRNA levels as a first-order rate equation with a constant K_{exp}*, suggesting a logarithmic decline in transcript levels. Moreover, to verify that all tested CRISPR-GEMs abide by similar kinetics, we measured the mRNA levels of the various dCas9 fusions (**Fig. 5B**) which indeed followed a similar trend that could fit a logarithmic first order model (K_{eli}=1.109 d^{-1}; R^2>0.96 for every treatment). Therefore, we proceeded to measure mRNA levels for every target gene over multiple timepoints ranging between day 0.5 and Day 5 post-transfection (**Fig. 5C**). Interestingly, while the expression of CRISPR-GEMs was similar

across target genes and different CRISPR-GEMs, we observed slightly different kinetics between the various conditions. First, we saw that the peak of repression for MLNR occurs at day 2, while for DKK1 repression peaks at day 3, and for IGFBPL1, it depends on the effector. Second, we observed that certain effectors showed repression of target genes within 12 hours, while others, such as SETDB2, would sometimes exhibit a prolonged lag before repression could be discerned. Additionally, this lag may be accompanied by an increase in mRNA levels of the target gene. Next, to better understand the kinetic advantage of novel CRISPR-GEMs, we proceeded to fit a first-order model to the semi-logarithmic part of the measurements (timepoints of 0.5-3 days) for every gene (**Fig. S3**), thus allowing to quantify the impact of every CRISPR-GEM on the rate of target gene expression by comparing them to dCas9 or KRAB. Across the three target genes we measured, we were able to demonstrate higher K_{exp}* with SIRT6, HDAC8 or KRAB than with dCas9, but not with SETDB2. In fact, almost in all cases, the K_{exp}* values of KRAB were lower than SIRT6 and HDAC8, suggesting that they are more potent than KRAB in repressing their target genes at days 0.5-3.

Correlations between changes in gene expression and changes in histone marks suggest that H3K9 modifications may precede changes in gene expression.

To gain insights into the relationship between chromatin marks and gene expression, we calculated Spearman's rank correlation coefficients between changes we observed in chromatin marks and gene expression (**Fig. 5D**). We hypothesized that the calculated coefficients for active marks would be positive, while it would be negative for repressive marks, demonstrating an inverse correlation. Indeed, the expression of DKK1 on day 2 mildly correlated with H3K27ac

and H4K16ac marks (rho values of 0.57 and 0.61, respectively; non-significant), and the expression of IGFBPL1 on day 2 strongly correlated with H3K9ac and H3K27ac marks (rho values of 0.82 and 0.61, respectively; $P<0.05$ and non-significant, respectively). However, on day 4 post-transfection, we observed mostly weak correlation between chromatin and gene expression. Previous studies showed that certain epigenetic changes precede changes in gene expression (23,24,53, PMID: 33770473), thus we hypothesized that the expression levels on day 4 may better correlate with chromatin marks at day 2. This hypothesis was supported by the correlation coefficients at the MLNR locus and showed that gene expression at day 4 correlated well with H3K9ac, H3K27ac and H4K16 marks at day 2 (rho values of 0.71, 0.5 and 0.57, respectively; non-significant). Similarly, in the DKK1 and MLNR loci, we observed that early H3K9me3 marks strongly correlate with gene repression on day 4 (rho values of -0.8 and -0.9, respectively; non-significant and $P<0.05$, respectively). Interestingly, we also observed that H3K9me3 marks strongly correlate with DKK1 expression at day 4 (rho of -0.9; $P<0.05$), while at the MLNR locus, we measured a strong correlation at day 10 post-transduction with H3K9me3 marks (rho of -0.9; $P<0.05$). These findings led us to propose a model (**Fig. 5E**) which suggests that in some genes, changes in certain epigenetic markers (namely, H3K9 modifications), may precede changes in gene expression, as observed in the case of DKK1 and MLNR as well as previous studies. Conversely, changes in other marks (H3K27ac and H4K16ac) may sometimes reflect a contemporary state of transcription. Previous studies showed that different genes may have a different mechanism of epigenetic regulation (13,53). Our data suggest that the expression of DKK1 and IGFBPL1 mostly correlate with the acetyl marks we

tested at day 2, while changes in the expression of MLNR correspond better with changes in the levels of H3K9me3.

DISCUSSION

The field of epigenetics is riddled with many observations that are seemingly contradictory (9). One of the major obstacles that hinder our progress toward answering these questions stems from our fundamental inability to distinguish between cause and effect in a system that is highly convoluted and comprised of multifarious components. Thus, it is necessary to develop tools that are capable of editing specific chromatin marks in a targeted and efficient fashion. In this study, we used the CRISPR/Cas9 platform to develop three novel epigenome editors: dCas9-HDAC8, dCas9-SIRT6 and dCas9-SETDB2, and demonstrated their ability to target and modify gene expression and histone marks in three different loci. Although we were unable to demonstrate a uniform outcome for all three genes, we were able to observe unique kinetics for the individual CRISPR-GEMs that were characterized in this study. We also showed that various targetable domains, including the dCas9 infrastructure itself, may have an effect on gene expression and/or chromatin marks regardless of having an active catalytic domain, thus justifying the inclusion of dCas9 and catalytic mutants in these experiments. Lastly, we were able to draw meaningful correlations between transcription and chromatin marks, which supported previous evidence strongly linking H3K9 marks to promoter activity (3), as well as suggesting that changes in gene expression are subsequent to changes in certain epigenetic marks while concomitant with others (23,24,53).

The discovery of CRISPR and its application in mammalian cells led to a substantial surge in the development of targeted gene editing technologies alongside an increasing utility of the CRISPRa and CRISPRi platforms. However, these platforms, whose main focus is to potently alter gene expression, recruit endogenous machinery to drive a potent transcriptional change, thereby affecting a multitude of epigenetic marks. For example, the commonly used KRAB domain that is derived from the ZFP10 protein, is known to recruit KAP1, which in turn provides a scaffold for a repressive machinery that recruits HP1, SETDB1 and multiple other enzymes, including several proteins from the HDAC family (74-76). These components are capable of driving epigenetic changes on their own merit. For example, HP1 can propagate heterochromatin formation and enrichment of H3K9me3 through recruitment of SUV39H1 (79). In this study, we saw a significant deposition of trimethyl marks by dCas9-KRAB in early timepoints (day 2 post-transfection), however these changes in chromatin marks only correlated with gene expression in later time points (day 4 post-transfection and day 10 post-transduction). Prominently, the effect of dCas9-KRAB was significantly potent in the latest time point (day 10 post-transduction), suggesting there is a potentiation of its repressive effect over time. These observations can be explained by Bintu et al. who used a three-state kinetics model to describe the repression by the KRAB domain, indicating a quick reversible repression and a slow transition to an irreversibly committed state (78). Furthermore, our results also agree with previous observations showing that deposition of H3K9me3 marks was not sufficient to drive gene repression (52). These observations were made at days 3-4 post-transfection and can be similarly observed in this study. Looking at measurements that were taken at early time points, we were able to demonstrate that H3K9me3 levels correlated with gene expression only at day 4

post-transfection or day 10 post-transduction in two of the three tested loci. Due to the inherent limitations of testing more time points and additional loci, we cannot rule out additional hypotheses regarding gene repression by dCas9-KRAB and dCas9-SETDB2. For example, it is unclear if the repression is directly correlated with H3K9me3 levels or there is also a threshold that needs to be met for repression to take place. Moreover, since we observed only weak methyltranseferase activity by dCas9-SETDB2 (in comparison to dCas9-KRAB), it is possible that the repression by KRAB is supported or even dependent on recruitment of endogenous co-factors, such as HP1. Finally, although the KRAB domain in dCas9-KRAB is relatively small, it forms a large complex by recruiting multiple proteins. There is no known KRAB mutant that can mimic its steric hindrance without performing its enzymatic activity, thus mitigating our ability to estimate the effect of its steric hindrance on transcription.

Thus far, only a limited number of publications showed effective deposition of epigenetic marks, mostly targeting acetyl groups on H3K27 (37,49) and methyl groups on H3K4 (50,51,53), H3K9 (52), H3K27 (52,53,80) and H3K79 (50). Interestingly, among the few epigenome editors that were tailored for gene repression, a common challenge was to distinguish between the catalytic effect they directly carry on gene expression and chromatin marks versus confounding effects they may carry through steric hindrance or recruitment of various endogenous factors (49,52). To account for this issue, we included in our experimental design a comprehensive set of controls, including dCas9, dCas9-KRAB and CRISPR-GEMs that possess mutations in their catalytic domains. In multiple cases we could observe patterns in the data that applied to all CRISPR-GEMs (or to a specific CRISPR-GEM and its mutant) which could be attributed to

aforementioned reasons. For example, Fig.2C demonstrates a ~50% depletion of H3K9ac levels by every dCas9 fusion at day 2 post-transfection, raising the possibility that dCas9 competes with endogenous factors that maintain H3K9Ac levels at this locus. Notably, similar phenomena were observed previously: for example, O'Geen et al. showed that SUV[SET]-dCas9 and Ezh2-dCas9, as well as mutants of the latter, reduced HER2 mRNA levels, but did not result in enrichment of methyl marks at H3K9 or H3K27 (52). In contrast, Dorighi et al. showed that expression of genes by Mll3/4 remained intact even when its methyltransferase activity was abrogated (15). Moreover, Polstein et al. demonstrated that merely the targeting of dCas9 to gene promoters could increase chromatin accessibility and expression of these genes (81). Collectively, these examples may provide an explanation to results in this study that demonstrated significant changes to gene expression or chromatin marks by dCas9 or by CRISPR-GEMs that possess mutated catalytic domains, therefore emphasizing the importance of including catalytically functional and non-functional CRISPR-GEMs to separate cause from effect. Nonetheless, there may still be additional challenges, such as effectors that carry an effect through their non-catalytic domains as exemplified by Poleshko et al., who showed that abolishing the deacetylase activity of HDAC3 did not impair its effect on cardiac myocyte differentiation (82).

In addition to confounding effects by steric hindrance, another major challenge in previous studies was to link changes in gene expression to deposition of repressive marks. For example, O'Geen et al. showed that the enrichment of canonical repressive marks, such as di- or tri-methylation of H3K9 and H3K27, was not sufficient for gene repression (52). In this study,

we employed multiple methods to tackle this conundrum. First, we characterized the changes in gene expression and chromatin marks over multiple time points. Second, we utilized statistical methods to draw correlation levels between gene expression and chromatin marks over multiple timepoints. Third, we performed a comprehensive kinetic analysis that included dCas9 to account for steric effect on transcription. This combined approach allowed us to take into consideration changes that develop over time, resulting in unbiased correlations which support a model where certain histone marks precede changes in gene expression, as was previously shown (23,24,53, PMID: 33770473). Prominently, our results indicate that H3K9 modifications demonstrate strong correlation between gene expression at day 4 and chromatin marks at day 2 post-transfection in certain genes, while in other case, the correlation is stronger when the gene expression at day 2 is correlated with ChIP data at day 2, suggesting that these chromatin-transcription dynamics may be gene-dependent. While these correlations resulted in valuable insights regarding each gene, it proved difficult to discern universal patterns across all genes. This may be in part due to the limited ability to test many more chromatin marks in different genes, however it is supported by previous observations, which suggested there may be different models of epigenetic regulation across different genes. For example, Braun et al. were able to induce expression using the BAF domain in only one gene out of six that they tested (53), and O'Geen et al. who showed that certain subgroup of genes necessitated dCas9-KRAB for long term repression, while another subgroup required dCas9-Ezh2 (80), both supporting the hypothesis of context-dependent epigenome editing, thereby providing a possible explanation as to why IGFBPL1 mainly correlated with acetyl marks, while DKK1 and MLNR also correlated well with H3K9me3 marks.

It was previously shown that different epigenetic domains possess vastly different kinetics (78). By testing multiple effectors, they showed that the repression kinetics of HDAC4 and KRAB are both quick, but the level of cells that commit to an "irreversibly repressed" mode is substantially higher with KRAB. This observation aligns with data in our study, which showed that HDAC8 has fast and non-committing kinetics, leading to the strongest gene repression at day 2 alongside depletion of acetyl marks in tested loci, while the effects of SIRT6 and KRAB on chromatin were prominent at days 2 and 4, but their strongest impact on gene expression showed mostly later at days 4 and 10, respectively. This aspect of kinetics has often been overlooked in similar studies, although it can prove to be an important factor when deciding which epigenome editor to use. For example, if a certain application necessitates fast repression, it may be more beneficial to use HDAC8 or SIRT6 over KRAB, or alternatively, to use a combination of CRISPR-GEMs to take advantage of the individual strengths of every tool.

Since this study ventured into a fairly uncharted territory of epigenome editing, these experiments surely have multiple limitations: (a) The chosen domains did not undergo optimization, and it is very possible that later studies can employ directed-evolution methods to increase their potency; (b) Furthermore, due to technical difficulties of performing ChIP with small number of cells, or declining expression levels at later time points, we had to change the delivery method between days 4 and 10 (c) Due to the laborious nature and technical complexity of these experiments, we tested only one locus per ChIP-qPCR, thus possibly overlooking changes in other loci (d) Due to endogenous feedback loops, changes in later time points are

more prone to bias, especially by effectors that are less potent, thus mitigating our ability to understand long-term kinetics of certain CRISPR-GEMs.

In summary, we created and comprehensively characterized three novel CRISPR-GEMs based on the methyltransferase SETDB2 and the two proteins from the HDAC family, HDAC8 and SIRT6. While the widely used tool for targeted gene repression dCas9-KRAB demonstrated superior repression under constitutive expression at a late time point (day 10), transient expression experiments revealed that dCas9-HDAC8 demonstrated the strongest repression at day 2 in all three genes, and dCas9-SIRT6 had the strongest repression at day 4 in 2 of the 3 tested genes. Furthermore, despite modest gene repression and deposition of H3K9me3 by dCas9-SETDB2 at days 4 and 10, it was difficult to conclude that the latter was mediated by its catalytic activity, since both the wildtype and the catalytically mutated fusion often showed similar results. Nonetheless, comparison of changes to H3K9me3 levels by dCas9-KRAB at different time points, suggested that its effect on histone marks precedes its effect on gene expression. Similarly, early results with the dCas9-HDAC8 and -Sirt6 fusions, suggested that H3K9Ac marks and to a lesser degree H4K16Ac, precede changes in gene expression as well. Moreover, we were able to draw strong correlations between gene expression and specific chromatin marks that are gene dependent. Overall, these new tools provide diversity to the relatively small toolkit of epigenome editing tools. They introduce aspects of variegated kinetics, and expand the available infrastructure to tackle burning questions in the field of epigenetics, such as the endeavor to distinguish between correlation and causality between chromatin marks

and gene expression, or the attempt to identify chromatin marks that are predictive of gene expression levels, namely “the epigenetic code”.

Acknowledgements

This work was supported by an Allen Distinguished Investigator Award from the Paul G. Allen Frontiers Group, the Thorek Memorial Foundation, US National Institutes of Health (NIH) grants R01DA036865, R21DA041878, P30AR066527, R41GM119914, U01HG007900, UM1HG009428, and the Duke-Coulter Translational Partnership.

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