Engineering Open Chromatin with Synthetic Pioneer Factors:

Enhancing Mammalian Transgene Expression and

Improving Cas9-Mediated Genome Editing in Closed Chromatin

by

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ABSTRACT

Chromatin is the dynamic structure of proteins and nucleic acids into which eukaryotic genomes are organized. For those looking to engineer mammalian genomes, chromatin is both an opportunity and an obstacle. While chromatin provides another tool with which to control gene expression, regional density can lead to variability in genome editing efficiency by CRISPR/Cas9 systems. Many groups have attempted to de-silence chromatin to regulate genes and enhance DNA's accessibility to nucleases, but inconsistent results leave outstanding questions. Here, I test different types of activators, to analyze changes in chromatin features that result for chromatin opening, and to identify the critical biochemical features that support artificially generated open, transcriptionally active chromatin.

I designed, built, and tested a panel of synthetic pioneer factors (SPiFs) to open condensed, repressive chromatin with the aims of 1) activating repressed transgenes in mammalian cells and 2) reversing the inhibitory effects of closed chromatin on Cas9-endonuclease activity. Pioneer factors are unique in their ability to bind DNA in closed chromatin. In order to repurpose this natural function, I designed SPiFs from a Gal4 DNA binding domain, which has inherent pioneer functionality, fused with chromatin-modifying peptides with distinct functions.

SPiFs with transcriptional activation as their primary mechanism were able to reverse this repression and induced a stably active state. My work also revealed the active site from proto-oncogene MYB as a novel transgene activator. To determine if MYB could be used generally to restore transgene expression, I fused it to a deactivated Cas9 and targeted a silenced transgene in native heterochromatin. The resulting activator was able to reverse silencing and can be chemically controlled with a small molecule drug.

Other SPiFs in my panel did not increase gene expression. However, pretreatment with several of these expression-neutral SPiFs increased Cas9-mediated editing in closed chromatin, suggesting a crucial difference between chromatin that is accessible and that which contains genes being actively transcribed. Understanding this distinction will be vital to the engineering of stable transgenic cell lines for product production and disease modeling, as well as therapeutic applications such as restoring epigenetic order to misregulated disease cells.

DEDICATION

To my family,

For their constant support,

Unrelenting pride,

And unconditional love.

I love you all so much.

To my friends,

Both near and far,

Who regardless of the distance

Have always been there for me.

To my clan,

For gathering around me at Thanksgiving and

Being there when I come home-

You are my life.

To Scott,

My biggest fan

And constant companion.

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LIST OF ABBREVIATIONS

AAP- activation associated peptide

CMV- cytomegalovirus

CR- chromatin remodeler

Gal4- Gal4 DNA binding domain

HAT- histone acetyltransferase

NLS- nuclear localization signal

ORF- open reading frame

PF- pioneer factor

PolII- RNA polymerase II

PRC- Polycomb repressive complex

SPiF- synthetic pioneer factor

TAD- transcriptional activation domain

UAS- upstream activation sequence

CHAPTER 1

INTRODUCTION†

1.1 Unlocking Access to DNA in Chromatin

The genomes of eukaryotic cells are condensed into a complex, organized nucleo-protein structure known as chromatin. Chromatin dynamically regulates gene expression by locally altering the density of DNA packaging and associations with chromatin remodeling factors (Bintu et al.; Libbrecht et al.; Bowman and McKnight). Tightly compacted areas of chromatin, known as heterochromatin, directly inhibit associations between DNA and transcriptional machinery by preventing the recognition of DNA binding sites (Johnson et al.; Janssen et al.). This closed state is characterized by silencing marks, repressive protein collocation, and a density of linker histones that together form condensed structures that are difficult to disrupt (Figure 1.1) (Arrighi and Hsu; Peng; Erdel and Rippe). The DNA within is generally inaccessible to DNA binding proteins, thus prohibiting active gene expression. Open chromatin, or euchromatin, is characterized by an accessible, loose structure, and activation-associated chemical modifications (Figure 1.1) (Wolffe and Urnov). This non-compacted structure allows access for transcriptional machinery and is thus associated with active transcription.

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As synthetic biology advances, its practitioners are constantly seeking out new chassis in which to execute increasingly intricate designs and with which to manufacture more complex products, such as therapeutic antibodies. This has naturally led to the engineering of mammalian cell lines, including human lines to construct disease models and therapeutic molecular machines. While the complexity of this chassis may be desirable in that it provides more opportunities for engineering, increased system complexity also increases variability in engineering outcomes. This is evidenced by the impacts of chromatin on engineered mammalian cell lines. While site-specific chromatin manipulation provides an additional layer of gene expression control, native chromatin can interfere with transgenic systems. Gaining reliable, controlled access to the (epi)genome is an important step toward realizing the full potential of synthetic biology in mammalian chassis.

A perennial source of variability that thwarts this reliable access, is the natural condensation of chromatin around inserted transgenes (Meyer). Native defense mechanisms often epigenetically silence inserted genetic material, posing a challenge to the construction of stable mammalian cell line (Leung and Lorincz; Ross et al.; Ellis; Ngai). Part of the key to unlocking chromatin will be to develop a suite of tools that can reliably reverse transgene silencing. We must also understand the impact that chromatin has on editing tools such as small-RNA-guided nucleases (CRISPR/Cas9), as well as protein-DNA binding nucleases such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZF) that all rely directly on DNA binding interactions to achieve editing.

To unlock access to the genome, we need to understand the answers to a few vital questions: 1) What specific features of chromatin impact DNA accessibility to endogenous and exogenous effectors? 2) How do these features perturb stable engagement of gene-editing enzymes with their targets? And, 3) how can we

manipulate chromatin to overcome transgene silencing and inhibition of gene-editing activity?

Much work has already been done to understand the first two of these questions. This chapter will review that work by addressing 1) what is known about how the structural elements of chromatin affect accessibility and 2) how chromatin impacts transgene expression and CRISPR/Cas9 function as well as the few studies that have attempted to improve CRISPR/Cas9-mediated editing in heterochromatin. I will then review a series of major methods employed by the cell to open chromatin that may be harnessed by engineers to do the same in order to regulate gene expression and chromatin state. I review the mechanism behind each method, its potential to be engineered, and previous attempts to do so. Finally, I propose remaining challenges to the opening of chromatin as well as important design parameters that warrant further investigation. Several of these native chromatin-opening mechanisms are underexplored or have never been the subject of any molecular engineering. Testing and comparing these chromatin-opening approaches, as well as addressing nuanced design parameters outlined at the end of this chapter, set up the motivation for the remained of this work.

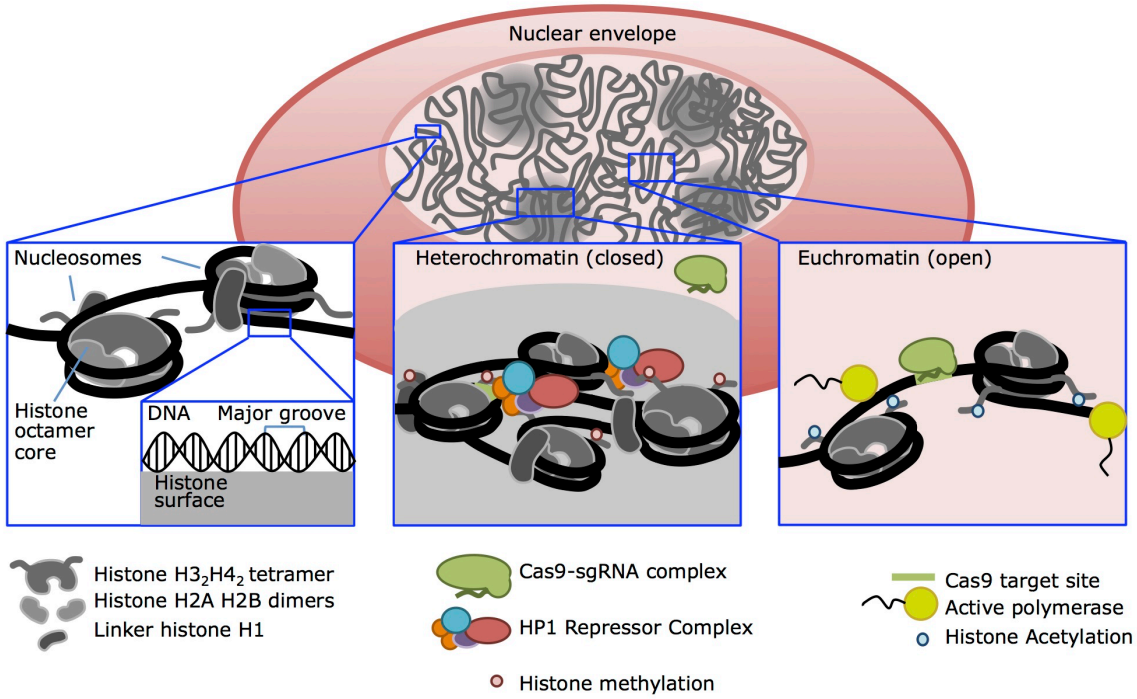

Figure 1.1: Chromatin architecture impacts accessibility. The core structure of chromatin is the nucleosome, an octamer of distinct histone proteins, around which DNA is wound. Highly compacted areas of chromatin are characterized by the localization of repressor complexes, repressive histone tail modifications, and linker histones that bring adjacent nucleosomes into close contact. This dense structure creates a liquid-like compartment through phase separation that precludes the interaction of DNA binding proteins such as editing endonucleases with DNA. Loosely packaged chromatin or euchromatin allows for active transcription of DNA and interaction with binding factors. Histone tail acetylation in euchromatin recruits these associated factors. (Reprinted with permission from Chemical Engineering Progress (CEP). Copyright © 2018 American Institute of Chemical Engineers (AIChE))

1.1.1 Core Structural Elements of Chromatin Impact Accessibility

The primary structural unit of chromatin is the nucleosome. Nucleosomes are comprised of a 147 base pair length of DNA wound around an octamer of four core histone proteins (Figure 1.1). Protruding from the histone $H3_2H4_2$ tetramer are unstructured amino acid tails that can be chemically modified with either activating or silencing marks (Figure 1.1). Arrays of nucleosomes can be assembled into higher order structures through small-scale interactions such as the binding of linker histones near the nucleosome dyad axis (Zhou et al.) or co-localization of repressor

complexes (Fan et al.). These structures may be further organized and compacted through interactions with distant genomic domains through phase separation and the weaving of chromosomal structures through interactions of chromatin with the nuclear envelope (Figure 1.1) (Erdel and Rippe). DNA at the histone-octamer interface is shielded from the nuclear environment. Furthermore, bending of the DNA around the histone complex distorts the width of the major groove in the double helix, resulting in a suboptimal fit for ZF proteins that interact with B-form DNA (Figure 1.1) (Luger et al.). Through this mechanisms nucleosome positioning dynamically regulates the genome (Bai and Morozov) as dense local nucleosome occupancy on the chromatin fiber precludes DNA accessibility to transcription factors (TFs) and other DNA binding proteins involved in transcription, replication and repair.

Nucleosome positioning dynamically regulates the genome. Dense local nucleosome occupancy on the chromatin fiber precludes transcription factors (TFs) and other DNA binding proteins involved in transcription, replication, and repair from accessing DNA. In assays where nucleases are used to cut DNA within whole nucleosomes, high levels of cleavage reproducibly distinguish open chromatin regions, whereas closed chromatin regions show low levels of cleavage (Tsompana and Buck). Physical interactions between endogenous nuclear proteins and DNA block the nucleases from accessing the DNA, further supporting the concept that access to DNA is influenced by chromatin structure.

How then, within this dense throng, do TFs and other DNA binding proteins still ultimately find their binding domains? The congregated and compacted nature of the eukaryotic genome is a dynamic physical barrier to any process that requires accessible DNA. Nucleosome positioning is regulated by several factors that determine local accessibility. Some DNA sequences directly inhibit or, conversely, favor nucleosome occupancy (Struhl and Segal). Additionally, nucleosomes bound to

DNA can be displaced by ATP-dependent chromatin remodelers or certain classes of pioneer transcription factors (PFs) that can regulate nucleosome position (Struhl and Segal). This displacement of nucleosomes directly increases accessibility to DNA binding factors. For example, high nucleosome density at promoter regions directly blocks the initiation of transcription (Lorch et al.) while depletion of nucleosomes at promoters facilitates RNA polymerase II binding and thus the initiation of transcription (Ichikawa et al.). While dynamic regulation of nucleosome occupancy allows the cell to regulate transcription, it more generally increases DNA accessibility for any binding protein, making nucleosome depletion an attractive option for opening chromatin.

1.1.2 Opening Chromatin to Activate Gene Expression

The site-specific opening of chromatin to activate gene expression is an invaluable tool for mammalian synthetic biologists. Orchestrating the activation of specific genes within the cell allows us to alter cell type (Black et al.), replicate gene expression profiles to create disease models (Hurtado Del Pozo et al.), and shift metabolic loads to increase product yields. Epigenetic activators can themselves be used as components of constructs for biosensing, computing, or other applications such as toggle switches or represilators (Kramer et al.; Perez-Carrasco et al.; Lei). Furthermore, these activators can be used to return gene expression to a desired state, either by reversing abnormal silencing in diseased cells (such as cancer) or reversing the undesired silencing of transgenes.

Targeted opening of chromatin in order to control gene expression state has been successfully achieved with two mechanisms: the deposition of activation-associated histone modifications and the localization of expression enhancing TFs. Functional domains behind both mechanisms can be fused to DNA binding domains

to locally increase gene expression at desired target sites, as described below. Whether this increase of expression and potential histone modification also increases accessibility for gene editing proteins is an area of active research (see Chapter 4).

Significant progress towards reliable, targeted gene activation through epigenetic control has been made, but a few key factors remain to be fully addressed. Foremost is the difficulty of inducing sustained activation after transient interaction with an activator. In other words, full remodeling of the chromatin from heterochromatin to a sustainable (wider-reaching) euchromatin state is still a challenge. This may be due to several factors including the putative phase-separated nature of heterochromatin, which favors reformation of dense chromatin after slight disruption (Erdel and Rippe). Heterochromatin spreading may also be a contributing factor (Greenstein and Al-Sady). The performance of targeted activators themselves may also be chromatin context-dependent. For example, Cano-Rodriguez *et al.* found that the endogenous chromatin microenvironment inhibited a targeted histone methyltransferase (Cano-Rodriguez et al.). Systematic studies at target sites with thoroughly mapped chromatin features will need to be conducted to elucidate the sources of such context dependence. Finally, many natural mechanisms of chromatin opening are under-utilized by biological engineers. This work seeks to address that gap in research.

1.1.3 Chromatin as a Barrier to Genome Editing

Chromatin prevents gene-editing enzymes from stably engaging with their targets. Since their discovery in the 1970s, prokaryotic proteins that bind to specific DNA sequences and carry out site-specific DNA cleavage — e.g., Type II restriction endonucleases (Loenen et al.) — have inspired the development of customizable nuclease-based systems that cut and alter DNA sequences in living eukaryotic cells.

Among these is the newcomer Cas9. This genetic editing tool is particularly attractive because only a short (~20 base pair [bp]) single-stranded guide RNA (sgRNA) is required to direct the Cas9 endonuclease to a specific location in the genome. Synthetic biologists and cell engineers have designed many alternate CRISPR-Cas9 systems that harness the highly specific and customizable binding properties of Cas9-sgRNA complexes.

Cas9 binding and endonuclease activity can be disrupted by nucleosome occupancy in vitro and in vivo. (Hinz et al.; Isaac et al.; Horlbeck et al.) The link between chromatin inaccessibility and Cas9 inhibition has been made in murine embryonic stem cells (Wu et al.), human cancer cells (Chari et al.; R. M. Daer et al.) and even zebrafish (*Danio rerio*). While other nucleases such as TALEs may also be perturbed by heterochromatin (Bultmann et al.), Cas9-based systems show a pronounced variability in robustness across genomic loci in different eukaryotic models. Variability in Cas9 editing can be linked to variance in chromatin architecture across the genome (Singh et al.). The context-dependence of Cas9 editing must be overcome for Cas9 to be the broadly effective, flexible tool genomic engineers require. To this end, scientists have investigated CRISPR-Cas9 activity within chromatin to identify critical design parameters for editing efficiency.

The primary design element of any CRISPR-Cas9 system is the sgRNA. In vivo studies have shown that sgRNA selection impacts editing efficiency in heterochromatin (R. M. Daer et al.; Uusi-Mäkelä et al.) and that sites with increased editing efficiency are often positively associated with areas of active transcription or open chromatin (Uusi-Mäkelä et al.). The distance between nucleosomes and the sgRNA recognition site has been shown to impact editing (Isaac et al.) — areas with high nucleosome occupancy are more likely to exhibit variability in editing across nearby target sites.

Cas9 protein concentration may also be an important parameter for improving editing in heterochromatin. Sampling of DNA binding sites by the Cas9-sgRNA complex is slowed in heterochromatin, suggesting that increasing protein concentration may increase sampling and successful binding events (Knight et al.). Likewise, editing is also delayed in heterochromatin in a concentration-dependent manner (Kallimasioti-Pazi et al.).

A few attempts have been made to engineer the limited intrinsic chromatin-opening ability of Cas9 proteins. Barkal et al. report that a catalytically dead Cas9 (dCas9) can induce chromatin accessibility as measured by DNAse (i.e., a nuclease that degrades DNA) hypersensitivity and gene activation at 16 previously inaccessible genomic loci in mouse embryonic stem cells (mESCs) (Barkal et al.). However, previous work shows that Cas9's ability to open chromatin is highly dependent on local chromatin structure, which was not investigated through either the interrogation of histone post-translational modifications (PTMs) or nucleosome positioning in Barkal et al. Their dCas9 system was also genomically integrated, possibly yielding higher dCas9 expression levels than by transient transfection.

Another group, Chen et al., used SpCas9, derived from Streptococcus pyogenes, to assist in opening chromatin for FnCas9, a novel RNA-guided nuclease (Chen et al.). This approach targets multiple SpCas9s between 7 bp and 50 bp from FnCas9, presumably forcing open local chromatin (although alterations to chromatin structure were not determined in their study). This approach does improve FnCas9 function, but the mechanism and efficacy across cell types and loci remains unclear. Although Cas9 design parameters can be manipulated in some contexts, inhibitory nucleosome occupancy remains an obstacle in many cases. Guide RNA target sites may be limited for certain applications and, although high concentrations of Cas9 may be able to surmount chromatin inhibition (Kallimasioti-Pazi et al.), this may be

both difficult to achieve and undesirable in vivo, where the host immune response to Cas9 must be taken into account (Charlesworth et al.). We ultimately need to remodel local chromatin structure to reduce variability and increase efficiency of Cas9-mediated editing throughout the genome.

1.2 Tools for Opening Chromatin

Editing the epigenome in order to harness chromatin as another layer of control for eukaryotic gene expression is a topic of growing interest (Park et al.). While dynamic regulation of epigenetic state has been achieved (Bintu et al.; Park et al.) harnessing this ability to improve Cas9 function remains a nascent area of research. Earlier studies showing that nucleosomes inhibit Cas9, were also able to reverse this inhibition with chromatin remodelers known to displace nucleosomes, suggesting a mechanism for engineering Cas9-accessible chromatin (Isaac et al.; Horlbeck et al.). We have also tested targeted activators as a means of reversing chromatin inhibition of Cas9 (R. Daer et al.). Such targeted activators have already shown promise for selectively remodeling genomic structures (P. Liu et al.) in a more controlled manner than is possible with epigenetic drugs, which induce genome wide disruptions to chromatin structure rather than intentional changes at a specific locus. *In vivo*, the cell utilizes a suite of native mechanisms to carefully tune accessibility and expression in the dynamic chromatin environment. Here, I explore these mechanisms, previous attempts at harnessing these cellular mechanisms that may assist in engineering chromatin (Table 1.1) and their potential to induce Cas9-accessible chromatin states. The next sections describe the native mechanisms of chromatin opening (Figure 1.2),previous attempts at harnessing these cellular mechanisms that may assist in engineering chromatin (Table 1.1), and their potential to generate Cas9-accessible chromatin states

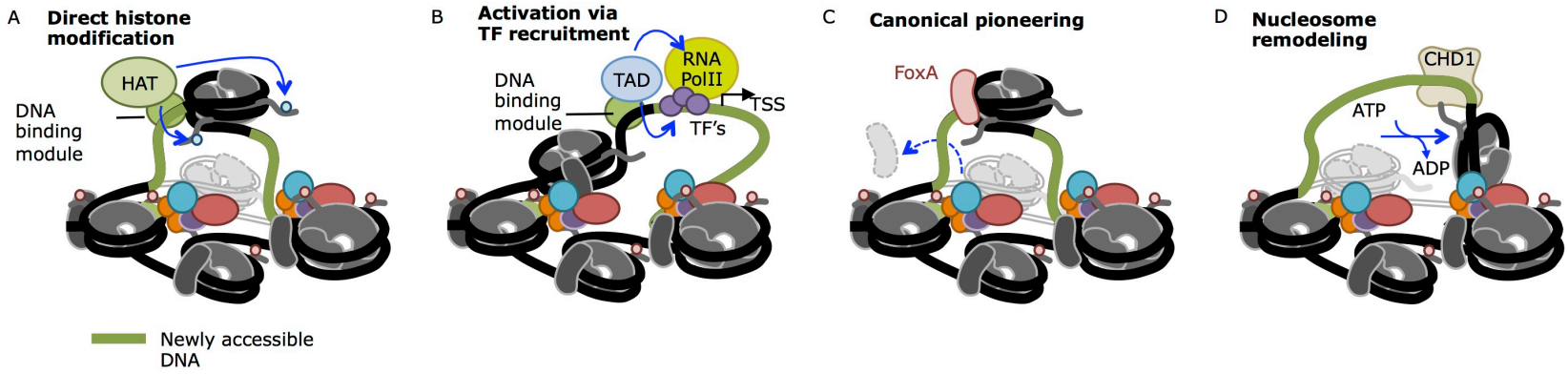

Figure 1.2: Native mechanisms for disrupting heterochromatin (A) Targeting of direct histone modifiers to specific sites allows activation-associated histone tail modifications to be locally deposited, inducing the opening of chromatin. HAT = histone acetyltransferase. (B) Site-directed activators induce chromatin opening and the activation of transcription by recruiting additional transcription factors and polymerases. TAD = transcriptional activation domain; TSS = transcription start site. (C) FOXA is shown as an example of a pioneer factor with structural homology to linker histones; this homology enables disruption of condensed nucleosome organization and increased DNA accessibility. (D) ATP-motor driven nucleosome remodeling complexes directionally shift, slide, or replace nucleosomes in order to modulate local chromatin accessibility. The "DNA binding module" represents any peptide (e.g. zinc finger or TALE) or complex (e.g. deactivated Cas9-sgRNA) that can be fused to an effector module (e.g. HAT or TAD) to target it to a specific location in the genome. (Reprinted with permission from Chemical Engineering Progress (CEP). Copyright © 2018 American Institute of Chemical Engineers (AIChE))

Table 1.1 Many groups have engineered targeted chromatin opening

Domain	General Function*	Effect(s) on Chromatin	DNA Binding Domain(s)	Model System(s)	Citation(s)
CBP	DHM; Histone acetyltransferase	+H3Kac	Gal4;MLL	• Human caner cells (U2OS) • Mice	Martinez-Balbas et al. 1996; Santillan et al. 2006
Dot1L	DHM; Histone methyltransferase	+H3K79me	LexA;MLL	• Yeast • Human cancer cells (U937)	Stulemeijer et al. 2011; Okada et al. 2006; Chory et al. 2018
GCN5	DHM; Histone acetyltransferase	+H3Kac	MLL	• Mice	Santillan et al. 2006
Meisetz	DHM; Histone tri-methyltransferase	+H3K4me3	Gal4	• Mice testes	Hayashi et al. 2005
P300 CD	DHM; Histone acetyltransferase	+H3K27ac	LexA; dCas9	• Hamster cells (CHO-K1) • Human cancer cells (HEK293T)	Kwaks et al. 2005; Hilton et al. 2015
PCAF	DHM; Lysine aceytltransferase	+H3Kac	Gal4; MLL	• Mouse melanoma (B78) • Mice	Krum et al. 1998; Santillan et al. 2006
PRDM9	DHM; Histone methyltransferase	+H3K4me3	dCas9	• Human cancer cells (HEK293, HeLa, A549, A2780, C33a)	Cano-Rodriguez et al. 2016
SUVH4	DHM; Histone-lysine N-methyltransferase	+H3K9me	TALE	• Murine Neuroblastoma (Neuro 2a)	Konermann et al. 2013
TgSET8	DHM; Histone methyltransferase	+H4K20me	TALE	• Murine Neuroblastoma (Neuro 2a)	Konermann et al. 2013
p65	TAD	Unknown	ZF; TALE; dCas9	• Rat heart myoblast (H9c2[2-1]) • Human fibroblasts (BJ) • *C. Elegans* • Human cancer cells (HEK293, HEP3B, A375) • Yeast • *Drosophila melanogaster* cells (S2R +) • Murine neuroblastoma (Neuro 2a)	Liu et al 2001.; Maeder et al. 2013; Perez-Pinera et al. 2013; Konermann et al. 2015
SAM (VP64, p65, HSF1)	TADs	Unknown	dCas9	• Human cancer cells (HEK293T, A375)	Konermann et al. 2015
VP64	TAD	DNA demethylation, +H3K27ac, +H3K4me	ZF; TALE; dCas9	• Human cancer cells (HeLa, HEK293T, K562, Hep3B, H9c2[2-1]) • Human fibroblasts (BJ) • Mouse fibroblasts (NIH3T3, PMEG-H) • Yeast • *Drosophila melanogaster* cells (S2R +) • Murine neuroblastoma (Neuro 2a) • Murine embryo	Beerli et al. 2000; Liu et al. 2001; Ji et al. 2014; Zhang et al. 2011; Maeder et al. 2013; Perez-Pinera et al. 2013; Male et al. 2013; Farzadfard et al. 2013; Chen et al. 2013; Hu et al. 2014; Jachowicz et al. 2017
VPR (VP64, p65, Rta)	TADs	Unknown	ZF; TALE; dCas9	• Human cancer cells (HEK293) • Yeast • *Drosophila melanogaster* cells (S2R +) • Murine neuroblastoma (Neuro 2a)	Chavez et al. 2015
CHD1	CR	Directional nucleosome sliding	Ume6 yeast DNA binding domain	• Yeast	McKnight et al. 2016
Ldb1 self-association domain	CR	Forced looping between promoter and enhancer	ZF	• Embryonic stem cells (G1E)	Deng et al. 2014
mSWI/SNF (BAF)	CR	-H3K27me, +H3K4me3	dCas9	• Mouse embryonic stem cells	Braun et al. 2017
223 yeast chromatin modifiers	Various functions	Unknown	ZF	• Yeast	Keung et al. 2014

*DHM = direct histone modifications; CR = ATP-dependent chromatin remodelers; TAD transcriptional activation domain; CD catalytic domain

1.2.1 Direct Histone Modifiers as Chromatin Opening Tools

For many TFs an exposed DNA consensus sequence is not sufficient to initiate binding in chromatin; posttranslational histone modifications (PTMs) work in concert with TFs to open chromatin (Guertin and Lis). Certain combinations of posttranslational histone modifications promote an accessible chromatin state as characterized by increased DNAse accessibility and actively transcribed genes (Bannister and Kouzarides). Previous work has shown the efficacy of engineered Direct Histone Modifying proteins (DHMs) for inducing an active, accessible chromatin state at a specific genomic target site (Figure 1.2a) (de Groote et al.).

1.2.1.1 Targeted Histone Methylases and Acetylases

Two major classes of DHMs have been engineered to date: histone methyltransferases and histone acetyltransferases. Both directly modify the histone tails of nucleosomes thereby altering local charge interactions that may disrupt histone-DNA binding interactions. They also serve as chemical marks to recruit transcription factors and other chromatin remodeling machinery. By fusing histone-modifying domains to DNA binding domains, targeted epigenetic manipulation can be achieved.

A variety of targeted DHMs have been used to modify local histone methylation. A few targeted demethylases have been engineered to deplete repressive histone methylation marks (Yokoyama et al.; Fukushige et al.). A fusion of histone demethylase KIAA1718 to a Gal4 DNA binding domain increased expression through the depletion of repressive histone methylation. (Yokoyama et al.) Depletion of these repressive marks is most likely directly responsible for induction of an active state by KIAA1718, as mutant KIAA1718 lacking demethylation activity does not enhance transcription (C. Huang et al.). Along with

histone methylation erasers, histone methylation writers have also been engineered to open chromatin. Several groups (Table 1.1) have developed targeted histone methylases capable of increasing local expression and adding active histone modifications (Stulemeijer et al.; Okada et al.; Cano-Rodriguez et al.; Hayashi et al.).

Histone acetyltransferases have also been used to engineer targeted DHMs. Several groups have effectively used fusions of EP300 to both LexA and dCas9 to increase local expression and deposit H3K27ac at promoter regions, which precludes the deposition of repression-associated H3K27me (Kwaks et al.; Hilton et al.). A variety of other histone acetyltransferase DHMs have been engineered to modulate expression state and modify local chromatin both *in vivo* and *in situ* (Krumm et al.; Martinez-Balbas; Santillan et al.).

1.2.1.2 Histone Modification Crosstalk Impacts DHM Function

Histone modifications display a certain level of crosstalk (Fischle et al.). Certain modifications may only be deposited if other modifications are pre-existing on the histone tail (Fischle et al.; McGinty et al.). The complexity of histone modification crosstalk is an important design parameter to consider when engineering open chromatin. For example, Cano-Rodriguez et al. found the chromatin microenvironment of their target sites, specifically levels of DNA methylation, impacted the ability of their DHM to deposit H3K4me and induce activation. Furthermore, H3K79me co-localization appeared to assist in stability of H3K4me transmission. Histone modification co-dependence has been shown for other engineered DHM domains as well (McGinty et al.; O'Geen et al.).

While silencing can be reversed using targeted histone modifications, doing so in a heritable manner that does not required the continued action of DHMs remains a

challenge. Persistence of open chromatin may be useful for engineering stable cell lines or for using Cas9 at low concentrations and thus a slow sampling rate. Several groups have managed to achieve this over a few generations of cell doubling (Kramer et al.), although persistence of activation may, again, be dependent on the co-localization of other histone modifications (Cano-Rodriguez et al.; Rivenbark et al.; X. Xu et al.).

1.2.2 Targeted Transcriptional Activation Domains

Although direct histone modification may encourage an increase in expression and directly alter chromatin structure, targeted recruitment of activating transcription factors has been a more popular approach for inducing strong increases in expression (Figure 1.2b). Two domains have been particularly well studied- Herpes simplex virus protein vmw65 (VP16) and nuclear factor NF-kappa-B p65 subunit (p65) encoded by the human *RELA* gene. VP16 subunits can be effectively fused together (i.e. VP64, VP160, etc.) along with a DNA binding domain to induce an increase in expression (Table 1.1) (Cheng et al.; Polstein et al.; Perez-Pinera, Ousterout, et al.; Tanenbaum et al.). While many studies show that VP16-based systems are able to increase expression across biological systems, few have looked at its structural impact on chromatin. Two independent groups have shown that VP64-dCas9 recruits remodeling factors and has been linked to increased chromatin accessibility and to the deposition of activating histone marks, including H3K27ac and H3K4me (Black et al.; Konermann, Brigham, A. E. Trevino, et al.). More wide scale effects that may increase accessibility such as nucleosome depletion have not been investigated.

P65 based systems have been harnessed in combination with VP64 by several groups to induce high levels of activation necessary to reprogram cells (Y. Zhang et

al.). However, p65-based systems have an undetermined effect on chromatin structure and accessibility. Some research suggests that excessive levels of transcription such as those induced by p65 may initially inhibit Cas9 editing due to Polymerase crowding (Clarke et al.; R. Daer et al.). Thus, when engineering accessibility with activators it may thus be important to consider their broader effect on the local chromatin environment. Histone modifications as well may not always be enough to induce an accessible state. Therefore, we propose two under-explored but promising strategies that leverage other endogenous tools for chromatin opening.

1.2.3 Canonical Pioneer Factors

Pioneer factors (PFs) are a unique class of non-enzymatic transcription factors able to bind DNA in heterochromatin and initiate the transition to an open state without the use of ATP or other cofactors (Figure 1.2c) (Zaret and Carroll). They engage sterically hindered DNA (that is bound to nucleosomes) by disrupting histone-DNA binding (Zaret and Carroll). PFs are crucial to cell development and reprogramming, serving as the initial foot-in-the-door for other TFs and DNA binding proteins (Iwafuchi-Doi and Zaret). While PFs have not been extensively investigated or manipulated as fusion protein tools, this well-characterized and highly conserved class of proteins has the potential to be tapped as a new resource for genomic engineering.

1.2.3.1 Structure and Function of Canonical Pioneer Factors

Pioneer factors begin the cellular cascade that transitions a heterochromatic locus to an open state of active transcription (Zaret and Carroll). PFs are unique in their ability to directly bind DNA bound to nucleosomes for a stable period before

activation and before other factors are recruited, imparting the necessary competence to initiate an open state (Zaret and Carroll). This transition initiation function is crucial to the dynamic regulation of the eukaryotic genome and is thus well conserved. Yeast Gal4 usurps endogenous chromatin remodelers through DNA-binding in order to displace histones at promoters and activate transcription (Kang et al.; Owen-Hughes and Workman). PFs are vital in initiating differentiation during the development process in both *Drosophila* and humans as well (Nien et al.; Liang et al.; Foygel et al.; Pan and Schultz). Iwafuchi-Doi and Zaret provide a useful compendium of verified pioneer factors across species, although more and more PFs are still being discovered (Oldfield et al.; Iwafuchi-Doi and Zaret).

Of these validated pioneer factors, Forkhead box protein A1 (FoxA1) is by far the most well-studied. It's structure and mechanism of nucleosome displacement serve as a model for general PF function. Early research on FoxA1 showed that the purified protein was able to bind target sites on nucleosomal DNA in a non-ATP dependent manner, opening previously closed areas of chromatin through the displacement of nucleosomes (Cirillo et al.). How is FoxA1 able to exert this type of remodeling power without catalytic energy expenditure? Further research showed that FoxA1 in fact has a preference for binding in nucleosome occupied sites as opposed to most TFs, which are inhibited by nucleosomes (Z. Li et al.). Indeed, FoxA1 binds quite tightly as indicated by its slow movement in chromatin and binds without the need for any histone PTMs (Sekiya et al.).

This unusual affinity between a TF and nucleosomes is partially revealed in the crystal structure of FOXA1, which has a winged helix motif with high homology to the DNA binding domain of linker histones H1 and H5 that also bind nucleosomes with great affinity (Clark et al.; Cirillo et al.). Linker histones favor chromatin compaction by contorting linker DNA and bringing distant nucleosomes into contact

(Cirillo et al.). FoxA's winged helix motif, however, has a slightly higher binding affinity to DNA than linker histones, allowing the protein to displace them by (Cirillo et al.). This winged helix motif binds the DNA helix along the long axis, leaving the other side still bound to core histones (Clark et al.). By binding simultaneously to the DNA and the core histones, FoxA1 disrupts local internucleosomal interactions between linker histones and the nucleosome core that stabilize chromatin higher-order structure (Schalch et al.; Lisa Ann Cirillo et al.). This disruption of condensed chromatin structures enhances accessibility, allowing other transcription factors to bind and stimulate transcription (Iwafuchi-Doi et al.). Through this mechanism, PFs can also bookmark enhancer regions putting them in a poised state by loosening chromatin and preventing the deposition of repressive histone modifications by occupying potential histone modifier binding sites (Cirillo et al.).

1.2.3.2 Pioneer Factors in the Complex Chromatin Environment

Pioneer factors interact dynamically with chromatin, both by shifting nucleosomes and through the occupation of potential chromatin effector binding sites (Zaret and Carroll; Cirillo et al.). PFs are impacted chromatin in several ways. Firstly, the are uniquely attracted to nucleosomes, running contrary to the dogma that nucleosomes are exclusively repressive as dense nucleosome occupancy at enhancer regions may stimulate PF recruitment (Barozzi et al.). PFs also interact with histone PTMS and other chromatin modifications such as DNA methylation, which is known to decrease FoxA1 binding (Sérandour et al.). Other histone PTMs are known to inhibit the PF function, such as inhibition of Oct3/4, Sox2, and Klf4 by H3K9me during fibroblast differentiation (Soufi et al.). Reducing the presence of H3K9me allows remodeling by these PFs to continue, adding another layer of control to the cellular reprogramming process of which PFs are an integral part (Soufi et al.). Enrichment of

H3K9me2 is also negatively associated with FoxA1 recruitment, further highlighting the need to consider the larger chromatin environment when engineering open states (Lupien et al.).

PFs also exert an influence on chromatin structure and histone modifications. H3K9ac deposition follows or may be concomitant with FoxA1 binding, opposing any deposition of repressive H3K9me (Taube et al.). Other activation-associated modifications such as H3K4me are also associated with PF binding at enhancers (Sérandour et al.; Lupien et al.; Heinz et al.). Deposition of such modifications, coupled with linker histone displacement further serves to bookmark enhancer regions and prevent repressive modifications, potentially allowing for a longer-term open chromatin state (J. Xu et al.; You et al.).

1.2.3.3 Pioneer Factors as Tools for Engineering Open Chromatin

While human PFs have to date not been engineered as DNA-binding protein fusions, the idea has been put forth by previous reviewers of the topic: "Conceivably, such chromatin-opening domains could be transferred to other factors and augment their regulatory function" (Zaret and Carroll). Yeast pioneer factor Gal4 has been extensively used in engineering, but primarily for its DNA binding domain. Erkine and Gross have shown that its histone binding domain in isolation does deplete histones at a target site, giving a precedence for isolating PF functional groups for engineering (Erkine and Gross). Other PF functional domains such as the winged-helix domain of FOXA1 have been identified (Cirillo et al.) providing us a tool kit to begin working with and targeting to loci of interest with DNA binding domains. Indeed previous work suggests that continuous FOXA PF binding is not required to maintain local nucleosome organization (X.-Y. Li et al.; Thomassin et al.) or in other words that we

can produce a primed chromatin state with only a transient induction, leaving the site open for Cas9.

1.2.4 ATP-Dependent Chromatin Remodelers

Chromatin remodelers (CRs) exert a powerful effect on chromatin structure by directly displacing nucleosomes in a reaction driven by ATP. Unlike PFs, the displacement of nucleosomes by CRs is driven by an ATP-dependent process that is currently still the subject of debate, as opposed to energetically favored structural binding (Figure 1.2d).

1.2.4.1 Structure and Function of ATP-Dependent Remodelers

CRs are typically large multi-subunit complexes, powered by an ATP-hydrolysis motor, that shift, remove, or exchange nucleosomes from DNA (Clapier et al.). Together with sequence-specific TFs CRs orchestrate the precise positioning of nucleosomes in eukaryotic genomes (Clapier et al.; Bowman and McKnight). While the ATP-hydrolysis motor is a common function among CRs their effects on nucleosome positioning differ drastically (Hota and Bartholomew). For example, in the switch/sucrose non-fermentable (SWI/SNF) system, the ATPase motor's helicase motif binds to nucleosomal DNA, disrupting histone DNA binding, while auxiliary subunits bind to superhelical structures to act as a counter grip for the motor to displace nucleosomes (X. Liu et al.). While some CRs remove nucleosomes, others slide nucleosomes along the DNA (Figure 1.3). For example, ISWI remodelers pull DNA through the nucleosome and out of the exit site during remodeling (Deindl et al.). Still others exchange histones of the nucleosome for new histone varieties (Figure 1.3) (Narlikar et al.). While the exact mechanism that drives the ATPase

motor is still debated, the structural evidence that does exist for CRs suggests a series of distinct subunits associate with unique functions open to isolation and engineering. Although the exact mechanism that drives the ATPase motor is still debated, evidence suggests that distinct subunits are associated with unique functions that can be isolated and engineered.

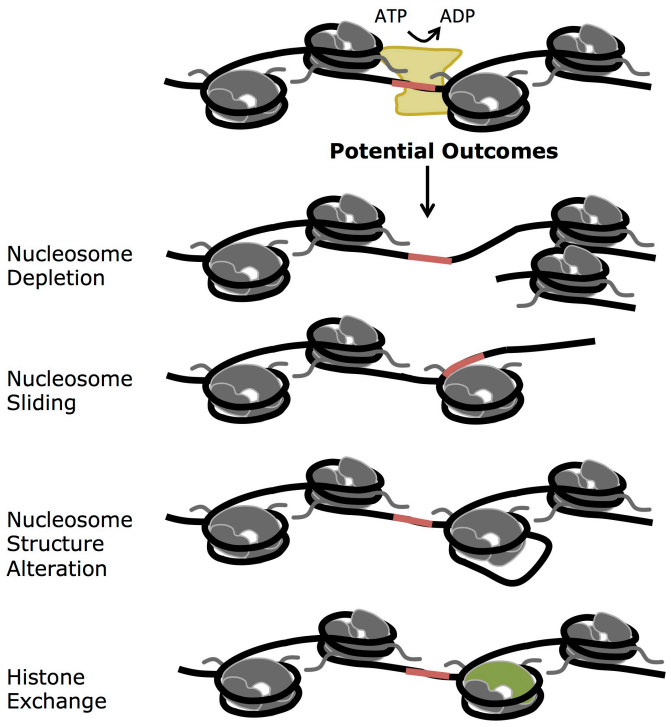

Figure 1.3: Potential outcomes of ATP-Dependent chromatin remodeling. ATP-dependent chromatin remodeling complexes (in yellow) catalyze a reaction that can result in several outcomes. Nucleosomes may be locally depleted through removal or by sliding them along the DNA strand. The physical structure of the local chromatin can also be disrupted by remodeling of the nucleosome-DNA interaction or the physical exchange of nucleosomes to alternate histone variants that are more permissive to a euchromatin state.

1.2.4.2 Nucleosome Remodelers as Tools for Opening Chromatin

Pioneering efforts have been made to stimulate chromatin remodeling at targeted sites. Keung et al, who screened a library of 223 yeast chromatin-regulating

domains (many of which were remodelers) fused to a DNA binding domain to determine their relative effects on expression (Keung et al.). They identified a large number of domains that successfully increased expression at the target locus. McKnight et al fused the nucleosome sliding domain Chd1 to a yeast DNA binding domain, representing one of the few successful examples of engineered sequence-targeted remodeling (McKnight, Tsukiyama, et al.). Their targeted remodeler successfully added nucleosomes to the target site, inducing a closed chromatin state. These works serves as a proof of concept for engineering chromatin states with CRs.

While McKnight et al. demonstrated gene repression in their CHD1-based system, theoretically this directional sliding remodeler could also induce an open state by pushing nucleosomes further downstream away from the promoter (Table 1.1). This is often its native function; in mouse embryonic fibroblasts Chd1 evicts nucleosomes from promoters to allow for PolII entry into the site, providing a precedence for this domain to induce an open state that may facilitate an open state amenable to editing (Skene et al.). CRs such as CHD1 could be used to expose DNA to Cas9 for genome editing, because CRs do not directly initiate transcription but instead physically open chromatin. CHD1 has been shown to detach two turns of DNA from the nucleosome as it positions its ATP-driven motor for catalysis, suggesting a loosening, as well as a nucleosome-sliding function (Farnung et al.).

Braun et al. have also achieved targeted activation with chromatin remodelers, although not through the construction of direction fusions (Braun et al.). Instead, they utilize a dCas9-MS2 anchor system by which they can induce the recruitment of the SWI/SNF (BAF) chromatin remodeling complex to a specific site. While they did not examine the effects of recruitment on nucleosome positioning, they did see increases in transcription as well as effective Polycomb Repressive Complex II (PRC2) antagonism as shown by the deposition of activating H3K4me3

marks and the depletion of H3K27me3 typically deposited by PRC2. Although activation via this system proved to be transient, this study is unique in its inducible mechanism for targeted remodeling.

Can we separate these useful functions of loosening DNA and sliding nucleosomes to be individual tools for chromatin opening? Many remodelers systems including Chd1 and ISW complexes have HAND-SANT-SLIDE domains with more distinct known functionality. The architecture of the HAND-SANT domains suggests their involvement in a loosening mechanism whereby DNA is pushed into the nucleosome entry site to move it circum the nucleosome (Dang and Bartholomew). Interestingly, SANT-SLIDE deletion removes directionality of nucleosome sliding, suggesting an involvement in sliding (McKnight, Jenkins, et al.). Deletion of all three of these domains reduces rates of remodeling drastically, suggesting their functions greatly aid the ATPase-motor in remodeling (Mueller-Planitz et al.).

While the domains of interest explored above effectively remodel local chromatin, few have attempted broad scale disruption of higher-order chromatin structure with a sequence-specific fusion (Table 1.1). Although this approach may be too disruptive for many applications, it may be useful when targeting Cas9 to several loci across an entire gene. Deng et al achieved this sort of large scale remodeling by fusing the Lbd1 domain with a DNA binding domain (Deng et al.). Lbd1 forced contact between the targeted promoter and upstream enhancer regions to activate transcription. This type of long-distance remodeling and activation may be useful to disrupt board areas of dense heterochromatin that inhibit editing across an entire coding sequence.

1.3 Innovations in Chromatin Opening

Cells use several mechanisms in concert to regulate the opening and closing of chromatin. Though progress has been made toward engineering each of these mechanisms in isolation, synergistic approaches may be able to more effectively tune chromatin states. How do we begin to design strategies to open chromatin that harmoniously and effectively use multiple chromatin effectors? Can we do this in a manner that decouples opening from increases in expression that may preclude effective editing? To do so would support efficient editing across the genome without inducing pleiotropic effects on metabolism and regulation.

1.3.1 Effectively Combining Chromatin Opening Strategies

One of the challenges in designing robust and reliable chromatin remodeling is crosstalk between different remodeling activities. For instance, histone modifications can alter the chromatin landscape in ways that promote or inhibit the function of CRs. Therefore, it is important to consider the synergistic or inhibitory relationship between different chromatin features. Cooperative interactions that drive the opening of chromatin suggest the potential for engineering effective systems to expose DNA to nuclease-mediated sequence editing.

The targeted deposition of histone modification has been successfully engineered on several occasions, suggesting it as a relatively easy strategy to use in concert with other mechanisms of chromatin opening. For example, through frequent transient interactions, CRs scan the biophysical potential of nucleosomes to be displaced. Most of these encounters do not lead to remodeling; the outcome of the interaction depends on increased binding affinity to chromatin that is mediated through histone PTMs and the colocalization of PFs (Erdel et al.). Histone acetylation

typically precedes chromatin remodeling, therefore DHMs (such as p300) can prepare a target site for remodeling (Huang et al.; Chatterjee et al.).

Activators have also been successfully used in concert with native remodeling machinery. VP16 interacts with the SWI/SNF complex to promote transcription (Neely et al.). This relationship was leveraged by Gutiérrez et al, who targeted a Gal4_VP16 fusion to a target site in order to recruit SWI/SNF and induce remodeling (Gutiérrez et al.). This approach has been validated for other CRs as well; VP16 and INO80 work in concert to enhance chromatin mobility and remodeling (Neumann et al.).

There is also some evidence that suggests the utility of PFs in assisting in remodeling. PFs assist in readying chromatin for transcription and remodeling. Linker histone H1 inhibits the activity of certain CRs (Hill and Imbalzano). PFs such as FoxA1 can directly displace H1, providing a mechanism by which PFs and CRs may be used in sequence to engineer an accessible chromatin state.

Synergistic strategies may also help overcome expansive heterochromatin environments that are spread over several kilobases of inter- and intra-chromosomal neighborhoods. Multi-dimensional chromatin structures are regulated by combinations of factors that affect accessibility, suggesting the need for a combination of factors to disrupt these structures (Buenrostro et al.). For example, the regulation of boundaries between open and closed chromatin relies on both histone PTMs and nucleosome arrangement (Chai et al.). Moreover, these boundaries can be highly distinct, forming liquid-like compartments through mechanisms akin to phase separation making them difficult to disrupt (Erdel and Rippe). *In vitro* evidence suggests that differences in nucleosome density (as regulated by PTMS, PF presence, etc.) contribute to phase separation (Yamamoto and Schiessel). Due to the expansive, dense nature of these liquid-like compartments combinatorial approaches

to opening may be necessary. Native regulation of these liquid-like compartments relies of interactions between CRs and siRNAs, suggesting avenues of exploration for combinatorial engineering of large-scale heterochromatin disruption (Wan et al.).

1.3.2 Decoupling Chromatin Remodeling from Expression

Structural opening of chromatin and the initiation of transcription are two distinct events, yet their occurrence are often linked. This may present an issue when we wish to engineer a chromatin state that increases accessibility to genetic editing machinery. An increase in transcription may not be a desirable outcome for engineered cells. Whether knocking out a gene with an undesirable gene product or editing a region that is associated with large-scale effects on cellular function, care must be taken to expose rather than activate DNA. Additionally, high levels of transcription may actually inhibit editing. Recent evidence suggests that Pol II (associated with high levels of transcription) physically displaces SpCas9 endonucleases (Clarke et al.). This is consistent with work showing that the use of p65 activators, which recruit Pol II, may initially inhibit rather than enhance editing, even if chromatin is being opened (R. Daer et al.).

How then can we decouple opening from enhancement? Is there an accessible intermediate state between closed and active chromatin? Research suggests that a "poised" state may strike a balance between chromatin closure and active transcription. This Goldilocks state is characterized by the colocalization of opposing factors such as repressive and active histone PTMs, repressive histone marks with PFs, or repressive proteins and transcription factors (Sérandour et al.). For example, FOXA1 depletes local nucleosomes and recruits other nuclear proteins to establish a poised expression-competent state (Zaret and Carroll). The threshold for activation

of the gene is now much lower; with nucleosomes already removed from the DNA, only the displacement of a few repressors is necessary to induce activation. This less dense, easily activated state developed by FOXA1, makes the gene poised for activation. Likewise, specific combinations of activation associated PTMs can push a state from poised to active (Creyghton et al.). Deposition of histone PTMs may, if carefully designed, also be able to induce a poised state without increases in expression.

Chromatin is a dynamic, DNA-shielding barrier that is natively regulated by a host of mechanisms. These mechanisms are not beyond our control and may be engineered to specifically target genomic areas of interest to increase the effectiveness of genetic editing. The interplay of these many epigenetic mechanisms suggests that synthetic multimodal systems may be the key to gaining complete access to eukaryotic DNA for genome engineering.

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CHAPTER 2

USE OF MYB AS A NEW SYNTHETIC ACTIVATOR TO ENHANCE TRANSGENE EXPRESSION WITHIN REPRESSED POLYCOMB CHROMATIN[†]

Epigenetic silencing of transgenes through chromatin packaging has been a persistent issue for the development of transgenic mammalian cell lines. Endogenous mechanisms are known to induce a closed chromatin state around foreign DNA before and after it has been integrated into a host cell's genome. Scientists are interested in reversing this silencing, but a lack of *a priori* knowledge of the chromatin features at transgenes hinders the rational design and application of effective strategies for transcriptional activation. Here, we systematically tested activation-associated DNA elements and proteins in transfected plasmid DNA and at epigenetically silenced chromosomal transgenes. We demonstrated that placing DNA elements that are targeted by MYB (c-myb) and p65 upstream of a minimal promoter enhance expression from transfected plasmid DNA. To regulate the expression of chromosomally integrated transgenes, we used proteins fused to the Gal4 DNA binding domain or dCas9/sgRNA. Three activation-associated peptides, p65, VP64, and MYB, sustained reactivation of transgene expression over 15 cell divisions in an immortalized human cell line (HEK293). Activity of the MYB fusion was inhibited by celastrol, a drug that blocks interactions between MYB and the p300/CBP histone acetyltransferase complex. Single-site targeting via dCas9-MYB was sufficient

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to activate transgenes within ectopic Polycomb heterochromatin and at a different site that had undergone position effect silencing. We demonstrate the utility and flexibility of cis-regulatory elements and fusion proteins derived from natural gene regulation systems to enhance expression from epigenetically silenced transgenes. DNA motifs for p65 and MYB can be added to the transgene itself, or the activating proteins can be targeted to transgenes without enhancers to stimulate gene activation. This work has implications for determining the most appropriate strategy to enhance gene expression specifically in Polycomb-repressed chromatin.

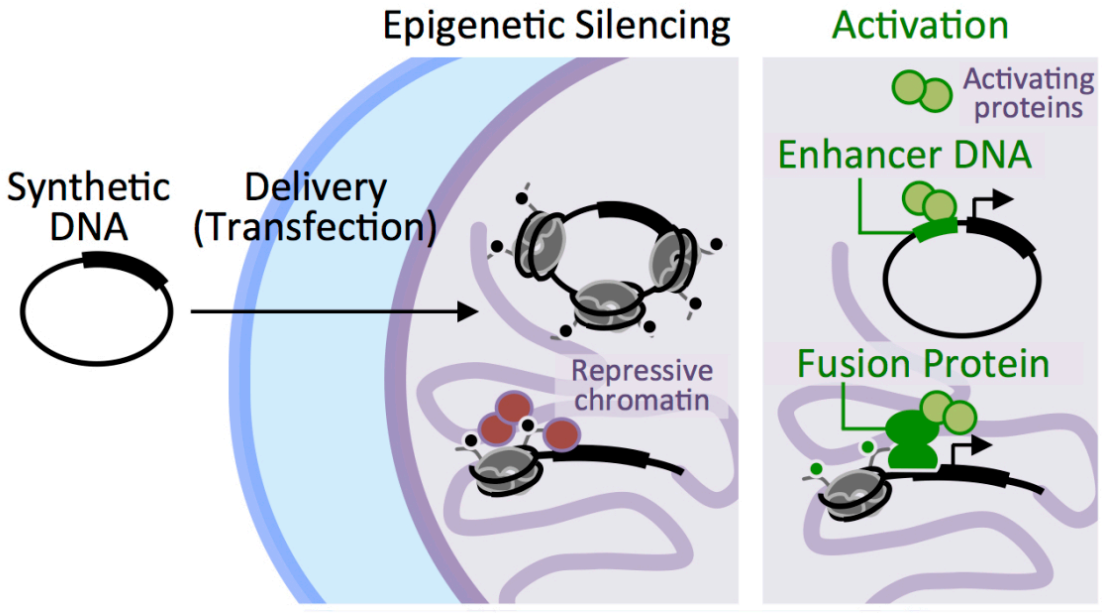

Figure 2.0: Targeted enhancers can be used to reverse transgene silencing. After the delivery of transgenic material into the cell, transgenes can either be stably integrated or maintained in the cell as plasmids. In both scenarios, heterochromatin may form around the transgenic material as a natural defense mechanism against foreign (viral) DNA. This accumulation of nucleosomes and repressive proteins prohibits active gene expression. To reverse this in a targeted manner, two general approaches can be used. DNA sequences that recruit enhancers may be integrated into the original construct. Here we test several mammalian enhancers on plasmid DNA to compare their ability to increase expression of plasmid-borne transgenes. Alternatively, DNA sequence-specific fusion proteins with activation-associated domains may be able to reverse silencing of chromosomally integrated transgenes.

2.1 Background

The advancement of cell engineering requires robust and reliable control of endogenous and synthetic genetic material within living cells. A lack of tools for enhancing the expression of transgenes in mammalian cells currently limits effective gene regulation across contexts. The rapid formation of heterochromatin around transgenic material in mammalian cells limits our ability to express foreign DNA for the production of therapeutic proteins and the development of engineered mammalian systems for biosensing and computing (Brooks et al.; Suzuki et al.). Integrated transgenes are often silenced by the same mechanisms that serve as a cellular defense against viral insertion into the genome (Leung and Lorincz; Ross et al.; Ellis). Nucleation of heterochromatin around transgenic material can be initiated and sustained by both promoter methylation (Brooks et al.; Ellis) and various histone modifications (Ross et al.; Suzuki et al.). For example, MyD88 pathway-mediated silencing of transgenes leads to an accumulation of repressive H3K9me on newly bound histones (Suzuki et al.; Gong et al.). Silencing of transgenes may also be Polycomb-mediated, where Polycomb repressive complexes deposit H3K27me3 on histones to establish a silenced state (Erhardt et al.; Dufourt et al.; Otte and Kwaks). The diversity and persistence of transgene silencing has led to the development of tools for mammalian cell engineering specifically aimed at combating heterochromatin.

Recruiting activators to a specific locus in order to reverse epigenetic silencing can be achieved either by including an activation-associated cis-regulatory DNA sequence within the construct itself, or through the targeting of engineered fusion proteins to the silenced transgene. Both natural and synthetic cis-regulatory motifs

that recruit activators have been used (Johansen et al.; Cheng and Alper; Zimmerman et al.; Wen Wang et al.) to help increase transgene expression as an alternative to viral promoters that are prone to methylation and silencing (Brooks et al.). Previous screens by ourselves and other groups (Roberts et al.; Saxena et al.; Cheng and Alper) have identified mammalian activation-associated cis-regulatory elements that recruit endogenous factors to increase the expression of epigenetically silenced transgenes, including motifs for nuclear factor Y, CTCF, and elongation factor alpha (EF1-α) (Zimmerman et al.; Wen Wang et al.). The underlying regulatory mechanisms are not entirely understood, since in this case efficient screening for functional sequences has been prioritized over dissecting the mechanism of individual elements.

Fusion proteins that target activation-associated domains to transgenes can also be used to reverse silencing. Targeted activators such as VPR, SAM, and SunTag (Chavez et al.; Konermann et al.; Y.-H. Huang et al.) are composed of transcriptional activation domain (TAD) peptides, including Herpes simplex virus protein vmw65 (VP16) and nuclear factor NF-kappa-B p65 subunit (p65). Site-specific targeting of VP64 (4x VP16) has been used to increase endogenous gene expression, and remodels chromatin through the accumulation of activation-associated histone modifications (H3K27ac and H3K4me) (Konermann et al.; Black et al.; Gao et al.). Likewise, p65-based systems are very effective at restoring both endogenous (Chavez et al.; Zhang et al.) and transgenic (Daer et al.) gene expression, but have an undetermined effect on chromatin structure and accessibility.

Significant progress towards transgene reactivation has been made so far, but several important gaps remain. First, several natural mechanisms of activation are still under-investigated by biological engineers. For instance, chromatin remodelers shift, remove, or exchange nucleosomes (Clapier et al.), and pioneer factors increase

DNA accessibility in closed chromatin by displacing linker histones (Clapier et al.; Zaret and Carroll; Magnani et al.). Second, the parameters for stable transgene activation are not yet fully defined. So far, at least two studies have demonstrated prolonged enhancement of transgenes (10 to 25 days) via targeted fusion proteins alone (Kramer et al.) or in combination with flanking anti-repressor DNA elements (Kwaks et al.). Neither study evaluated the chromatin features at the target genes prior to their reactivation, therefore the context in which expression enhancement occurred is uncertain. Finally, the performance of targeted activators can be context-dependent. Catalytic domains used for site-specific chromatin remodeling (Santillan et al.; Hilton et al.; Kwaks et al.), may be inhibited by pre-existing chromatin features that vary across loci. For example, Cano-Rodriguez *et al.* constructed a targeted histone methyltransferase fusion and found that the endogenous chromatin microenvironment, including DNA methylation and H3K79me, impacted the ability of their fusion to deposit H3K4me and induce activation (Cano-Rodriguez et al.). Similarly inconsistent performance has been shown for other fusions that generate H3K79me and H3K9me (McGinty et al.; O'Geen et al.). Systematic studies at loci with well-defined chromatin compositions are needed to fully understand mechanisms of chromatin state switching.

Here, we expand previous work where we had identified cis-regulatory sequences that enhanced expression from plasmid-borne transgenes (Zimmerman et al.). To regulate expression of chromosomally inserted transgenes, we compare targeted proteins that represent diverse activities: transcriptional activation through cofactor recruitment, direct histone modification, and nucleosome repositioning and displacement. We focus on reversal of silencing within Polycomb heterochromatin, which is known to accumulate at transgenes that are integrated into chromosomes (Erhardt et al.; Dufourt et al.; Otte and Kwaks) and is widely distributed across

hundreds or thousands of endogenous mammalian genes that play critical roles in normal development and disease (Otte and Kwaks; Aloia et al.; Poynter and Kadoch). We report that recruitment of p65 and MYB-associated components via a cis-regulatory element or fusion proteins enhances expression from epigenetically silenced transgenes. MYB-mediated activation within Polycomb heterochromatin relies on interactions with p300 and CBP. Our results have implications for determining the most appropriate strategy to enhance gene expression, specifically within Polycomb-repressed chromatin.

2.2 Results

2.2.1 Identification of Activation Associated Peptides

We surveyed public data to identify epigenetic enzymes and other proteins that are associated with transcriptional activation, and therefore might effectively disrupt repressive Polycomb chromatin. Polycomb-enriched chromatin typically includes Polycomb Repressive Complex 1 (PRC1: RING1A/B, PCGF1–PCGF6, CBX2, PHC1–PHC3, and SCMH1/2) (Schuettengruber et al.), PRC2 (EZH1/2, EED, Suz12, and RBBP4/7) (Schuettengruber et al.), H3K27me3, histone deacetylation, H2AK119ub1, and lncRNAs (Simon and Kingston; Schuettengruber et al.). Each activation-associated peptide (AAP) generates modifications of histone tails either by intrinsic catalytic activity or the recruitment of chromatin-modifying co-factors. In order to predict how these AAPs might influence Polycomb heterochromatin, we searched the STRING protein-protein interaction database for binding partners and their associated chromatin-modifying activities (Figure 2.1).

The AAPs fall into six general categories. The transcriptional activation group, (NFkB)-p65 and the MYB (c-myb) transcriptional activation domain (TAD), includes proteins that recruit RNA Polymerase II (PolII) and p300/CBP, respectively. These AAPs have no known intrinsic gene regulation activity, and therefore rely upon the recruitment of other proteins to stimulate transcription (Beerli et al.; P. Q. Liu et al.; Weston and Michael Bishop). We also included the recombinant TAD VP64 (four tandem copies of VP16), a popular component for synthetic activators. Histone modifications deposited by the co-activators that are recruited by these three domains are primarily associated with activation.

The histone acetylation (HAT) group includes ATF2, P300, and KAT2B. These peptides acetylate H3K27. In particular, p300 is associated with the recruitment of CBP and other co-activators that generate the activation associated mark H3K4me (Vo and Goodman). The histone H3 methyltransferase (H3 MT) group and the H4 methyltransferase (H4 MT) group include proteins that are either Mixed-Lineage Leukemia (MLL) complex components or SET proteins. SETD7 deposits the activation-associated modification H3K4me, but its regulatory impact may vary based on local DNA methylation, which can enhance or impede co-recruitment of repressive cofactors. The histone H4 methyltransferase PRMT5 induces histone acetylation that is associated with DNA methylation in some contexts (Zhao et al.). Still, PRMT5 primarily acts as an activator.

The final two groups, chromatin remodelers (CR) and pioneer factors (PF) represent activities that are relatively underexplored in the design of fusion-protein regulators. SMARCA4 is a chromatin remodeler that relies on an ATP-dependent reaction to shift the position of nucleosomes at a target site (Antonysamy et al.). It does not mediate the deposition of histone modifications, but is associated with CBP recruitment that evicts Polycomb-associated histone modifications (Alver et al.). PFs

are represented in our library by FOXA1, a winged-helix protein that displaces linker histones from DNA to facilitate a transition to open chromatin (Clark et al.). In general, PFs bind to DNA within heterochromatin and do not catalyze histone post-translational modifications (Magnani et al.).

Several of the AAPs in our panel are associated with the eviction of Polycomb repressive complexes (PRCs) from endogenous genes. Accumulation of the chromatin remodeling protein SMARCA4 (BRG1) leads to the loss of PRCs at *Pou5f1* in mouse cells (Kadoch et al.) and at *INK4b-ARF-INK4a* in human malignant rhabdoid tumor cells (Kia et al.). In the latter case, KMT2A (MLL1) also participates in PRC depletion. ATF2 interacts with a kinase that generates H3S28p, which antagonizes PRC binding (Lau and Cheung; Gehani et al.; Josefowicz et al.). Acetylation and methylation at H3K27 are mutually exclusive (Tie et al.; Englert et al.), therefore the AAPs associated with H3K27ac (p65, MYB, ATF2, P300, KAT2B) might contribute to PRC eviction (Figure 2.1). None of the AAPs in our panel are associated with enzymatic erasure of H3K27me3.

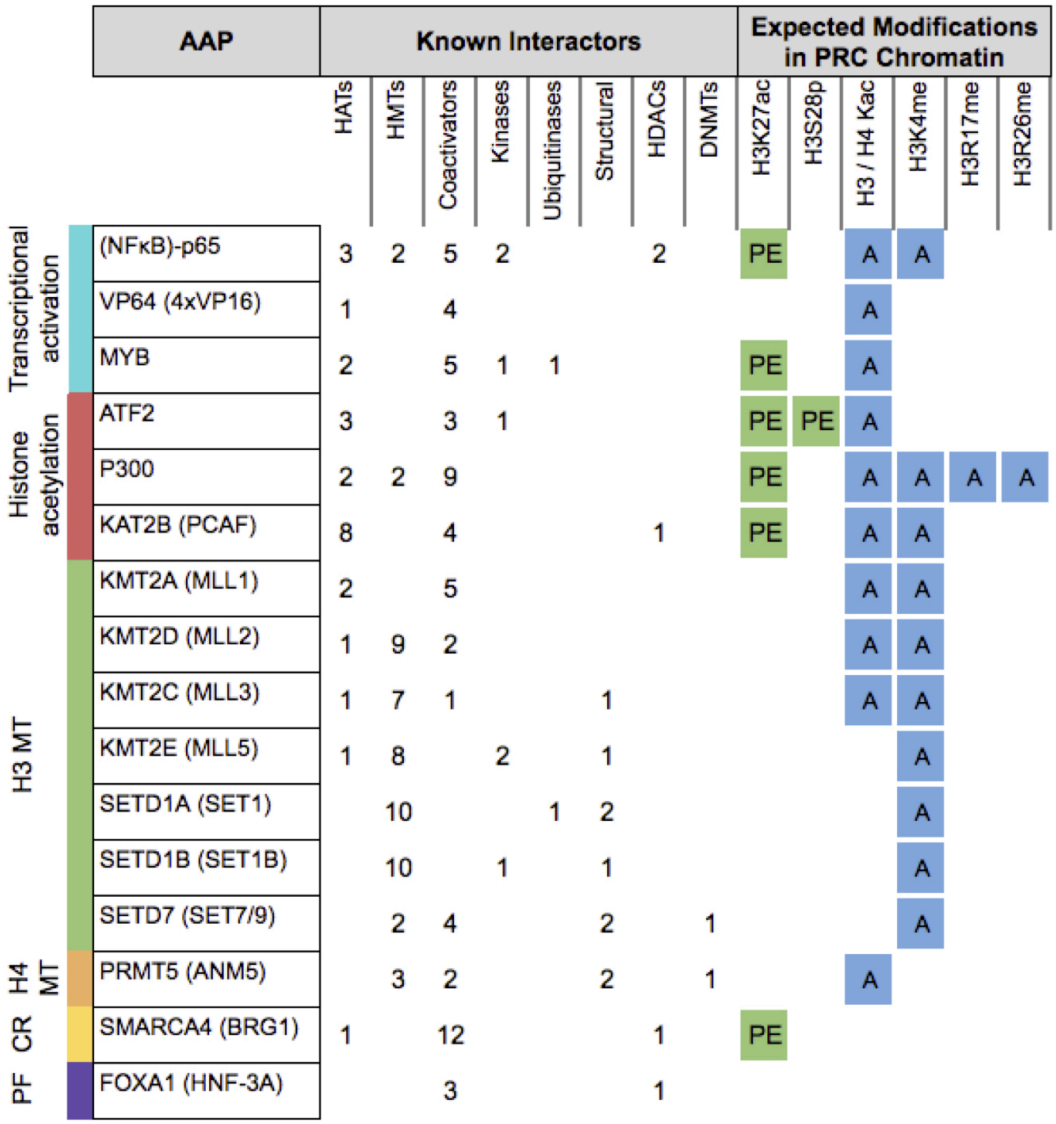

Figure 2.1: Activation-associated histone modifications associated with activation-associated peptides (AAPs) used in this study. Interaction partners determined by STRING analysis are listed in Appendix A, Table A1. Previous work that characterized each AAP is cited in Appendix A, Table A2. H3 MT = histone H3 methyltransferase, H4 MT = histone H4 methyltransferase, CR = chromatin remodeler, PF = pioneer factor, PE = Polycomb eviction, A = transcriptional activation.

2.2.2 Cis-regulatory elements recognized by transcriptional activators p65 and MYB enhance expression from an extra-chromosomal transgene

First, we used enhancer DNA elements to regulate expression from transiently transfected plasmid DNA. Work from our group (Christensen et al.) and others (Gracey Maniar et al.; Riu et al.) has shown that plasmid DNA becomes occupied by histones, which may contribute to transgene silencing in human cells. In a previous study, we used DNA sequences that were known targets of endogenous activation-associated proteins to reduce silencing of a *luciferase* reporter gene (Zimmerman et al.). Here, we tested additional motifs (Figure 2.2a) that are recognized by AAPs from the transcriptional activator group in our panel: MYB and p65 (Figure 2.1).

One of three MYB enhancer variants or the p65 enhancer was placed in either a forward or reverse orientation upstream of an EF1a promoter and a *luciferase* reporter (Figure 2.2b). PC-3 (human prostate cancer) cells were transfected with each plasmid as described previously (Zimmerman et al.). The highest levels of enhanced expression were observed for MYB variant A (4.5-fold, $p = 0.03$) or p65 (5-fold, $p = 0.08$) placed in the reverse orientation (Figure 2.2c). Interestingly, switching the orientation of these motifs eliminated the enhancement effects. Nonetheless, these results suggest that cis-regulatory elements from the p65 and MYB systems can be used to attract endogenous transcriptional activators to a synthetic promoter to drive transgene expression.

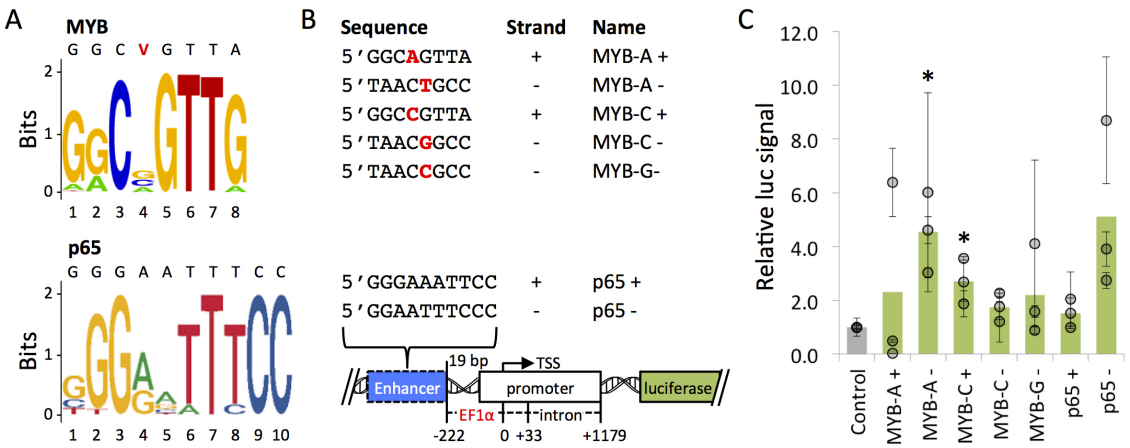

Figure 2.2: Luciferase expression from MYB- and p65-enhancer constructs. A) Enhancer motif logos for MYB and p65 were generated by JASPAR (Mathelier et al.). The MYB sequence includes a variable site (V) equally occupied by A, C, or G nucleotides. B) *Luciferase* reporter constructs (center) included one of the enhancer sequences (MYB-A +, etc.) 19 bp upstream of an EF1α promoter, or no enhancer (Control). C) Luciferase assays were carried out using PC-3 cells transfected with Lipofectamine-plasmid complexes. For each transfection, luminescence (luc signal) values were measured in triplicate and normalized to the average signal from the Control. Circle = mean normalized signal from a transfection, error bars = standard deviation. Wide bars represent the average of three transfections. Asterisks (*) = $p < 0.05$ for the experimental average, relative to the Control average.

2.2.3 Identification of fusions with robust activity within Polycomb heterochromatin

Next, we asked whether the individual peptides MYB and p65, as well as other AAPs could enhance transgene expression in the absence of a specific enhancer sequence. To determine AAP activity within silenced chromatin, we targeted AAP fusion proteins (Figure 2.1) to a chromosomal *luciferase* reporter that had been previously targeted by Polycomb repressive complexes (PRCs). The AAP open reading frames (ORFs) encode catalytic subunits or full length proteins (Figure 2.3) that have been shown to support an epigenetically active state in various prior studies (Beerli et al.; P. Q. Liu et al.; Zobel et al.; Kawasaki et al.; L. Yang et al.; Milne et al.; Nishioka et al.; Antonysamy et al.; Clark et al.; Serandour et al.; W. Wang et al.). All of these ORFs exclude DNA binding and histone binding domains,

except for the ORF encoding FOXA1 which has a catalytic domain that requires histone interactions. We cloned each ORF into mammalian vector 14 (MV14) (Figure 2.3) to express a Gal4-mCherry-AAP fusion. The Gal4 DNA binding domain serves as a module to target AAPs to UAS sequences in the transgene, while the mCherry tag allows for protein visualization and quantification of the activator fusion.

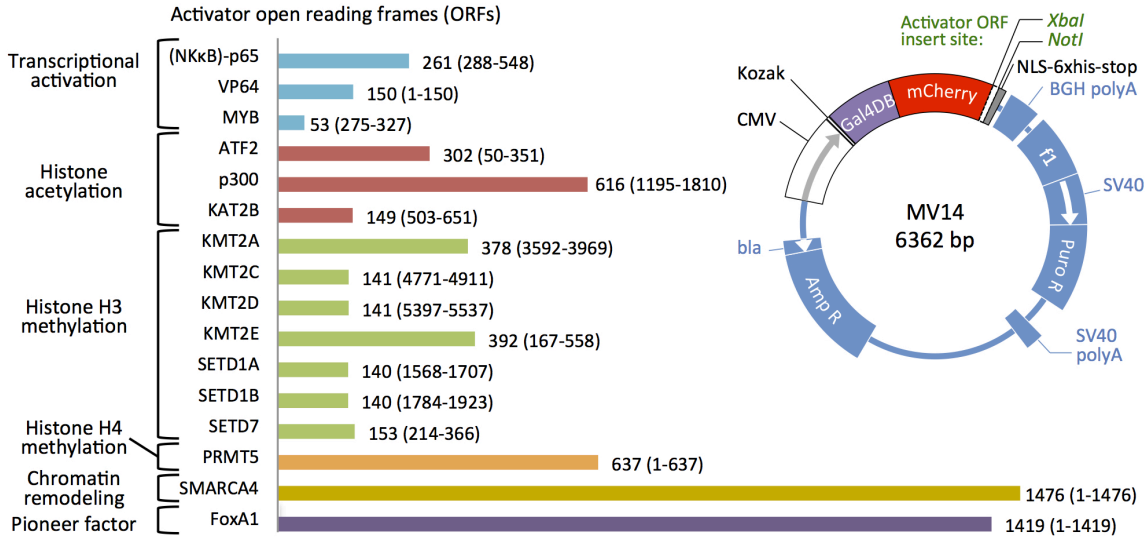

Figure 2.3: Design and construction of activation-associated peptide (AAP) -Gal4 fusions. Amino acid lengths are indicated as well as domain location within the full-length wild-type sequence (Appendix A, Table 2A). ORFs were cloned into MV14 to express a Gal4-AAP fusion protein from a cytomegalovirus (CMV) promoter. Gal4-AAPs are expressed with a C-terminal nuclear localization signal (NLS) and a 6X histidine tag. MV14 expresses puromycin resistance to enable selection of Gal4-AAP positive cells.

We tested all sixteen Gal4-AAP candidate fusion activators at repressive chromatin in HEK293 (human embryonic kidney) cells. The HEK293 cell line Gal4-EED/luc, carries a stably integrated *firefly luciferase* transgene with an upstream Gal4UAS (*Gal4UAS-Tk-luciferase*) (Figure 2.4a) (Hansen et al.; Daer et al.). The cells also carry a *TetO-CMV-Gal4EED* construct, which encodes a Gal4 DNA-binding domain (Gal4) fused to an embryonic ectoderm development (EED) open reading

frame under the control of TetO-CMV promoter (Figure 2.4a). Expression of the Gal4-EED fusion protein is controlled by a Tetracycline repressor (TetR). The addition of doxycycline (dox) to cultured Gal4-EED/luc cells releases the TetR protein from *TetO-CMV-Gal4EED*, initiating expression of Gal4-EED. Gal4-EED binds to the Gal4UAS site upstream of *luciferase*, recruiting PRC2 to the reporter. Expression of *luciferase* is switched from active to silenced through accumulation of Polycomb chromatin features, which have been detected by chromatin immunoprecipitation (ChIP) experiments: EZH2, Suz12, CBX8, depletion of H3K4me (Hansen et al.), and gain of H3K27me3 (Hansen et al.; Daer et al.). This system allows us to test the activity of Gal4-AAPs with a priori knowledge of the chromatin environment at the target gene.

Gal4-EED/luc cells were treated with dox for 48 hours to induce Polycomb heterochromatin at the *luciferase* transgene. Afterwards, dox was removed and cells were grown for four days without dox to allow for Gal4-EED depletion. Cells were then transfected with individual Gal4-AAP plasmids. *Luciferase* expression was measured 72 hours post transfection.

Three of the sixteen Gal4-AAP-expressing samples showed increased luciferase levels compared to a mock-transfected control (Lipofectamine reagent only) ($p < 0.05$) (Figure 2.4a). Lack of enhanced luciferase expression for the other fusions could have been due to strong inhibition by PRC complexes or failure of the AAPs to function as Gal4 fusions at the UAS site. Therefore, we also tested the activities of the fusion proteins within open chromatin. We used a parental HEK293 cell line, Luc14, that carries the *firefly luciferase* construct (*Gal4UAS-Tk-luciferase*) but lacks the *TetO-CMV-Gal4EED* repression cassette (Figure 2.4b) (Hansen et al.). *Luciferase* is constitutively expressed at high levels in Luc14.

We found a similar trend of expression enhancement at open chromatin in Gal4-AAP-expressing cells (Figure 2.4b), where only three Gal4-AAP fusions were

able to stimulate expression when positioned at the promoter-proximal UAS (Figure 2.4b). In both chromatin states, AAPs from the transcriptional activation group (Figure 2.1, Figure 2.3) significantly increased expression compared to a mock transfection control ($p < 0.05$) by up to five fold. Our results are consistent with previous studies where p65, VP64, or MYB stimulated gene expression from a promoter-proximal site (P. Q. Liu et al.; Beerli et al.; Weston and Michael Bishop). Here, we have demonstrated activities of these proteins within highly PRC-enriched chromatin.

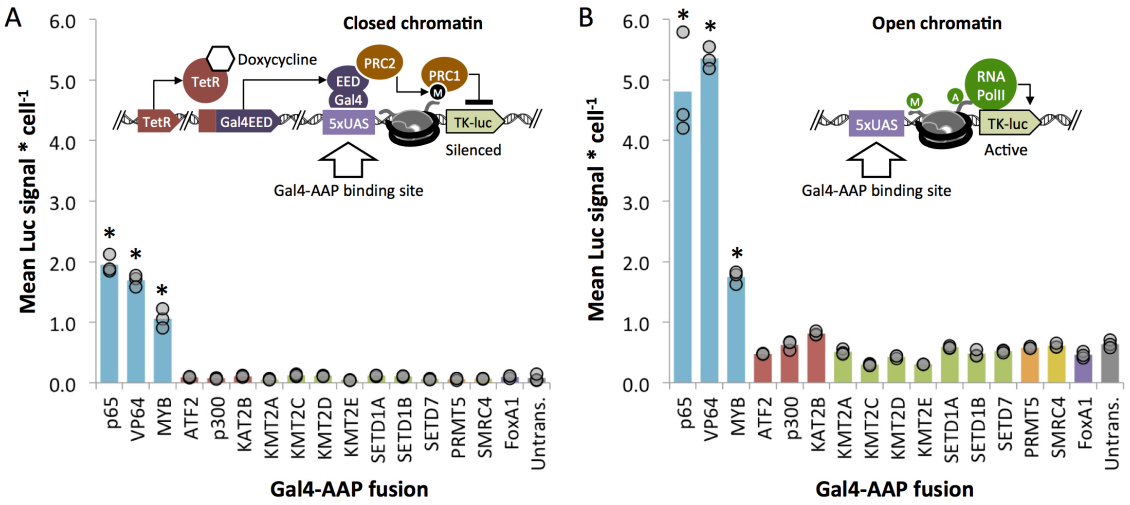

Figure 2.4: Measurement of *luciferase* reporter expression within closed or open chromatin after exposure to Gal4-AAP fusions. A) In Gal4-EED/luc HEK293 cells, PRC is recruited to the Tk-luciferase reporter gene via Gal4-EED (induced by dox). Treated cells were transfected with each Gal4-AAP fusion plasmid. Seventy-two hours post transfection luciferase signal was measured. Each circle in the bar graph shows the mean luciferase (Luc) signal for a single transfection, divided by cell density (total DNA, Hoechst staining signal). Bars show means of three transfections. Asterisks (*) = $p < 0.05$ compared to untransfected cells. B) The same procedure was carried out for unsilenced *Tk-luciferase* (in Luc 14 cells).

2.2.4 Fusion-induced activation is sustained after loss of the Gal4-AAP transactivator

The results so far were obtained at a single time point after Gal4-AAP expression. We were interested in determining whether transgene activation within Polycomb chromatin is stable or is transient and susceptible to eventual re-silencing (Pirrotta). To investigate this question, we performed time-course experiments to measure expression from re-activated *luciferase* over time. We induced Polycomb heterochromatin in Gal4-EED/luc cells as described for the previous experiments. Twenty-four hours post transfection with one of the strong activators, Gal4-p65, -VP64, or -MYB, cells were grown in medium supplemented with 10 µg/mL puromycin to select for Gal4-AAP positive cells. Seventy-two hours post transfection, we measured *luciferase* expression, Gal4-AAP mRNA levels, and mCherry fluorescence from a sample of each transfected culture. The cells were then passaged in puromycin-free medium to allow for loss of Gal4-AAP, sampled every four days (approximately three generations), and the same three measurements (luciferase, Gal4-AAP mRNA, and mCherry) were repeated at each time point.

We found that transient induction by Gal4-AAPs was sufficient to induce mitotically heritable reactivation of *Tk-luciferase* in Polycomb heterochromatin. For all three Gal4-AAP fusions, *luciferase* expression was significantly increased at most time points and at 456 hours ($p < 0.05$) compared to a mock transfection control (Lipofectamine reagent only) (Figure 2.5a). In two of the three additional trails, Gal4-p65 and Gal4-MYB showed at least ~2-fold enhancement at 360 hours (Appendix A, Figure A1). Steep declines of Gal4-AAP mRNA and mCherry fluorescence after 72 hours (Figures 2.5b, 2.5c) confirmed the transient presence of the transactivators. Therefore, enhancement of luciferase expression persisted long

after depletion of each Gal4-AAP, suggesting heritable epigenetic memory of the activated state.

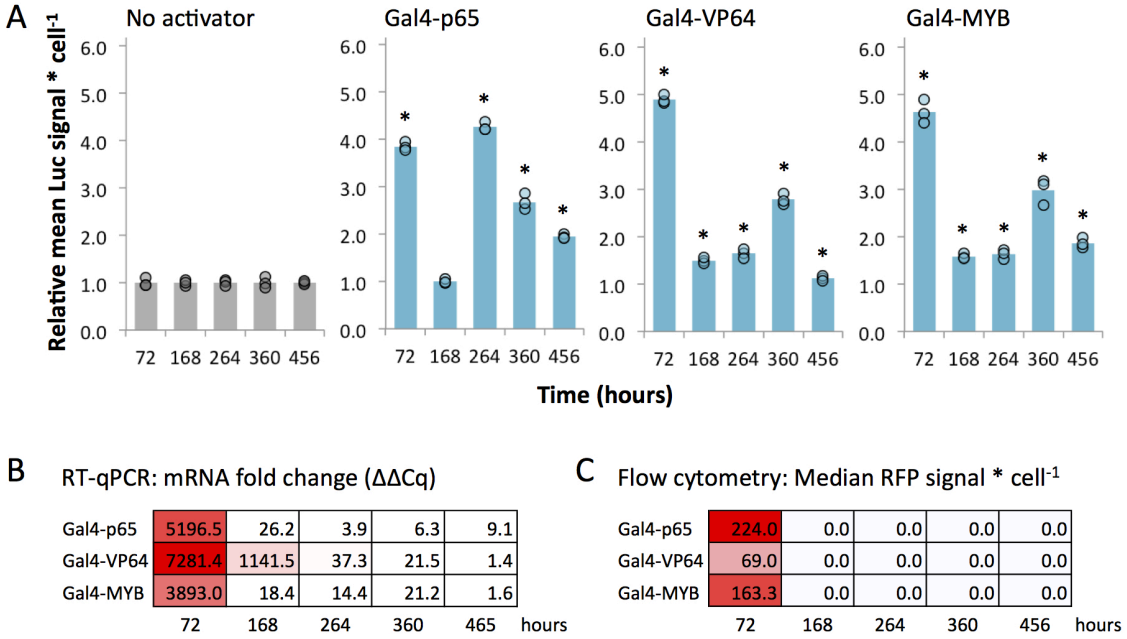

Figure 2.5: Expression of Polycomb-repressed *Tk-luciferase* over time after expression and loss of Gal4-p65, Gal4-VP64 or Gal4-MYB. A) Gal4-EED/luc cells were treated with dox to induce Polycomb chromatin, transfected with a Gal4-AAP plasmid, and grown under puromycin selection (10 µg/mL). At 72 hours post transfection, cells were sampled for luciferase (Luc) assays, passaged in puromycin-free medium, then sampled 168, 264, 360, and 465 hours post transfection for additional Luc assays. Mean Luc signal per cell is presented as described for Figure 4, except individual values (circles) at each time point are normalized by the mean of the "No activator" negative control. Asterisks (*) = $p < 0.05$ compared to the negative control. Results from replicate trials are shown in Appendix A, Figure A1. B) Reverse transcription followed by quantitative PCR (RT-qPCR) with primers against mCherry was used to determine Gal4-AAP transcript levels. "mRNA fold change" represents the Cq value normalized by the Cq of a housekeeping gene (*TBP*), and relative to mock-transfected "No activator" cells (Lipofectamine reagent only), log2 transformed. C) Flow cytometry of mCherry signal (red fluorescent protein, RFP) was used to determine Gal4-AAP protein levels. Data in B and C were generated from one set of transfections in A. For other samples, cells were visually inspected for RFP to verify the loss of Gal4-AAP.

2.2.5 MYB-mediated activation within closed chromatin requires interactions with a histone acetyltransferase

Next, we used specific chemical inhibitors to probe the mechanism of MYB-driven enhancement. The TAD core acidic domain of human MYB (D286-L309) included in our Gal4-MYB fusion construct is known to interact with a protein heterodimer of p300 and CBP (Appendix A, Figure 2A). A single base pair mutation within the MYB TAD domain (M303V) disrupts p300 recruitment and subsequent activation by MYB indicating that this recruitment is crucial to activation by MYB (Sandberg et al.; Pattabiraman et al.). The p300/CBP histone acetylation complex deposits H3K27ac in opposition to H3K27me3 induced by PRC2 (Raisner et al.; Ogryzko et al.). Therefore, induced activation within Polycomb heterochromatin may be driven by histone acetylation.

To test this idea, we treated cells with two compounds that are known to disrupt the activity of the MYB/p300/CBP complex. Celastrol is a minimally toxic pentacyclic triterpenoid that directly inhibits the MYB/p300 interaction, by binding to the KIX-domain of CBP which serves as a docking site for the formation of the MYB/p300/CBP complex (Coulibaly et al.; Uttarkar, Piontek, et al.; Denis et al.; Uttarkar, Dassé, et al.) (Figure 2.6a). C646, a pyrazolone-containing small molecule, binds the p300 catalytic domain and thus directly and selectively inhibits p300 HAT activity regardless of its association with MYB (Figure 2.6a) (Y.-M. Wang et al.; Oike et al.; Bowers et al.). These compounds allow us to resolve the roles of complex assembly and p300-mediated histone acetylation during Gal4-MYB-mediated activation.

Gal4-EED/luc cells were treated with dox to induce Polycomb chromatin and transfected with Gal4-MYB as described for previous experiments. We treated these

cells with 5 μM celastrol or 5 μM C646 for six hours. MTT assays indicated no toxicity to HEK293 cells at this concentration (Appendix A, Figure A3). We expected luciferase assays to show a decrease in Gal4-MYB-induced expression in drug-treated cells compared to an untreated control. We observed a significant ($p < 0.05$) decrease in *luciferase* expression in celastrol-treated cells, but not in C646-treated cells (Figure 2.6b). This result suggests that Gal4-MYB activity requires MYB TAD and p300/CBP assembly, while p300 HAT activity is dispensable. The other two strong activators, Gal4-VP64 and -p65, were insensitive to celastrol and C646 (Figure 2.6b), indicating a p300/CBP-independent mechanism for these two fusions.

In a time-course experiment using celastrol, we observed that Gal4-MYB-mediated activation is reversible. Eighteen hours after removal of celastrol from Gal4-MYB-treated cells, *luciferase* expression levels increased ($p < 0.05$ compared to repression at t = 6), nearly restoring expression to original levels (t = 0) (Figure 2.6c). Re-addition of celastrol led to a loss of Gal4-MYB induced expression (Figure 2.6c).

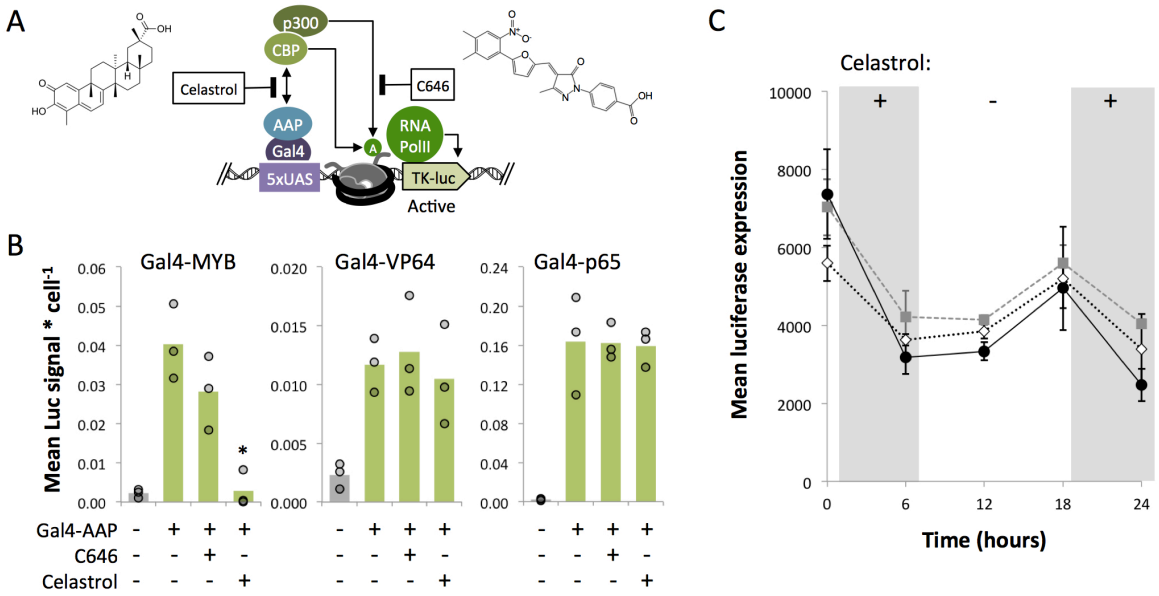

Figure 2.6: Celastrol disrupts Gal4-MYB-mediated activation of *luciferase* in closed chromatin. A) The p300/CBP complex acetylates histones via the catalytic HAT domain of p300 and/or CBP (Ogryzko et al.). Celastrol inhibits the recruitment of p300/CBP by MYB by binding a docking domain in CBP that facilities complex assembly (Uttarkar, Dassé, et al.; Uttarkar, Piontek, et al.). C646 disrupts histone acetylation by binding the active site of p300 (Bowers et al.). B) Seventy-two hours after Gal4-EED-mediated repression of *Tk-luciferase* and transfection with Gal4-AAPs, cells were treated with either 5 μM celastrol or 5 μM C646 for six hours and collected for luciferase assays. Mean luciferase (Luc) signal per cell is presented as described for Figure 4. Asterisks (*) = $p < 0.05$ compared to Gal4-MYB without drug treatment. C) Luc measurements were carried out in Gal4-MYB-expressing cells after removal (-) and re-addition (+) of celastrol. Each series represents an independent transfection. Point = mean of three luciferase assays, bars = standard error.

2.2.6 MYB-mediated activation in Polycomb heterochromatin relies upon proximity to the transcriptional start site

Next we asked whether MYB-mediated activation at transgenes is context dependent. We leveraged the flexible dCas9/sgRNA system to target the MYB TAD to several sites along the *luciferase* transgene (Figure 2.7a). To do so, we targeted sites at different positions within the *Tk-luciferase* gene. We also tested the MYB TAD

at a different transgene, *CMV-GFP* in HEK293, that had become silenced after several passages (C. Liu, unpublished).

We induced Polycomb heterochromatin in HEK 293 Gal4EED/luc cells with dox, followed by washout of dox to allow Gal4-EED depletion as described above. We transfected the cells with one of four dCas9-MYB constructs, each carrying a different sgRNA targeted at the *luciferase* transgene. After 72 hours, we tested *luciferase* expression and found that dCas9-MYB targeted nearest the transcription start site (+9) was able to restore levels of expression similarly to Gal4-MYB (Figure 2.7b). In induced Polycomb heterochromatin we observed clear position effects, as the downstream target sites show levels of activation significantly lower than Gal4-MYB ($p < 0.05$).

After determining the viability of dCas9-MYB to act as an activator for silenced transgenes in a defined chromatin environment, we wanted to test this domain against endogenous heterochromatin at the *CMV-GFP* transgene. The construct, *GFP* under the control of a CMV promoter, was inserted via Cas9-mediated HDR into a non-protein-coding region of the HEK293 genome (HEK293 site 3 (Tsai et al.)). We transiently transfected the cells with dCas9-MYB constructs, each carrying one of four different sgRNAs targeted upstream, within the promoter, or in the coding region of the transgene. Seventy-two hours post transfection, we used flow cytometry to measure GFP fluorescence compared to a mock-transfected control (Lipofectamine reagent only). We found that GFP fluorescence was significantly higher ($p < 0.05$) in all dCas9-MYB-expressing cells regardless of sgRNA position (Figure 2.7c), indicating that MYB-mediated activation does not require proximity to the TSS in all contexts.

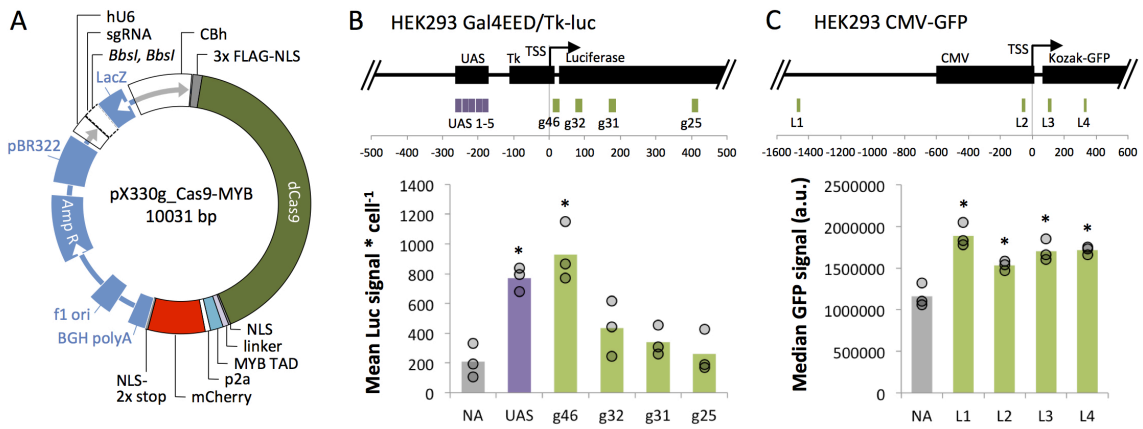

Figure 2.7: dCas9-MYB's ability to enhance expression in induced Polycomb heterochromatin is dependent upon distance from the promoter. A) Expression vector pX330g_dCas9-MYB was constructed from vector pX330A_dCas9 (a gift from Takashi Yamamoto, Addgene plasmid #63598) to co-express a dCas9-MYB fusion protein and mCherry from a CBh promoter. Single-stranded guide RNA sequences (Appendix A, Table A3) were cloned into the BbsI sites and expressed from a hU6 promoter on the same vector. B) We targeted dCas9-MYB to four locations (g46, g32, g31, g25) across the *Tk-luciferase* transgene in silenced Gal4-EED/luc cells. Mean luciferase signal per cell is presented as described for Figure 4. The control (grey bar) is a mock-transfection with Lipofectamine (No Activator, NA). C) We targeted dCas9-MYB to four sites (L1-4) across a chromosomal *CMV-GFP* transgene in HEK293 cells. Seventy-two hours post transfection with dCas9-MYB/sgRNA vectors or mock transfection, we measured GFP fluorescence via flow cytometry. Circle = median GFP fluorescence value from one transfection, 10,000 cells; bars = means of three transfections. In B and C, asterisks (*) = $p < 0.05$ for experimental mean compared to the NA control mean.

2.3 Discussion

We have demonstrated that DNA enhancer elements and fusion proteins derived from endogenous mammalian systems can be used to support strong expression from transgenes. Furthermore, we have successfully demonstrated long-term reactivation of a transgene that had been previously silenced by ectopic Polycomb heterochromatin. Transient induction of activation by Gal4-AAPs is sufficient to maintain an active state over nearly fifteen generations of cell division. These results have exciting implications for achieving reliable expression of synthetic DNA in engineered cells, as well as our understanding of inherited chromatin states.

Our results also suggest that the mechanism of artificial transgene reactivation within Polycomb heterochromatin requires assembly of transcription initiation complexes. From STRING analysis, we found no clear pattern of histone modifications to distinguish the inactive Gal4-AAPs from activators that were able to enhance expression in Polycomb heterochromatin (Figure 2.1). We observed that several of the fusion proteins did not restore expression from the Polycomb-repressed *luciferase* transgene in HEK293 cells (Figure 2.4a). Thus, Gal4-tethered proteins might be functional but not sufficient, are non-functional (sterically hindered), or require positioning within non-coding DNA such as enhancer elements. For instance, a p300 fusion has shown strong activation of *MyoD* and *Oct4* when positioned 5-20 kb upstream at an enhancer (Hilton et al.). Future work could be done to systematically test the AAPs at endogenous enhancers.

Upon further investigation we determined that assembly of the MYB TAD with P300/CBP is critical for Gal4-MYB-mediated activation within Polycomb chromatin. Inhibition of p300 HAT activity via C646 did not disrupt Gal4-MYB function (Figure 2.6b). Furthermore, the Gal4 fusion that included only the p300 HAT domain failed to activate Polycomb-repressed *Tk-luciferase* (Figure 2.3, Figure 2.4). Therefore the p300 catalytic domain alone is neither necessary nor sufficient to reverse epigenetic silencing under the conditions tested here. CBP, which is also a histone acetyltransferase, might compensate for p300 in C646-treated cells (Ogryzko et al.). Celastrol inhibits the interaction of p300/CBP with MYB by binding to the CBP KIX domain (Uttarkar, Dassé, et al.; Uttarkar, Piontek, et al.; Coulibaly et al.; Denis et al.), and completely reduces Gal4-MYB activity (Figure 2.6b). In contrast to C646, celastrol may disrupt the recruitment of both HAT enzymes, p300 and CBP.

In contrast to Gal4-MYB, the Gal4-p65 and -VP64 fusions showed robust activation of PRC-silenced luciferase in the presence of both inhibitors (Figure 2.6b).

Although VP64 (VP16) and p65 are known to interact with p300/CBP, they also interact with the large multi-subunit Mediator complex to initiate transcription (van Essen et al.; Thakore et al.; Wei Wang et al.). Multiple interactions of Gal4-p65 and -VP64 with Mediator may allow these proteins to function independently of p300/CBP (Lecoq et al.). However in the case of Gal4-MYB, cooperative interactions between p300/CBP and Mediator (Z.-Q. Huang et al.; Haas et al.) may be necessary for gene activation. In our study, Mediator complex recruitment arises as a particularly potent mechanism of transgene reactivation in Polycomb heterochromatin (Haas et al.). Mediator is known to cooperatively regulate PRC2 repression (Fukasawa et al.) and certain Mediator subunits are directly involved in the removal of PRC2 from endogenous promoters (Englert et al.). Similarly, Mediator has an antagonistic relationship with the PRC1 repression complex (Lehmann et al.).

The inhibitor experiments also suggest a novel technique for chemically inducible gene regulation in mammalian cells. The ability to quickly toggle between enhanced and repressed states is a cornerstone technology for the control of engineered transgenic systems (Oakes et al.; Kramer et al.; Greber et al.). Current methods for toggling gene expression in mammalian cells employ drug-mediated transactivator localization, such as allosteric modulation of DNA-binding protein domains (Stanton et al.; Kramer et al.; Oakes et al.), blue light-responsive CRY proteins (Mansouri et al.), and chemically induced dimerization (CID) systems (Inobe and Nukina; Rivera et al.; DeRose et al.), or RNA interference to deplete the regulator (Greber et al.). To our knowledge, no systems currently exist where the transactivation module's activity (i.e., MYB-CBP binding) is modulated by a small molecule drug. Celastrol has a low toxicity and is in fact being explored as a therapeutic due to its positive effects on the immune system (Cascão et al.; Venkatesha et al.; Ju et al.). The concentration of celastrol that is sufficient to toggle

Gal4-MYB activity in Polycomb chromatin is well below any reported LD50 values for celastrol (Li et al.; Raja et al.; H. Yang et al.; Cleren et al.; Konieczny et al.). Finally, our work demonstrates the potential flexibility of MYB fusion proteins as transactivators. dCas9-MYB showed strong activation of previously silenced transgenes near two different promoter elements, *Tk* and *CMV*. *Tk* had undergone silencing by ectopic Polycomb chromatin, whereas *CMV* had become silenced by undetermined mechanisms. Interestingly, stimulation of expression from PRC-repressed *Tk* seemed to require TSS-proximal positioning of Gal4-MYB, whereas Gal4-MYB stimulated expression from both upstream (up to 1400 bp) and downstream (up to 350 bp) of the CMV TSS. Factors that might account for this difference include intrinsic differences in the core promoter sequences, the presence of cryptic enhancers at one promoter and not the other, and differences in chromatin structure. To our knowledge, our work represents the first use of MYB as a dCas9 fusion that can activate a transgene from proximal and distal locations.

2.4 Conclusion

In conclusion, we have determined a predominant role for p300/CBP-recruiting transcriptional activators in the reversal of Polycomb-mediated expression in the context of synthetic transgene regulation. In particular, we have expanded the characterization of the transcriptional activator protein MYB and its associated enhancer DNA sequence for applications in artificial gene regulation in mammalian cells.

2.5 Methods

2.5.1 Construction and Testing of Plasmids Containing MYB- and p65 Motifs

Plasmid construction, transfection of PC-3 cells, and luciferase assays were carried out as described previously (Zimmerman et al.). Briefly, cloning of double-stranded oligos was used to insert motifs 222 bp upstream of the transcription start site of an EF1a promoter at XbaI/SpeI. Plasmids were then transfected into PC-3 cells (ATCC, CRL-1435) using Lipofectamine LTX™ following the manufacturer's recommended protocols. Luciferase expression was measured 48 hours after transfection using a luciferase assay kit (Promega, Madison, WI). All luciferase values were normalized relative to the native plasmid control, which contained an unaltered EF1a promoter.

2.5.2 Construction of MV14 and Gal4-AAP Plasmids

We constructed mammalian expression vector 14 (MV14) for the overexpression of Gal4-mCherry-AAP fusion proteins in-frame with a nuclear localization sequence and 6X-histidine tag. First, plasmid MV13 was built by inserting a Gal4-mCherry fragment into MV10 (Tekel et al.) directly downstream of the CMV promoter. Next, MV14 was built by inserting a SpeI/PstlI (FastDigest enzymes, ThermoFisher Scientific) -digested gBlock Gene Fragment (Integrated DNA Technologies), which encoded a XbaI/NotI multiple cloning site, into MV13 downstream of mCherry. Ligation reactions included gel-purified (Sigma NA1111) DNA (25 ng linearized vector, a 2x molar ratio of insert fragments), 1x Roche RaPID ligation buffer, 1.0 µL T4 ligase (New England Biolabs), in a final volume of 10uL.

AAPs were cloned into MV14 at the multiple cloning site containing XbaI and NotI cut sites. AAPs were either ordered from DNASU in vectors and amplified using primers that added a 5' XbaI site and a 3' NotI site or ordered as gBlock Gene Fragments with the same 5' and 3' cutsites (Integrated DNA Technologies). Sequences in vectors were amplified with Phusion High Fidelity DNA Polymerase (New England BioLabs) and primers listed in Appendix A, Table A2. MV14 and AAP inserts were double-digested with FastDigest *XbaI* and FastDigest *NotI* (ThermoFisher Scientific) and then ligated with T4 DNA ligase (New England Biolabs). MV14_AAP plasmids are publically available through DNASU (Appendix A, Table A4)

2.5.3 Cell Culturing and Transfections

Luc14 and Gal4-EED/luc HEK293 cells were grown in Gibco DMEM high glucose 1× (Life Technologies) with 10% Tet-free Fetal Bovine Serum (FBS) (Omega Scientific), 1% penicillin streptomycin (ATCC) at 37 °C in a humidified 5% CO_2 incubator. Gal4-EED/luc cells were treated with 1 µg/mL doxycycline (Santa Cruz Biotechnology) for 2 days to induce stable Polycomb repression. Dox was removed and cells were cultured for another four days before being seeded in 12-well plates. Luc14 cells and dox-induced Gal4-EED/luc cells were seeded in 12-well plates such that cells reached 90% confluency for lipid-mediated transfection. Transfections were performed with 1 µg plasmid per well, 3 µL Lipofectamine LTX, and 1 µL Plus Reagent (Life Technologies) per the manufacturer's protocol. Seventy-two hours post transfection, cells were either collected for analysis or passaged further.

Puromycin selection was carried out on Gal4-AAP-expressing cells for the experiments represented in Figure 5 and Figure A1. Dox-treated Gal4-EED/luc cells were transfected in 12-well plates and then grown for 24 hours before the addition of

10 µg/mL puromycin (Santa Cruz Biotechnology) to Gibco DMEM high glucose 1× (Life Technologies) with 10% Tet-free Fetal Bovine Serum (FBS) (Omega Scientific), 1% penicillin streptomycin (ATCC). Cells were grown in puromycin containing media for two days before wash out.

2.5.4 Luciferase Assays

Luciferase assays were performed as previously described in Tekel *et al.* (Tekel et al.). In brief, a single well of cells from a 12 well tissue culture plate was collected per independent transfection in 1.5mL 1X PBS. Cells were loaded into 9 wells of a Black Costar Clear Bottom 96 Well Plates (Corning #3631). Three wells of cells were used to detect mCherry in order to quantify Gal4-AAP proteins. A 2X Hoechst 33342 stain (Invitrogen #H3570) was loaded into three more wells to stain nuclear DNA in order to quantify cell density. The final three wells were prepared with Luciferase Assay Buffer (Biotium #30085). Plates were scanned in a microplate reader (Biotek Synergy H1) to detect RFP (580 nm - 610 nm), Hoechst 33342 fluorescence (360 nm - 460 nm) and chemiluminescence from the same sample in parallel.

2.5.5 RT-qPCR

We prepared total RNA from ~1.0 x 10^6 cells (Qiagen RNeasy Mini kit 74104) and generated cDNA from 2 µg of total RNA and the SuperScript III First Strand Synthesis system (Invitrogen #18080051) in a reaction volume of 20 µL. Quantitative PCR (qPCR) was performed with universal primers against the mCherry portion of the Gal4-AAP fusions, or the *TATA binding protein* (*TBP*) housekeeping gene. Triplicate qPCR reactions (10 µL) each contained SYBR Green 1 2X master mix

(Roche), 2 µl of a 1:10 cDNA dilution, and 750 nM of each primer (forward and reverse, see Table A5). We calculated Mean Quantification Cycle (C_q) for three replicate wells per unique reaction. Change in gene expression level was calculated as $\Delta C_q = 2^{[\text{Mean Cp reference} - \text{Mean Cp target}]}$. Log2 fold change in gene expression was calculated as = $\log2(\Delta C_{q\ \text{transfected cells}} / \Delta C_{q\ \text{mock}})$.

2.5.6 Flow Cytometry

Cells were passed through a 35 µm nylon strainer (EMS #64750-25). Green fluorescent signal from GFP and red fluorescent signal from mCherry were detected on a BD Accuri C6 flow cytometer (675 nm LP filter) using CFlow Plus software. Data were further analyzed using FlowJo 10.5.3. One run (~10 000 live cells, gated by forward and side scatter) was completed per sample, allowing us to determine median fluorescence within the live cell population.

2.5.7 Construction of dCas9-MYB and Design of sgRNAs

We modified the vector pX330A_dCas9–1 × 4 (a gift from Takashi Yamamoto, Addgene plasmid #63598) by inserting a gBlock Gene Fragment (Integrated DNA Technologies) encoding the MYB TAD followed by a p2A signal (Z. Liu et al.) and *mCherry* after the *dCas9* ORF. The resulting vector expresses a dCas9-MYB fusion and mCherry as separate peptides from a single mRNA transcript. The vector and gBlock were digested with *FseI* (New England BioLabs) and FastDigest *Eco*RI (ThermoFisher Scientific) and ligated using T4 DNA Ligase (New England BioLabs). We named this new vector pX330g_dCas9-MYB. SgRNAs used in the study (Appendix A, Table A3) were designed using the CRISPR design tool at crispr.mit.edu. DNA

oligos were synthesized with BbsI overhangs for cloning into pX330g_dCas9-MYB (Integrative DNA Technology). Drop-in of sgRNAs followed the cloning protocol described in Cong *et al*. (Cong et al.).

2.5.8 Celastrol and C646 Treatments

Gal4-EED/luc cells were induced with dox and transfected as described above. Three days post transfection, cells were treated with either C646 (Selleck Chemicals) or Celastrol (Selleck Chemicals) diluted to a concentration of 5μM in Gibco DMEM high glucose 1× (Life Technologies) with 10% Tet-free Fetal Bovine Serum (FBS) (Omega Scientific). Cells were incubated with the drug for six hours before being washed and either harvested for a luciferase assay or grown further in drug-free media.

2.5.9 Statistical Analyses

The differences of means were calculated using the two sample, one-tailed Student's *t* test. For $p < 0.05$, confidence was 95% for 2 degrees of freedom and a test statistic of $t_{(0.05,2)} = 2.920$. To evaluate significance of Gal4-MYB induced activation after the removal of celastrol and its subsequent re-addition, a nest one-way ANOVA was used with 95% confidence and two degrees of freedom.

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CHAPTER 3

METHODS FOR VISUALIZING FUSION PROTEIN TOOLS IN MAMMALIAN CELLS

3.1 Background

Visualizing proteins is essential to our ability to engineer their functionality. Visualization of native proteins allows us to gain new information about their localization, expression, and longevity within the cell (Chudakov et al.; Rizzo et al.; Allen and Budowle). When working with engineered proteins expressed from transgenic material within the cell, visualization facilitates troubleshooting of those same parameters. Localization, expression, and longevity of proteins are all tunable elements in the design phase that we adjust to suit a certain need (Pleiss). This makes tracking them in the test phase especially crucial to determine if iteration of the design-build-test cycle is required. Visualization of proteins is a key testing-phase tool that facilitates this.

Historically, the use of isotope or colorimetric markers such as protein specific dyes has been used to quantify protein expression and location to particular organelles (Becker; Alturkistani et al.). While these approaches are still commonly used in histology, they lack the resolution required to provide quantitative data (low sensitivity) and often permeate the cell inducing mortality (destructive). This prohibits the tracking of protein expression and localization within the same cell over time. More advanced approaches typically involve the use of fluorescent labeling, which has the advantage of being usable in live cells (non-destructive) and showing protein localization to a high resolution (high sensitivity) (Rizzo et al.). This also mitigates safety concerns around the use of radiation and certain carcinogenic dyes.

Direct visualization also has some key advantages over the use of nucleic acid detection methods for expression tracking. While quantitative PCRs are an invaluable tool for tracking expression over time, they are necessarily destructive and lack the high-throughput capacity of methods such as flow cytometry. Fluorescent tags also provide spatial information that PCR methods cannot.

Transgenic systems can be visualized through fluorescence at various stages of expression. Here we investigate three facile approaches that can be used to visualize transgenic expression of targeted epigenetic effectors (Figure 3.1), comprised of a Gal4 DNA binding domain and activation-associated peptide (Gal4-AAPs). These fusion proteins were designed to open closed chromatin, a function that pioneer factors perform in native systems. In Chapter 2, we explored the ability of these fusion proteins to increase expression. We needed to use fluorescent labeling to quantify their expression so as to normalize the strength of their epigenetic effects by protein quantity. In Chapter 4, we discuss their ability to not only activate gene expression, but to develop a more accessible chromatin environment. With this dual functionality, we have decided to term these fusion proteins **S**ynthetic **Pi**oneer **F**actors (SPiFs). Visualization of SPiFs is crucial to the cell-sorting methodology used in Chapter 4 as well. This chapter details the fluorescent labeling approaches tested to visualize SPiFs 1) in live cells, 2) in parallel to assaying their epigenetic functionality, and 3) in high-throughput. All of these parameters are critical to our full investigation of SPiF functionality. For a full panel of the SPiFs used in these tests, see Chapter 2.

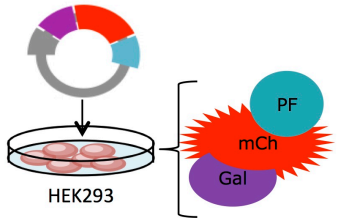
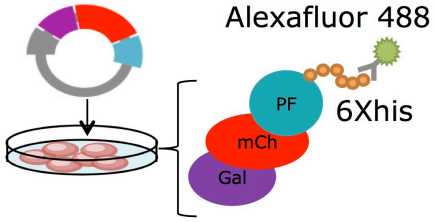
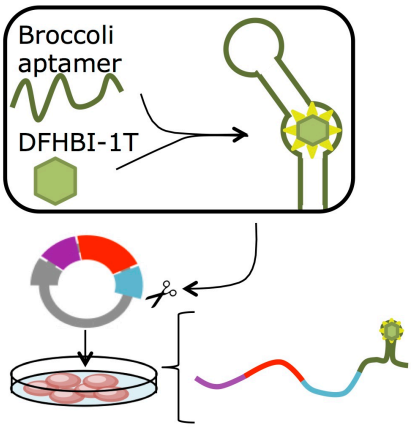
Detectable Fluorescent Element	Pros	Cons
Fusion Protein 	• Simple to integrate • Detectable in live cells • Cost effective	• Folding dynamics may vary with construct • High detection threshold
Tag-specific Antibody 	• Easy to change antibody target • Highly specific • Low background	• Costly • Loss of sample in fixation process • Induces cell damage
RNA Aptamer 	• Simple to integrate • Low metabolic burden • Direct visualization of expression	• Expensive dye • Induces cell damage • Visualizes RNA rather than protein • Rapid signal degradation

Figure 3.1: Detectable fluorescent elements used in fusion protein detection have unique pros and cons. Direct detection of a fluorescent protein fused to other construct elements is both facile and low cost, however, variations in folding dynamics may disrupt reliable detect in single cells. The use of tag-specific antibodies is costly but has a high degree of sensitivity (National Research Council et al.; Frerichs). Finally, the detection of fusion protein expression at the RNA level via the use of a fluorescent aptamer is low cost, both financially and metabolically, but does not provide spatial protein information.

The initial design of the SPiFs included an mCherry protein between the Gal4 DNA binding domain and terminal activation-associated peptide (Figure 3.1). This inclusion of a fluorescent protein within the fusion protein itself is an extremely common labeling practice in synthetic biology (Hoffman). The use of green fluorescent protein (GFP) as a fluorescent reporter and tag was the subject of the 2008 Nobel Prize in Chemistry (Shimomura). Since that time a rainbow of derivatives has been developed, allowing researchers to use multiple tags in parallel that do not have overlapping emission spectra (Shimomura; Follenius-Wund et al.). GFP-family proteins are typically comprised of a beta-barrel structure with an internal chromophore, making folding dynamics crucial to fluorescent functionality of the protein (Figure 3.1) (Orm et al.).

An external approach to fluorescent labeling is the use of fluorescently labeled antibodies targeted at tagged fusion proteins (Figure 3.1) (Griffiths). This approach allows the target of labeling to be easily changed without full reconstruction of your fusion protein. The labeling process is also highly specific but comes at both a high financial price and a cost to cell viability as permeabilization is necessary for antibody staining. However, this approach does allow for labeling regardless of most changes in fusion protein folding dynamics.

The final approach we investigated was the inclusion of a fluorescent RNA aptamer within the SPiF transcript itself (Figure 3.1) (Filonov et al.; Okuda et al.). This alternative to direct protein visualization still provides a proxy measurement for expression *in vivo* as well as duration of expression. RNA aptamers are also metabolically cheap and do not interfere with folding dynamics and fusion protein functionality. We explored this approach as an additional fluorescent monitor of fusion protein expression.

In this chapter, we compare three major visual methods to detect fusion protein expression by fluorescence, specifically in our SPiF system. Fluorescence detection serves as an important control parameter and should be detected in high-throughput, ideally in parallel to the effects of the fusion protein.

3.2 Results

We compared three major methods for fluorescent detection of fusion protein detection *in vivo.* Original construct design and structure for each SPiF can bee seen in Chapter 2.

3.2.1 Direct fusion of a fluorescent mCherry tag shows variability across constructs

Our initial approach was to use direct fusion of a fluorescent beta-barrel protein, mCherry, to our epigenetically functional protein domains. This approach requires no dyes, staining, or fixation and can be used in high-throughput via flow cytometry. The design incorporated an mCherry protein between an N-terminal Gal4 DNA binding domain and a downstream activation domain (Figure 3.2a). We first tested this design for visualization with the commonly used p65 activation domain. HEK293 cells were transfected and red fluorescence was visualized by flow cytometry (Figure 3.2b) and fluorescent microscopy (Figure 3.2c) at twenty-four and seventy-two hours post transfection, in order to optimize the time range for protein detection. With both methods we observed a detectable change in red fluorescence after seventy-two hours (Figure 3.2).

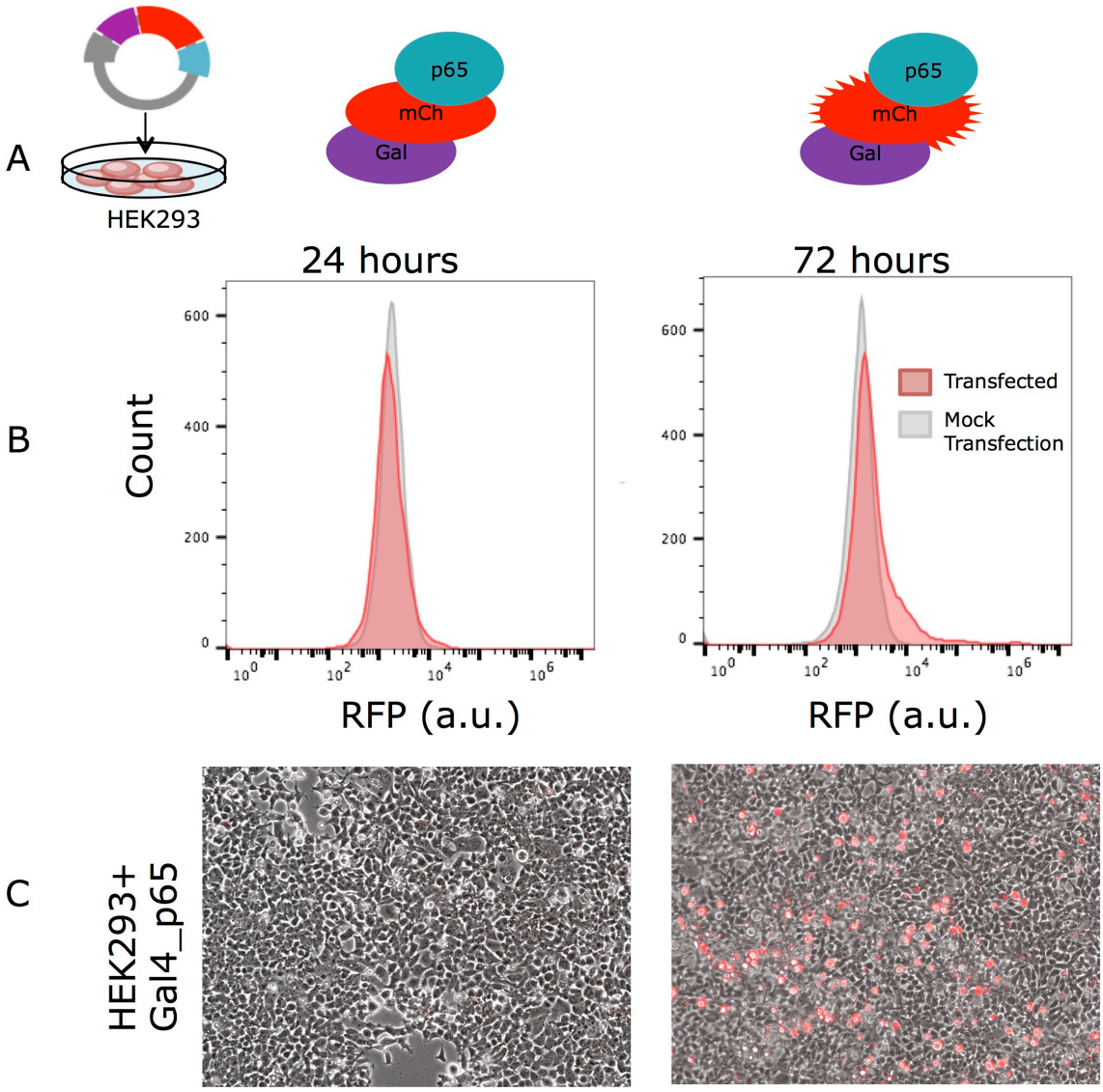


Figure 3.2: Visual detection of Gal4_mCherry_p65 fusion proteins. A) HEK293 cells were transfected with a plasmid expressing Gal4_mCherry_p65 (see Chapter 2 for plasmid map). Fusion proteins were detected via B) flow cytometry (each sample represented 10,000 lives cells) and C) fluorescent microscopy at 24 and 72 hours post transfection. mCherry expression was not evident until 72 hours post transfection via both methods.

Next, we tested a larger panel of our SPiFs to evaluate the ability of mCherry signal to be used as a proxy for expression seventy-two hours post transfection. Having a robust expression metric would allow us to normalize changes in *luciferase* expression induced by each SPiF in our HEK293 model system (see Chapter 2) to

relative loads of SPiF proteins within the cell. To this end, we ran luciferase assays (Figure 3.3a) in parallel with flow cytometry (Figure3.3b) to detect mCherry expression from each sample. Using this single-cell method, we did not observe correlations in activation strength with mCherry signal. Likewise, before harvesting we visualized mCherry expression via fluorescent microscopy (Figure 3.3c) and noticed high levels of variability across constructs, and across trials (data not shown).

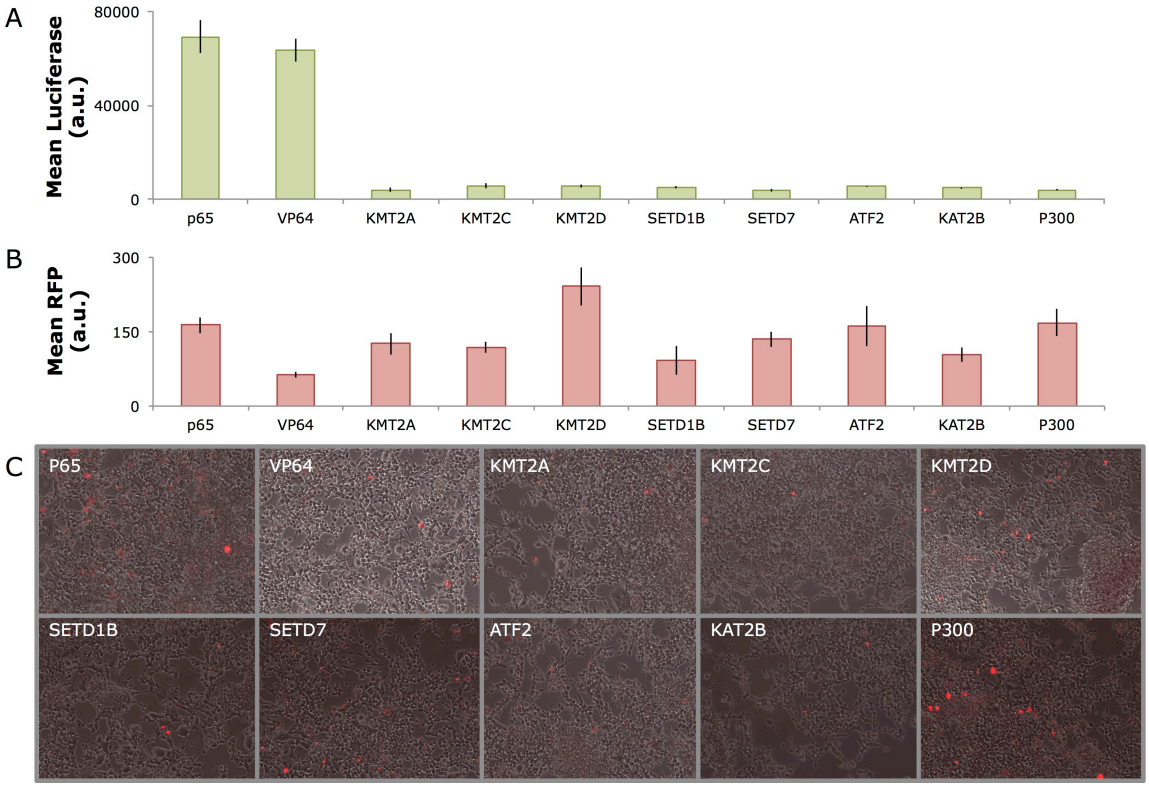


Figure 3.3: Fluorescent tag signal from SPiFs is inconsistent across SPiF function. Silenced Gal4-EED/luc HEK293 cells were transfected with individual SPiF plasmids. Seventy-two hours post transfection, A) luciferase expression was measured to determine the strength of SPiF activity at the reporter. B) mCherry expression was measured in parallel via flow cytometry. Each sample represented 10,000 lives cells. C) For each sample, mCherry expression was visually assayed via fluorescent microscopy. Error bars represent the standard deviation of three biological replicates.

3.2.2 Immunocytochemistry (ICC) yields low SPiF detection in live cells

After finding construct-dependent variability from our mCherry tags, we decided to explore a method of visual detection that does not rely on protein folding dynamics. Antibodies allow for highly specific tagging and detection of proteins. Each of our SPiFs is constructed with a terminal 6XHis tag. We used a fluorescently conjugated AlexaFluor 488 antibody specific to the 6XHis tag to visualize our SPiFs via flow cytometry seventy-two hours post transfection (Figure 3.4). Detection rates were extremely low using this method, most likely due to significant cell loss during antibody staining in a population with relatively low transfection rates (~20%).

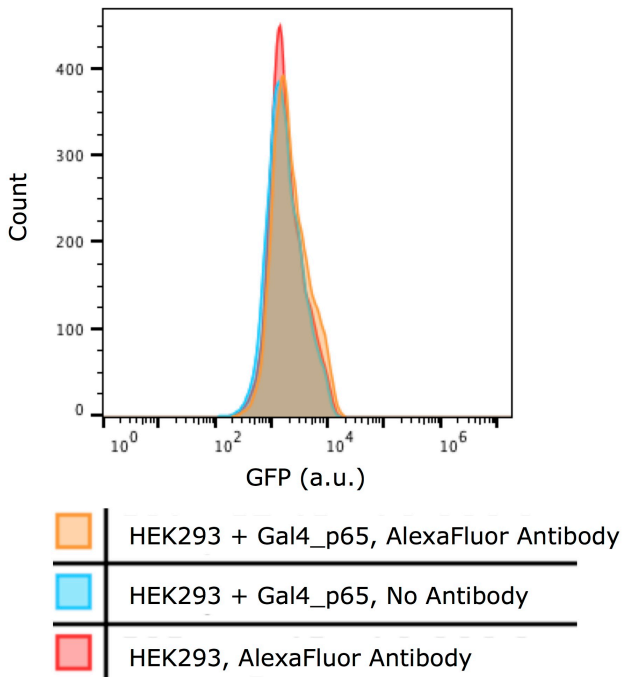


Figure 3.4: 6XHis tag specific AlexaFluor 488 antibodies do not provide sufficient signal of Gal4_p65 in HEK293 cells. Transfected and antibody-stained cells (yellow) only show slightly more GFP signal (from the 488 AlexaFluor) as compared to transfected cells without antibodies (blue) or mock transfected antibody-stained cells (red). Each sample represented 10,000 lives cells.

3.2.3 Broccoli aptamers are nearly undetectable from low copy number SPiF plasmid

RNA aptamers have a lower metabolic burden and reliable folding dynamics when positioned at the end of a construct (Di Palma et al.; Filonov et al.). We cloned in a 2xd-Broccoli aptamer at the C-terminus of our Gal4_p65 SPiF to see if detecting expression at the RNA level would be a more effective than the protein level where the large structure of the fusion protein may disrupt beta barrel formation. We transfected HEK293 cells with a Broccoli-modified Gal4_p65 construct as well as the original broccoli containing plasmid, PAVU6+27-F30-2xdBroccoli (Figure 3.5) (Filonov et al.). We then stained cells seventy-two hours post transfection for varying durations with two concentrations of DFHBI-1T dye to excite emission (Figure 3.5). We detected fluorescence with flow cytometry (Figure 3.5a) and visually through fluorescent microscopy (Figure 3.5b). In both cases, we found little to no detectable fluorescent signal as compared to the original plasmid.

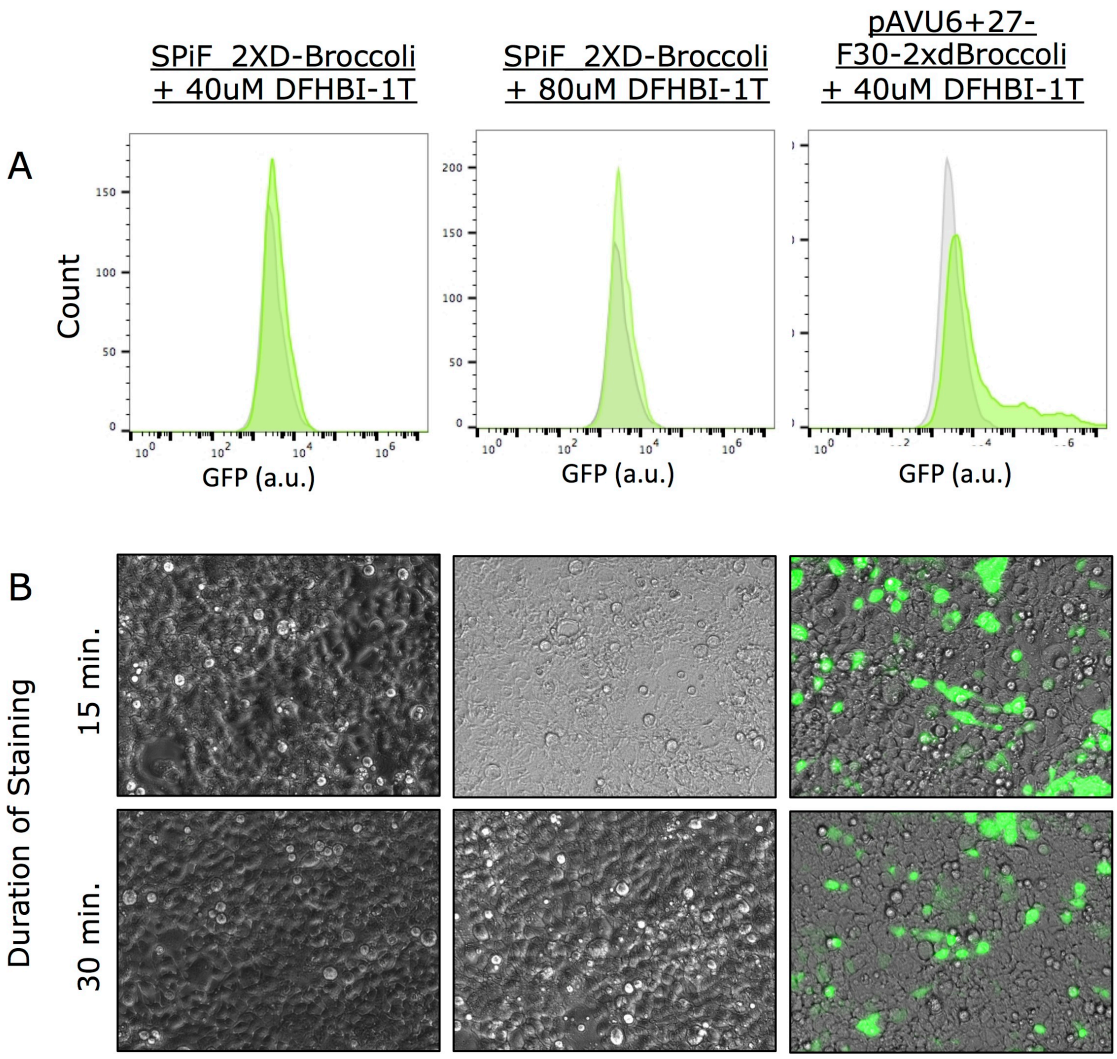


Figure 3.5: 2xd-Broccoli does not provide adequate signal for detection of low copy number SPiF plasmids via flow cytometry or fluorescent microscopy. We stained HEK293 cells transfected with either Broccoli-modified Gal4_p65 or PAVU6+27-F30-2xdBroccoli at two different concentrations of DHFBI-1T. A) We were not able to detect significant signal from SPiF containing cells, as opposed to a significant increase in fluorescence from PAVU6+27-F30-2xdBroccoli transfected cells as compared to a mock transfection control. B) We also stained cells for 15 and 30 minutes at two different concentrations of DFHBI-1T, but were unable to detect 2xd-Broccoli in Gal4_p65 transfected cells. Cells transfected with the original 2xd-Broccoli construct showed abundant signal at both concentrations.

3.2.4 Development of a high throughput, parallel detection assay for fluorescence and bioluminescence

Several projects in our lab require the parallel detection of a fusion protein's expression levels (as a normalizing factor) and their biological functionality, typically assayed by evaluating changes in expression at a *luciferase* reporter. In several cases, we found that low transfection rates and variable folding dynamics called for a reliable method to pool detection allowing us to surpass the minimum detection threshold of our BD Accuri C6 flow cytometer. To this end, we developed a plate-reader based method to effectively detect mCherry in both our SPiF constructs and other targeted epigenetic modifiers used in our lab (Tekel, Barrett, et al.). These include PcTF and its derivatives (Olney et al.; Tekel, Vargas, et al.) that our lab has previously tested within induced Polycomb heterochromatin (Figure 3.6a). By using a pooled cell method, we were able to yield detectable levels of mCherry in parallel with a luciferase assay on the same sample (Figure 3.6) (Tekel, Barrett, et al.). Hoechst nuclei staining was used as a proxy for cell count. We also determined plate reader detection limits (Appendix B) to ensure that the dynamic range of our measurements was fully detectable.

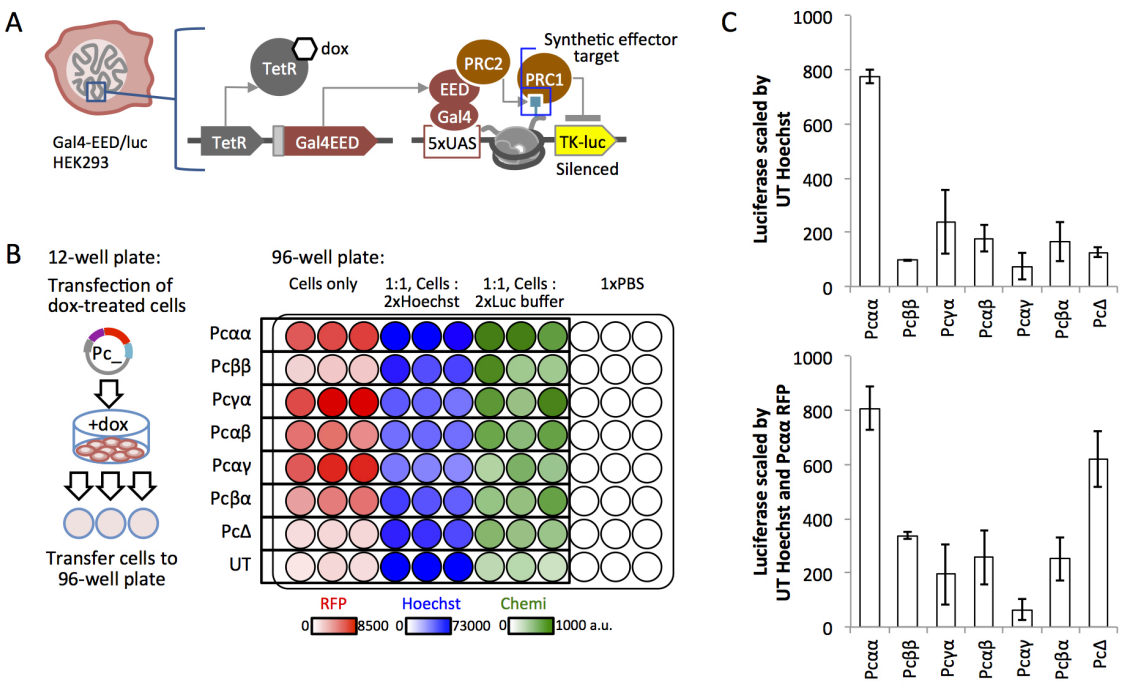


Figure 3.6: Microwell plate reader assay to determine fusion protein expression and regulation of a target reporter gene. (A) Gal4-EED/luc cells were treated with dox to induce ectopic recruitment of Polycomb repressive complex 1 (PRC1), and accumulation of H3K27me3 and PRC2 at a *luciferase* reporter. (B) After *luciferase* silencing, cells were transfected with each fusion-expressing plasmid (see Tekel et al. 2018), harvested, and aliquoted into a 96-well plate. In this procedure, RFP is used to determine fusion protein expression, signal from Hoechst 33342 DNA stain is a proxy for cell loading, and luciferase activity indicates gene expression induced by each Pc-activator fusion (Tekel, Barrett, et al.). (C) Bar charts show mean background-subtracted values from triplicate wells, normalized and scaled as described in Methods. Error bars, standard deviation; UT, untransfected cells. (This figure and its legend are reprinted here with permission from ACS Publications, Copyright © 2018 American Chemical Society Publications)

3.3 Discussion

In this chapter we investigated best practices for the parallel testing of epigenetically active fusion protein expression and function. We found that fusion proteins containing a medial beta-barrel protein suffered from inconsistent levels of fluorescence across construct types, potentially due to diverse protein folding dynamics. The C-terminal activation domains fused to the medial mCherry protein ranged in length from 53aa to over 1,000aa (see Chapter 2), however, we did not

find a correlation between activator size and mCherry signal degradation. Neither did mCherry signal correlate with an increase or decrease in epigenetic activity of the fusion protein as indicated by luciferase expression. In the future, the use of linkers or a p2a sequence to express fluorescent proteins from the same mRNA as the epigenetic fusion could help mitigate issues associated with direct fusion of a fluorescent tag (Chng et al.; Hadpech et al.).

Further work should be focused on determining the factors the impact fluorescent protein folding dynamics when they are incorporated as fusion proteins. We explored shifting the mCherry protein to the N and C termini with no conclusive results. For those aiming to visually track fusion proteins within the cell, the factors that impact folding will need to be further elucidated, as alternative methods that express the fluorescent marker separately will not suffice. Studies in crystallography on the changes to beta barrel structure with different N and C terminal fusions should be undertaken.

Given the low detection threshold of 2xd-Broccoli expressed from its original plasmid, we surmise that low transfection efficiencies of Gal4_p65, coupled with low plasmid copy number and cell loss during staining all contributed significantly to poor signal detection of SPiFs by this method. Cloning of 2xd-Broccoli into Gal4_p65 was verified by Sanger sequencing before transfection and the construct still increased luciferase expression significantly over a mock transfected control, indicating that the fusion protein itself was still viable in the cell despite a lack of 2xd-Broccoli detection. Likewise, ICC yielded low, although consistent detection of Gal4_p65 via 6XHis tag antibody staining. Although both methods have a high level of sensitivity for positive cells, neither provided robust detection of average fluorescence in a population with low positive cell density.

To this end we sought a method of fluorescence detection that pooled cells, allowing us to detect expression (fluorescence) in parallel with function (bioluminescence). We optimized a plate-reader based assay able to detect consistent variations in mCherry expression in parallel with cell density and bioluminescence, as an indicator of epigenetic effect in our transgenic HEK293 cell line (Tekel, Barrett, et al.). This work provides a facile pipeline for the high-throughput testing of epigenetically functional fusion proteins used throughout the rest of this work.

3.4 Methods

3.4.1 Cell culture and transfection

HEK293 Gal4-EED/luc cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% tetracycline-free fetal bovine serum and 1% penicillin and streptomycin at 37°C in a humidified CO_2 incubator. Prior to transfection, cells were plated in 12-well culture dishes at 40% confluency (~1.0E5 cells per well) in antibiotic-free growth medium. Transient transfections were carried out by adding 300 µl of DNA/Lipofectamine complexes to each well: 1 µg plasmid DNA or ddH_2O for mock transfections (10 µL), 5 µL of Lipofectamine LTX (Invitrogen #15338100), 285 µL of OptiMEM (Gibco #31985062). Plates were spun at 100 xg for 5 min to increase transfection efficiency and then incubated at 37°C in a humidified CO_2 incubator.

3.4.2 Flow cytometry

Cells were passed through a 35 µm nylon strainer (EMS #64750-25). Green fluorescent signal from GFP and red fluorescent signal from mCherry were detected

on a BD Accuri C6 flow cytometer (675 nm LP filter) using CFlow Plus software. Data were further analyzed using FlowJo 10.5.3. One run (~10 000 live cells, gated by forward and side scatter) was completed per sample, allowing us to determine median fluorescence within the live cell population.

3.4.3 Fluorescent microscopy

Forty-eight or seventy-two hours post transfection, cellular mCherry or GFP/broccoli signal was imaged in culture dishes on a Nikon Eclipse Ti wide field fluorescent microscope (MEA53100, filter G2E/C).

3.4.4 Luciferase assays

Luciferase assays were performed using Luciferase Assay Buffer (Biotium #30085) according to the manufacturer's instructions. In brief, a single well of cells from a 12 well tissue culture plate was collected per independent transfection. Cells were loaded into a Black Costar Clear Bottom 96 Well Plates (Corning #3631). Plates were scanned in a microplate reader (Biotek Synergy H1) set to detect chemiluminescence.

3.4.5 Broccoli aptamer cloning

The 2Xd Broccoli aptamer sequence housed in pAVU6+27-F30-2xdBroccoli (a generous gift from D. Menn) (Filonov et al.) was amplified with Phusion High Fidelity DNA Polymerase (New England BioLabs) and primers that facilitated the addition of a 5' XbaI and 3' NotI site. MV14 and the 2xd-broccoli amplicon were both digested with FastDigest *XbaI* and FastDigest *NotI* (ThermoFisher Scientific) and then ligated with T4 DNA ligase (New England Biolabs).

3.4.6 Broccoli staining

The lyophilized DFHBI-1T fluorophore (Lucerna, Inc.) was first diluted in DMSO to 50mM. For flow cytometry, cells were washed 48 hours post transfection with 1XPBS and then trypsinized with Trypsin-EDTA (0.25%), phenol red (ThermoFisher Scientific). Harvested cells were incubated in a 1XPBS solution with 4% 50mM DFHBI-1T for 30 minutes on ice. Cells were resuspended, strained, and analyzed via flow cytometry as described above. For microscopy, cells were washed 48 hours post transfection with 1XPBS. Fresh 1XPBS solution with 4% 50mM DFHBI-1T was added and cells were incubated on ice for 30 minutes. Cells were subsequently visualized in their dish via fluorescent microscopy as described above.

3.4.7 Immunocytochemistry staining

HEK293 cells (~$2x10^6$ adherent cells) were initially harvested by washing with 1XPBS and then trypsinizing them with Trypsin-EDTA (0.25%), phenol red (ThermoFisher Scientific). Cells were pelleted at room temperature by spinning at 1000 rpm for 3 minutes. Cells were then washed twice in ice-cold FACS buffer.

After preparing cells, a fixation solution of 4% paraformaldehyde was added to the cells and left to incubate for 12 minutes on ice. Cells were then immediately pelleted and washed twice in FACS buffer. Cells were then permeabilized with a fresh 0.5% Triton solution (Sigma-Aldrich) and incubated at room temperature for 30 minutes. Cells were then pelleted and the permeabilization solution was removed.

Cells were then prepared for staining with an AlexaFluor 488 conjugated 6XHis tag specific antibody (6x-His Tag Monoclonal Antibody (HIS.H8), AlexaFluor 488, ThermoFisher Scientific). During staining, samples were protected from light as much as possible. All staining was done in ice-cold FACS buffer. First, the antibody was diluted according to the manufacturer's protocols in 1XPBS. Diluted antibody

was added at a 1:100 ratio to cells suspended in FACS buffer. Cells were incubated with the diluted antibody for 30 minutes on ice. After staining, cells were washed twice with FACS FB-S buffer. Cells were resuspended, filtered, and run through flow cytometry as described above.

3.4.8 Plate reader assay

A single well of cells from a 12 well tissue culture plate was collected per independent transfection in 1X PBS. Cells were loaded into 9 wells of a Black Costar Clear Bottom 96 Well Plates (Corning #3631). Three wells of cells were used to detect mCherry in order to quantify Gal4-AAP proteins. Three more wells were loaded with cells and 2X Hoechst 33342 stain (Invitrogen #H3570) to quantify nuclear DNA as a proxy for cell density. The final three wells were loaded with cells and Luciferase Assay Buffer (Biotium #30085), prepared according to the manufacturer's instructions. Plates were scanned in a microplate reader (Biotek Synergy H1) to detect RFP (580 nm - 610 nm), Hoechst 33342 fluorescence (360 nm - 460 nm) and chemiluminescence from the same sample in parallel.

For each sample, luciferase (chemiluminescence), RFP, and Hoechst 33342, the average signal from the 1x PBS wells was subtracted from the value for every sample well. Next, the average background-subtracted luciferase value for untransfected (UT) cells was subtracted from each experimental luciferase value. UT-subtracted luciferase values were scaled by multiplying each value by [mean UT Hoechst / sample Hoechst], then by [mean RFP / sample RFP].

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CHAPTER 4

EXPLORING THE USE OF SYNTHETIC PIONEER FACTORS TO IMPROVE CAS9-MEDIATED GENOME EDITING IN POLYCOMB HETEROCHROMATIN

4.1 Background

In Chapter 2, we investigated the ability of Gal4-AAPs to increase gene expression at a target site in closed chromatin, but the issue of heterochromatin in mammalian genome engineering extends beyond transgene silencing. Prokaryotic CRISPR-Cas9 systems are increasingly popular as an easily programmable targeted endonucleases for rapid genome editing (Jinek et al.; Gilbert et al.). However, a dynamic and growing body of work suggests that heterochromatin inhibits Cas9 binding and cleavage. As described in Chapter 1, heterochromatin is a dense network of proteins and nucleic acids that structurally occludes particular enzymes, such as RNA polymerases. Its remodeling is associated with increased accessibility and often gene expression. While many efforts to implement Cas9 gene editing in eukaryotic cells have been successful, the fluctuating nature of the chromatin environment has lead to significant variations in editing efficiency across target sites, posing a continued barrier to reliable use (Jensen et al.; Horlbeck et al.; R. M. Daer et al.; X. Chen et al.).

Being bacterially derived, there was initially much skepticism about whether or not Cas9 systems would be transferable to eukaryotic chassis, especially mammalian ones where the full implications of facile genome editing for medical applications might be realized. However, as recently as 2013, Gilbert et al. demonstrated the ability of Cas9 to edit mammalian genomes. The robustness of this

ability and the parameters that need to be considered to facilitate this editing are still an area of active investigation.

Before editing can occur, the Cas9-guide RNA complex must bind to the target site. Several lines of evidence suggest that heterochromatin may block this initial step in editing. Multiple studies have found that off-target binding by nuclease-null or deactivated Cas9 (dCas9) occurs with a higher frequency in areas of open chromatin, as determined by DNaseI hypersensitivity (Singh et al.; Robertson et al.; Kuscu et al.). In addition, predictive tools to design guide RNAs yield better binding efficiencies when chromatin landscape data are taken into account (Singh et al.).

Direct research on the interaction between nucleosomes and Cas9-guide RNA complexes support these findings. In a series of *in vitro* studies using reconstituted nucleosomes on synthetic DNA, researchers found that nucleosome-bound DNA prohibited binding and cutting by Cas9 (Hinz et al.; Isaac et al.; Horlbeck et al.). Complete inhibition found in these studies is most likely a reflection of tighter binding between synthetic DNA sequences and nucleosomes as opposed to natural sequences which facilitate some level of chromatin breathing (Anderson and Widom; Partensky and Narlikar).

Within the *in vivo* context of transgenic mammalian cell lines, Cas9 inhibition has been demonstrated in multiple heterochromatin varieties. Our own previous work in facultative Polycomb heterochromatin (R. M. Daer et al.) demonstrated the sporadic editing capabilities of Cas9 within a short (100-300bp) genomic region. While some targets were completely blocked as compared to the open chromatin control, others showed only a minimal reduction in editing, suggesting that hyper local chromatin dynamics contribute to an overall decrease in editing efficiency (R. M. Daer et al.). Likewise, Chen et al. demonstrated a reduction of editing in ectopic constitutive HP1-mediated (X. Chen et al.). Again, within a relatively small region

(1500bp), all eight of their target sites showed varying amounts of reduction in editing as compared to an open chromatin state (X. Chen et al.). Blocking of Cas9-editing by heterochromatin has even been demonstrated in zebrafish (Y. Chen et al.) suggesting that the effects of heterochromatin on Cas9 are pervasive across *in vivo* contexts.

Cas9 access to DNA in heterochromatin can be described as sporadic, requiring multiple attempts at binding (Kallimasioti-Pazi et al.), and dependent on local sequence effects and nucleosome positioning. To this end, several studies have found that Cas9 is able to access target sites in closed chromatin and edit effectively (Yang et al.; Perez-Pinera et al.; Knight et al.; Polstein et al.). In fact, some groups have even used dCas9 as a tool to open up chromatin for increased accessibility, although systematic testing in areas of defined heterochromatin is lacking, as described in Chapter 1 (F. Chen et al.; Barkal et al.). The combination of these works with seemingly opposing conclusions testifies to the complexity of chromatin inhibition, and the need for tools that can facilitate editing in *all* chromatin contexts.

Research suggests that chromatin remodeling can reverse heterochromatin inhibition of Cas9. In their *in vitro* studies of the effects of nucleosomes on Cas9 binding Isaac et al. and Horlbeck et al. successfully reversed inhibition with nucleosome-sliding enzymes that improved Cas9 cleavage of DNA previously occupied by nucleosomes (Isaac et al.; Horlbeck et al.). In previous work (R. Daer et al.), we also attempted to reverse Polycomb-mediated inhibition of Cas9 through pretreatment with a targeted p65 activator that is known to significantly increase transgene expression in PRC2 heterochromatin. However, at several target sites we observed an initial drop in editing accompanied with a significant increase in transgene expression (R. Daer et al.). This is most likely due to Polymerase crowding, a phenomenon documented as inhibiting Cas9 editing elsewhere as well

(Kallimasioti-Pazi et al.), in which the density of polymerases associated with artificially high expression levels precludes Cas9 binding.

Together these works suggest a targeted approach to the remodeling of chromatin that does not necessarily increase expression. Canonically, opening chromatin suggests both an increase in accessibility and gene expression. However, even within native systems this correlation is not ubiquitous. The Goldilocks state for Cas9 in chromatin may be achievable if we can engineer analogues to the devices that facilitate "poised" chromatin in natural systems (Bernhart et al.; Lesch and Page; Rasmussen et al.). In mammalian systems, this role is filled by pioneer factors (see Chapter 1), a unique class of transcription factors that can access DNA in closed chromatin and facilitate a transition to an open state (Zaret and Carroll). This transition includes the formation of poised chromatin, in which pioneer factors co-locate repressors and activation-associated histone modifications to a single site (Sérandour et al.). In this "door ajar" state, the chromatin can be either pushed closed again or fully opened by the recruitment of transcriptional machinery. This suggests that pioneer factors may be able to facilitate a low point in the chromatin inhibition landscape for Cas9-mediated genome editing. To test this hypothesis, we investigated the ability of our Gal4-AAPs (see Chapter 2 for full list) that did not increase gene expression to instead increase Cas9-mediated editing efficiency in closed chromatin. We hypothesized that these expression-silent fusions were still epigenetically active, and that the inherent pioneer function of the Gal4 DNA binding domain, coupled with an epigenetic effector may act in a manner similar to native mammalian pioneers. To this end, we have named these Gal4-AAP fusions synthetic pioneer factors (SPiFs) for their ability to dynamically and separately regulate chromatin opening and gene expression activation.

In Chapter 2, we used a transgenic HEK293 cell line that allows us to control the chromatin state at a transgenic *luciferase* reporter with the addition of doxycycline (Hansen et al.; R. M. Daer et al.). As described in Chapter 2, doxycycline induces the accumulation of Polycomb heterochromatin at *luciferase*, a process that is controlled by the two major Polycomb group (PcG) complexes, Polycomb repressive complexes 1 and 2 (PRC1/2) (Kahn et al.). PRC2 deposits the repressive mark H3K27me3, which has been directly confirmed through chromatin immunoprecipitation (ChIP) (R. M. Daer et al.). This histone modification along with PcG complex localization induces nucleosome compaction and subsequent gene silencing (Jamieson et al.). It is this mechanism that leads to a chromatin state inaccessible to polymerases, native nucleosome remodelers, and CRISPR/Cas9 systems.

Here we investigate SPiFs as a method for improving Cas9 editing by artificially poising chromatin that has been previously inhibited by Polycomb-mediated heterochromatin (Figure 4.1). Polycomb is a relevant test bed, as PcG proteins are critical for gene expression control (Morey, Pascual, et al.; Morey, Santanach, et al.) and often involved in oncogenesis (van Lohuizen et al.; Jacobs, Scheijen, et al.; Jacobs, Kieboom, et al.; Schuettengruber et al.). They are also widely distributed across the genome in gene-enriched regions, where they confer an inherited form of gene silencing (Ingham). PcG proteins may also regulate higher-order chromatin structure and density through long distance interactions between PRC1 complexes (Entrevan et al.; Cabrera et al.; Isono et al.; Kundu et al.; Schoenfelder et al.). Therefore, Polycomb heterochromatin presents a major barrier to genome editing and serves as a good model to investigate the reversal of chromatin-mediated Cas9 inhibition.

Cas9-mediated genome editing is an increasingly important tool for biotechnology, and eventually medicine. Solving the issues of slow binding, varying editing efficiency, and site-specific blockage will be necessary to see Cas9 reach its full potential as a tool. Chromatin state plays a major role in each of these issues, making our ability to tune chromatin state accessibility for Cas9 critical. Here we explore the use of synthetic pioneer factors in optimizing the balance between heterochromatin and active euchromatin for Cas9 editing.

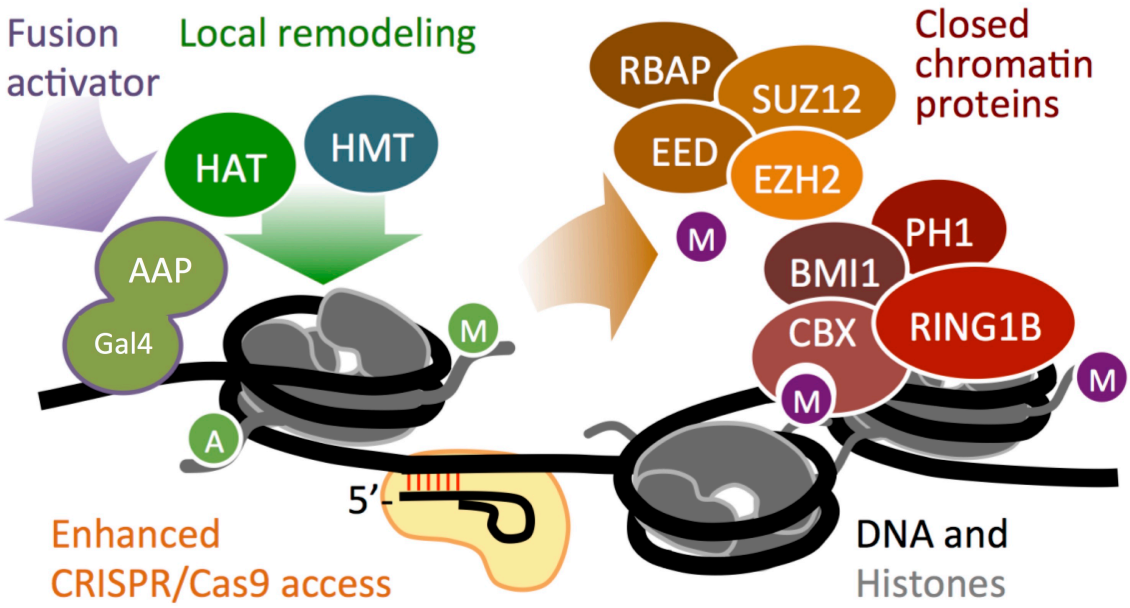

Figure 4.1: Synthetic pioneer factors increase accessibility to Polycomb heterochromatin. In the closed state, PRC2 generates the silencing mark H3K27me3 (purple M). This complex includes Suppressor of Zeste 12 (SUZ12), Embryonic ectoderm development (EED), Retinoblastoma-binding protein (RbAp), and Enhancer of zeste 2 (EZH2) (Tiwari et al.; Aoto et al.). PRC1 includes Chromobox protein homolog (CBX), Ring finger protein 1b (RING1B), and Polycomb group RING finger protein 4 (BMI1) (Tiwari et al.; Aoto et al.). This facultative heterochromatin inhibits Cas9 editing, along with DNA and RNA polymerases (X. Chen et al.; R. M. Daer et al.). Synthetic pioneer factors containing a Gal4 DNA binding domain (Gal4) and activation-associated peptide (AAP) can be used to recruit chromatin-remodeling proteins to a specific locus.(Polstein et al.) This targeted opening can induce the recruitment of chromatin remodelers (not shown), histone acetyltransferases (HATs), and histone methyltransferases (HMTs), that generate activation-associated modifications (green A, green M) (Racey and Byvoet; Rea et al.). This induces the remodeling of chromatin to support accessible DNA, and a Cas9-permissive state.

4.2 Results

4.2.1 SPiFs that increase expression do not improve Cas9 editing in closed chromatin

Here we explore the ability of SPiFs to reverse chromatin-mediated Cas9-inhibition. We used a cost-effective Sanger sequencing based method to evaluate the effects of SPiF+Cas9 co-treatment on editing efficiency in closed chromatin (Figure 4.2). We used doxycycline induction to recruit Polycomb heterochromatin to the *luciferase* transgene as described in Chapter 2. After silencing, we co-transfected with a Cas9-sg32 and Gal4-AAP plasmids, both targeted at the *luciferase* transgene (Figure 4.2). Forty-eight hours post transfection, cells were sorted to ensure that only cells transfected with both Cas9-sg32 (GFP labeled) and Gal4-AAPs (RFP labeled) were used for analysis (Figure 4.2). Genomic DNA was extracted and the editing target site was PCR amplified and sequenced using Sanger sequencing. The resulting sequences were analyzed using Synthego's Inference of CRISPR Edits (ICE) tool (Hsiau et al.), an open source software that utilizes a modified version of Tracking Indels by Decomposition (TIDE) algorithms (Brinkman et al.). This platform provides Cas9 editing efficiency data comparable to Next-Gen sequencing analysis (Hsiau et al.).

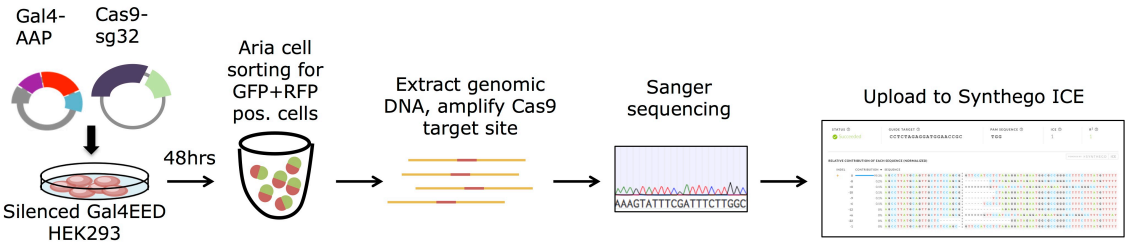

Figure 4.2: Synthego ICE method for high quality detection of Cas9 mediated editing after SPiF treatment. Silenced Gal4-EED/luc HEK293 cells were co-transfected with a Cas9-sgRNA plasmid (target site, g32) and Gal4-AAP plasmid (see Chapter 2). Cells were subsequently sorted to select for an entirely GFP/RFP positive population (containing both plasmids). Genomic DNA was extracted and editing target site DNA was amplified for Sanger sequencing. Sequences were then uploaded to Synthego ICE and compared to an unedited control to determine relative editing efficiencies across SPiF co-treatment types.

We transfected epigenetically silenced Gal4-EED/luc HEK293 cells with a pX330_g32 plasmid expressing a Cas9 protein and sgRNA32 (5' CCTCTAGAGGATGGAACCGC 3'), targeted ~100bp downstream of the luciferase transcription start site (Figure 4.3a). Site g032 was specifically chosen because previous work from our group showed that Cas9-mediated editing at this site was almost entirely blocked by heterochromatin but accessible to editing in open chromatin (R. M. Daer et al.). This previous work confirmed the inhibition specifically of SpCas9 from *Streptococcus pyogenes* by chromatin (R. M. Daer et al.). In Appendix C, we discuss work using the same method outlined in Figure 4.2 to establish chromatin inhibition of another Cas9 protein, FnCas9 (F. Chen et al).

In the same transfection reaction, we added a one of sixteen Gal4-AAP plasmids (1:1 stoichiometric ratio), targeted ~350bp upstream of the transcription start site (Figure 4.3a). Editing efficiency by SPiF co-treatment type was determined via Synthego ICE (Hsiau et al.). We compared editing with SPiF co-treatment to Cas9-mediated editing in non-silenced, fully open chromatin (in grey, ~60% editing efficiency) and fully silenced, closed chromatin (in black, ~7% editing) (Figure 4.3b). We found that Gal4 fusions to the histone acetylase KAT2B and chromatin

remodeler SMARCA4 restored editing to levels comparable to open chromatin. AAPs associated with significant increases in expression (p65, VP64, and MYB- see Chapter 2), did not significantly improve editing efficiencies (Figure 4.3b).

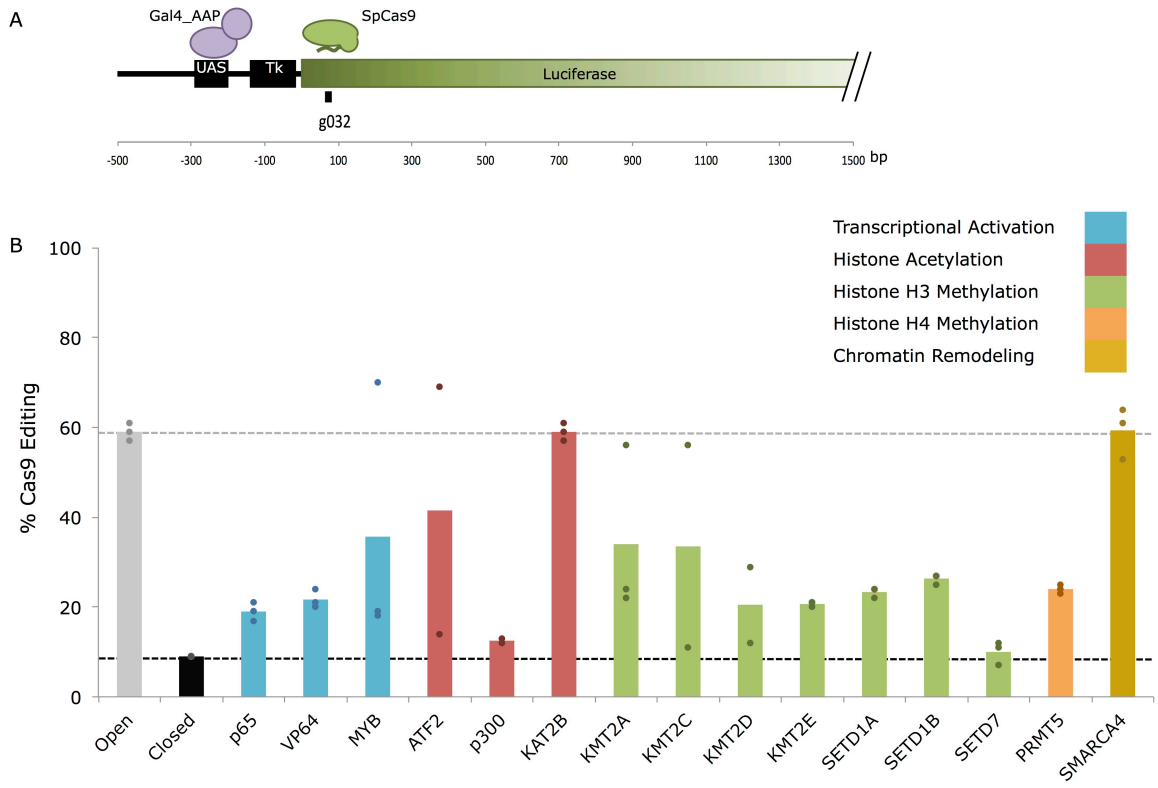

Figure 4.3: Cas9-editing efficiency in closed chromatin after SPiF treatment. A) Gal4_AAPs and Cas9-sg32 are simultaneously targeted to the epigenetically silenced luciferase transgene. Gal4 targets the AAP upstream of the editing site to induce changes to local chromatin. B) Percent Cas9-mediated editing efficiency at site g032 by SPiF co-treatment type. SPiF types are arranged by functionality of their AAP domains. Efficiency of Cas9 editing in open chromatin is indicated in grey. Efficiency of Cas9 editing in closed chromatin is indicated in black. Individual biological replicates are shown as single points, while bars represent median percent editing efficiency.

4.2.2 Indel types induced by Cas9 in re-opened chromatin vary by SPiF co-treatment

To further investigate the specific effects of each SPiF on Cas9-mediated editing, we compared the most common types of indels seen for each treatment (Figure 4.4). As expected when inducing non-homologous end joining (NHEJ) after Cas9-mediated cleavage, shorter indels and single-base pair mutations are most prevalent across treatment types. The relative proportions of indel types after SPiF treatment closely mirrored those seen in open chromatin (Figure 4.4). We also investigated the effects of SPiFs on homologous donor repair (HDR), the alternate repair pathway to NHEJ after Cas9-mediated cleavage, using a melt-curve analysis method (see Appendix C) (Miyaoka et al.; Smith et al.).

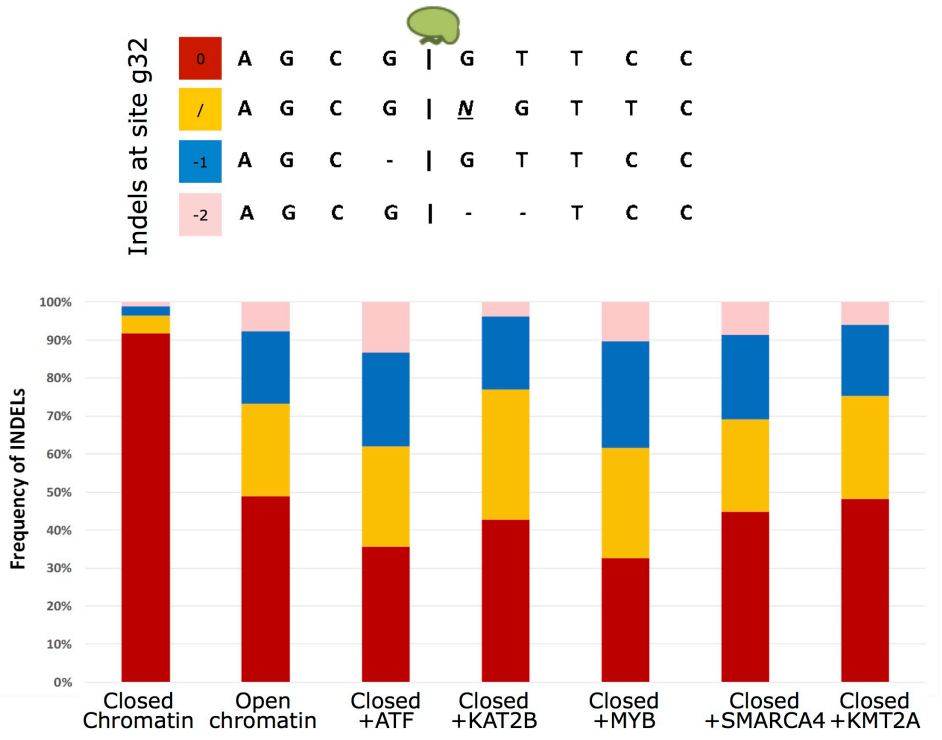

Figure 4.4: Most frequent Cas9-induced indels observed in closed chromatin after SPiF treatment. The three most common indel varieties induced at site sg032 are indicated in reference to the unedited sequence (red). All other indel varieties were present at a rate of less than 1% per biological replicate. Each bar represents the aggregate of three biological replicates.

4.2.3 Cas9 editing range in re-opened chromatin varies by SPiF co-treatment

Editing efficiency is only one Cas9-editing design parameter important to genetic engineering. Range of editing at a target site is critical to the subsequent repair process (Ochi et al.; Pannunzio et al.). We aggregated data on the range and density of indels from NHEJ after Cas9-mediated editing and SPiF co-treatment for three biological replicates (Figure 4.5). We found distinct variations in editing ranges based on SPiF co-treatment type. Gal4_SMARCA4, while restoring editing efficiencies to levels comparable with open chromatin (Figure 4.3b), yields a much narrower editing range than that seen in open chromatin (Figure 4.5). Likewise, while not fully restoring editing efficiency of Cas9 in closed chromatin, all SPiFs tested did restore some level of editing range as compared to closed chromatin (Figure 4.5).

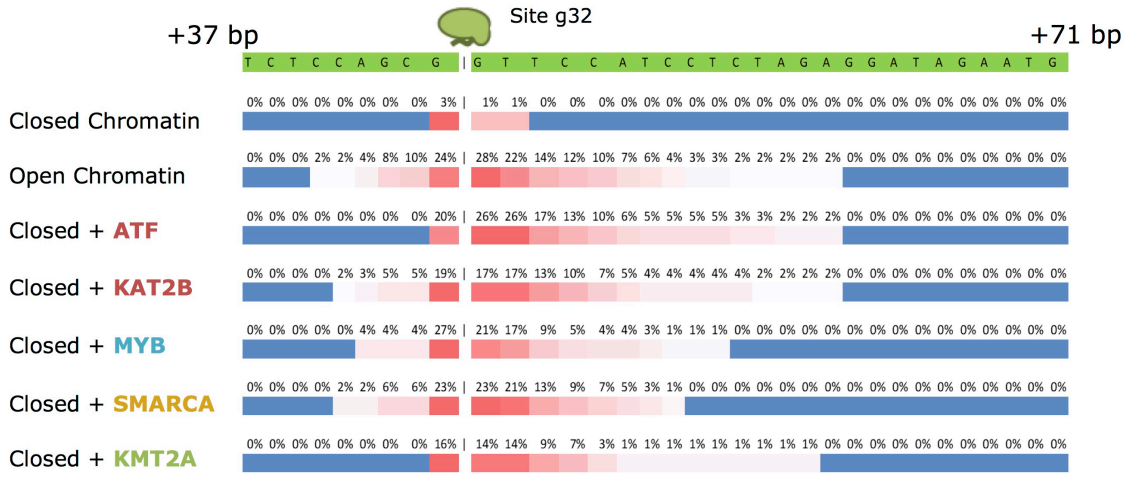

Figure 4.5: Spread and frequency of Cas9-mediated indels with SPiF co-treatment in closed chromatin. Range of indels induced by NHEJ after Cas9-mediated cleavage is shown for the sg032 target site in *luciferase*. Ranges of indels induced by NHEJ after Cas9-mediated cleavage and SPiF co-treatment are shown by SPiF treatment. Function of each SPiF is indicated with colored text according to the key in Figure 4.3 (red= histone acetylation, blue = transcriptional activation, yellow= chromatin remodeling, green= histone H3 methylation). Frequency of editing at each site is listed above each nucleotide position. A position was designated as affected by an INDEL based on sequence changes relative to the wild type sequence as reported by Synthego ICE alignment. Each individual heatmap represents the aggregate of three biological replicates.

4.2.4 Nucleosome Occupancy and Methylation sequencing (NOMe-seq) can be used to evaluate nucleosome position *in vivo*

After determining that SPiFs can have both a significant impact on chromatin accessibility (Figure 4.3b) and expression of the genetic material therein (Chapter 2), we wanted to ask what structural changes at the chromatin level contributed to these changes from a closed to open chromatin state. We explored in brief the depletion of histone modifications associated with Polycomb heterochromatin and the accumulation of activation-associated histone modifications deposited by Gal4_AAPs or their cofactors (see Appendix C). We did not find a correlation between SPiF function (either the induction of gene expression or accessibility) and changes in histone modifications; even those SPiFs that appeared to be non-functional induced some change to local chromatin modifications (Figure C2).

Being the core structural element of chromatin known to inhibit Cas9's access to DNA (Horlbeck et al.; Isaac et al.; Hinz et al.) we wanted to investigate changes in nucleosome density and position at site sg032 before and after SPiF treatment. Several methods exist for broadly determining nucleosome occupancy (Meyer and Liu). DNase sensitivity assays utilize an exonuclease to cleave exposed DNA in order to identify areas of open chromatin (Madrigal and Krajewski); however this approach is typically used to characterize chromatin density across large genomic regions as opposed to high-resolution nucleosome mapping. Similarly, ATAC-seq and DIVA-seq utilize the insertion of transposons or viral DNA to identify areas of chromatin with greater accessibility (Kumasaka et al.; Timms et al.). MNase-seq utilizes a Micrococcal endonuclease that directionally digests DNA until an obstruction, such as a nucleosome, is reached, allowing for higher-resolution mapping of local nucleosome density (Luo et al.; Nikitina et al.). While this method provides relative

sequence lengths based on average nucleosome occupancy, direct sequence mapping is difficult. A relatively new method known as Nucleosome Occupancy and Methylation sequencing (NOMe-seq) provides high-resolution data on nucleosome occupancy that can be directly mapped to DNA sequence position (Rhie et al.; Piao et al.; Lay et al.).

NOMe-seq utilizes a non-canonical GpC methyltransferase (M.CviPI) to methylate GpC sites that are unbound by nucleosomes, or other large obstructions such as transcription factors (Figure 4.6) (Rhie et al.). Bisulfite conversion induces mutations at unmethylated cytosines, allowing for direct mapping of nucleosome position (mutated cytosine bases) to the original sequence of interest (Figure 4.6). We are currently optimizing NOMe-seq as an approach to map nucleosomes at the *luciferase* transgene in open chromatin, closed chromatin, and SPiF-treated closed chromatin.

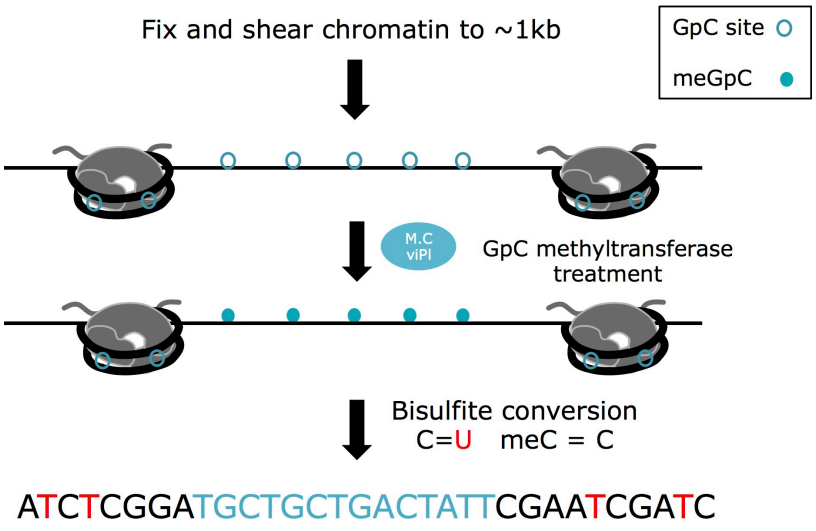

Figure 4.6: Nucleosome Occupancy and Methylation sequencing (NOMe-seq) can be used to determine nucleosome position after SPiF treatment in closed chromatin. Treated cells are lysed such that chromatin can be fixed and sheared into lengths of less than 1 kilobase. Treatment with the M.CviPI GpC methyltransferase induces methylation of exposed cytosine bases. In subsequent bisulfite conversion, unmethylated cytosines are converted to uracil, inducing mutations relative to the reference sequences after Sanger sequences. These regions of single base cytosine to thymine mutations indicate the presence of a nucleosome.

4.3 Discussion

Here we investigated the ability of SPiFs to improve Cas9-mediated editing in induced Polycomb heterochromatin. We hypothesized that a poised, permissive state between hyper compacted closed chromatin and active open chromatin would allow for increased editing efficiency by Cas9. Previous work (R. Daer et al.) with the SPiF Gal4_p65 established that gene expression activity and exonuclease accessibility must be considered as two separate parameters when opening chromatin. Hyper expression induced by Gal4_p65 actually reduced initial editing by Cas9 at several sites (R. Daer et al.). Further work should be done to fully understand the mechanism behind Polymerase crowding and inhibition of Cas9.

From our panel of SPiFs we were able to identify several "silent" SPiFs that do not increase local gene expression but do increase accessibility to the DNA for Cas9. Understandably, a Gal4 fusion to the chromatin remodeler domain SMARCA stored editing levels to those observed in open chromatin. However, the range of editing was less than that observed in other SPiF treatments, indicating that nucleosomes were potentially shifted rather than displaced. Further investigation into local nucleosome position after each treatment will be needed to fully assess changes to local chromatin and their impact on Cas9 editing.

The second SPiF that significantly increased Cas9 editing in closed chromatin contained a KAT2B histone acetylase domain. ChIP to assess the collocation of remodelers or other transactivators recruited by KAT2B should be performed to fully understand the mechanism behind opening. In general, functional assays to evaluate the chromatin landscape after treatment with each SPiF should be performed in order to understand the differences in chromatin composition that lead to either an active/inaccessible, inactive/accessible, or even active/accessible state. ChIP data in Appendix C suggest that histone modifications alone may not be predictive of one

state or the other. ChIP for polymerase colocation and full nucleosome mapping should be pursued. By looking at direct indicators of transcription and chromatin core structure we should gain more insight into Cas9 permissive chromatin architecture. These insights will in turn improve our ability to engineer mammalian cells with Cas9 systems.

Once Cas9 has access to its target DNA, other parameters become critical for effective editing. The range and variations of indels impact final editing outcomes. HDR and NHEJ pathways are in competition within the cell after Cas9 induces a strand break. Range, location, and fidelity of cleavage all impact which pathway will triumph (Miyaoka et al.). Whether the final desired outcome is a single base deletion or a full gene replacement, impacts the tuning of local chromatin state that will be required to reach acceptable levels of editing efficiency and range. Likewise, the ability to engineer accessibility without increasing expression levels will be of great value to any applications in a therapeutic setting. Genes targeted for therapeutic editing are often producing an undesirable gene product, expression of which should not be increased.

Refined opening of chromatin for Cas9 has several advantages in an *in vivo* setting. Whereas epigenetic drugs can open chromatin, site-specific approaches will not have the same broadly disruptive, unpredictable effects (Harrison). Many candidate diseases for genetic therapies such as neurodegenerative disorders or cancer are already characterized by unstable or disordered chromatin landscapes; avoiding compounding factors to epigenetic dysregulation is key (Verma; Coppedè; Kocerha and Aggarwal). Similarly, while Cas9 inhibition is not complete in closed chromatin, it is dose-dependent (Kallimasioti-Pazi et al.). Kallimasioti-Pazi et al. showed that by increasing Cas9 dosage, they were able to increase the speed and efficiency of Cas9-mediated cleavage in closed chromatin (Kallimasioti-Pazi et al.).

However, in many *in vivo* applications increasing Cas9 load will not be an option to overcome heterochromatin inhibition due to potential adverse host immune reactions (Hartweger et al.; Charlesworth et al.). Co-treating with effective epigenetic modifiers or modifying Cas9 directly with such pioneer domains, may allow us to reap the full benefits of its editing capabilities regardless of chromatin.

4.4 Methods

4.4.1 Cell culture and transfection

Cell culturing, silencing, and transfection of Gal4-EED/luc HEK293 cells were carried out as described in Chapter 2.

4.4.2 Aria cell sorting

Silenced and transfected Gal4-EED/luc HEK293 cells were collected 72 hours post transfection with Cas9_sg032 via standard trypsinization. Cells were diluted in ice-cold PBS + 20% FBS to ~10E7 cells per mL. At least 100,000 cells per sample were collected for genomic DNA extraction on a BD FAS Aria IIu Cell Sorter, with gate settings to collect only live cells with RFP/GFP double positive expression.

4.4.3 CRISPR editing analysis via Synthego ICE

Cells were co-transfected SPiF plasmids and pU6-(BbsI)_CBh-Cas9-T2A-EGFP (DNASU UnSC00746685) containing sgRNA sg032. Seventy-two hours post transfection, cells were harvested via standard trypsinization and run through Aria cell sorting.

After cell sorting, genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen). The editing target region was PCR amplified using GoTaq 2x Mastermix

(Promega) and primers 196 (5' cggaggacagtactccgctc 3') and 198 (5' ggcgttggtcgcttccggat 3') with the following cycling parameters: Cycle: 98°C /30 seconds, 35X (98°C/10 seconds, 67°C/ 30 second, 72°C/1min), 72°C 10 minutes, hold at 4°C. Amplicons were submitted for purification and Sanger sequencing to GeneWiz according to their protocols.

Once sequences were returned, they were uploaded alongside an unedited control sequence to Synthego's ICE Tool (https://ice.synthego.com/#/). Synthego ICE calculates percent editing across a sequence as well as frequency of indel variants across the range of edited sequence.

4.4.4 Nucleosome Occupancy and Methylation sequencing

NOMe-seq was performed using Active Motif's NOMe-seq kit (Active Motif, #54000). The manufacturer's protocol was followed, culminating in target region amplification via PCR and Sanger sequencing as described above. In brief, seventy-two hours post-transfection with an individual SPiF construct, cells are harvested and fixed using formaldehyde. This was followed by cell lysis, nuclei collection, and sonication to prepare strands of chromatin less than 1Kb long. The sheared chromatin was then treated with a GpC methyltransferase that non-canonically methylates cytosines. Chromatin cross-linking was reversed and DNA was purified for bisulfite conversion. Bisulfite conversion mutates the methylated cytosines such that the sites of methylation can be detected via sequencing. After bisulfite conversion, the DNA was desulfonated and purified for PCR and Sanger sequencing as described above.

The resulting sequences were analyzed for homology to an untreated control using Methyl Viewer (http://dna.leeds.ac.uk/methylviewer/), which visually indicates alterations to methylation. As nucleosomes preclude the GpC methylase, areas

lacking in conversion indicate the presence of a nucleosome; in this way the sequence mutations allow for visual mapping of nucleosomes across the sequence.

4.4.5 ChIP-qPCR

Chromatin immunoprecipitation qPCR was carried out as described in Daer et. al (R. Daer et al.). In brief, seventy-two hours post-transfection, cells were harvested for fixation with 1% formaldehyde (Thermo Fisher Scientific) in 1x Dulbecco's PBS. The reaction was quenched with 125 mM glycine (Sigma-Aldrich), followed by four washes with 1x PBS and Pierce Protease Inhibitors (Thermo Fisher Scientific).

Next, chromatin was prepared by lysing the cells and nuclei. The resulting chromatin was sonicated and diluted for immunoprecipitation with anti-H3K27me3 antibody (07−449, Millipore) or anti-H3K4me3 (ab8580, Abcam). After immunoprecipitation, crosslinking was reversed and DNA was prepared using the Genelute PCR Cleanup Kit (Sigma-Aldrich).

Quantitative PCR for the target site was performed with SYBR Green Mastermix (ThermoFisher Scientific) and previously published primers for the sg032 site in luciferase as well as a GAPDH control (R. Daer et al). To analyze ChIP-qPCR assay data, averages and standard deviations were calculated for each of 3 replicate IPs from a single chromatin preparation. For each cell/ treatment data set, %IP DNA for luciferase and GAPDH were normalized to %IP DNA for GAPDH.

4.4.6 Homology-directed repair melt curve analysis

Gal4-EED/luc HEK293 cells were silenced and transfected with SPiF constructs as described previously. Three days after initial transfection, cells were transfected again with Cas9_sg032 and a single stranded DNA oligo (ssODNA) with

complementarity to the sg032 cut site (5’ ggcgccattctatcctctagaggatggaacataaagctgatgcgctggagagcaactgcataaggctatgaa 3’). A ratio of 1ug Cas9 plasmid to 2ug ssODNA was used per transfection. Seventy-two hours post transfection, cells were harvested via standard trypsinization and genomic DNA extraction was performed using a QIAamp DNA Mini Kit (Qiagen).

Quantitative PCR was performed with SYBR Green Mastermix (ThermoFisher Scientific) and primers for the sg032 insertion site (p360: 5’ CGGCGCCATTCTATCCTCTA 3’ , p361: 5’ ATTCCGCGTACGTGATGTTC 3’) as well as primers for an unaffected TBP locus (TBPF: 5’ CAGGGGTTCAGTGAGGTCG 3’, TBPR: 5’ CCCTGGGTCACTGCAAAGAT 3’). The following cycling parameters were used: 95°C/ 3 min., 40x (95°C/ 10 sec., 57°C/ 10 sec, 72°C/ 10 sec), 72°C/ 3 min.

To analyze the data, we looked at the negative first derivative of the melting stage plot, which shows fluorescence (y-axis) over temperature (x-axis). Each sequence variant (unedited vs. those containing the ssODNA insert) melts at a different temperature, leading to distinct drops in the curve at different temperature intervals. By looking at the negative first derivative of this plot, we can see distinct peaks that correspond to each drop in the original plot. To compare the untreated to the treated samples, calculate $(Peakmax_{CRISPR} / Peakmax_{ref}) / (Peakmax_{untreated} / Peakmax_{ref})$ where the reference peaks (from the TBP melt curves) serve as loading controls. These values can then be compared across SPiF+CRISPR treatment types.

4.4.7 FnCas9 sgRNA cloning

Guide RNAs were cloned into the U6_FnCas9_sgRNA plasmid to direct FnCas9 to the silenced *luciferase* target site. Two sgRNA oligos with synthesized with overhangs for AarI cloning (in bold) (g32 F oligo Aarl: 5'

ACCGCCTCTAGAGGATGGAACCGC 3'; g32 R oligo AarI: 5'

AAACGCGGTTCCATCCTCTAGAGG 3'). These oligos were phosphorylated and annealed at 37°C for 30 min with T4 PNK (New England Biolabs). Following annealing, the oligo dimers and the U6_FnCas9_sgRNA backbone were both digested with AarI (ThermoFisher Scientific) and ligated with T4 ligase (New England Biolabs).

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CHAPTER 5

DISCUSSION

In this work we investigated the utility of synthetic pioneer factors (SPiFs) for controlling chromatin state in mammalian cells. We sought to control local chromatin compaction with two primary goals in mind, 1) epigenetically modulating gene expression and 2) increasing accessibility for genome editing tools such as CRISPR/Cas9.

Epigenetic control of gene expression has a wide range of uses. Here we focused on the reversal of transgene silencing to improve long term stability of exogenous gene expression in engineered cell lines. Gene expression control tools can themselves be incorporated in genetic devices, such as toggle switches or repressilators (Kramer et al.; Perez-Carrasco et al.; Rabajante and Babierra). In Chapter 2, we demonstrated the potential of the MYB TAD to be used as a toggle device under the control of small molecule drugs. Changes in MYB-induced expression were extremely rapid (2-3 hours) with the addition of celastrol, making it a good candidate for a switch in modular device design within mammalian cells.

Beyond *in vivo* engineering devices, epigenetic gene expression control has the potential to be used in medical applications from the production of model cell lines for research to *in situ* therapeutics. The same devices that can alter gene expression to match the profile of disease cell types for drug screens could be used to tune expression control to alter cell fate (Black et al.) either to reprogram diseased cells to a health state or control development for the growth of artificial tissues and organs. While *in situ* therapeutics and major organ development are still unrealized applications of epigenetic modulators, the refinement of these tools sets the groundwork for realizing these goals.

Secondly, the engineering of accessible chromatin, while less well researched, may be equally critical to the development of both reliable mammalian synthetic biology devices and *in situ* (epi)genetic therapeutics. Reliable access to DNA opens up the entire genome for editing and increases efficiency of editing at low concentrations of Cas9 protein (Kallimasioti-Pazi et al.), an important parameter for avoiding adverse immunological effects (Charlesworth et al.). Investigation of chromatin accessibility will also improve our general knowledge of chromatin structure, which is rapidly changing as the role of RNAs in chromatin is found to be increasingly important (Y. Li et al.; Guttman; Huang et al.) and our knowledge of the phase-like nature of chromatin develops (S. Liu et al.; Erdel and Rippe; Gibson et al.).

In Chapter 1 we outlined the set of potential tools available for the targeted modulation of chromatin. Synthetic biologists continue to use naturally occurring molecules as modular parts. Mammalian cells provide several mechanisms as inspirations for chromatin opening devices. These mechanisms are interconnected and interdependent, implying that more sophisticated design should utilize several mechanisms either sequentially or in parallel. Currently, the depth of these interconnections is underexplored and not yet amenable to engineering. Future work should aim to investigate co-dependence of various chromatin-opening mechanisms and underexplored mechanisms for controlling chromatin state, such as canonical pioneer factors.

In Chapters 2, 3, and 4 we explored the capabilities of a panel of synthetic pioneer factors (SPiFs) that utilized the functions for opening chromatin outlined in Chapter 1. We began in Chapter 2 by investigating the ability of these SPiFs to increase transgene expression in induced Polycomb heterochromatin. We found that SPiFs with transcriptional activation domains were the only group able to significantly

increase transgene expression, and that this increase in expression can be sustained after transient induction. This finding has implications for the engineering of stable mammalian cell lines.

Furthermore, among these activating SPiFs, the MYB TAD provides a novel tool for synthetic biologists. Its utility goes beyond that of simple gene expression enhancement, as its activating function can be rapidly controlled by the small, minimally toxic drug celastrol. The MYB TAD is functional when fused to multiple different DNA binding domains including Cas9, and can increase transgene expression in Polycomb heterochromatin as well as endogenous heterochromatin. Our results also suggest a cooperative, complex mechanism for chromatin opening as all activating SPiFs have no inherent catalytic activity and thus rely on the recruitment of cofactors to activate gene expression in closed chromatin. The identity of these cofactors and the specific changes to chromatin structure that each SPiF induces should be evaluated in future work. This could be achieved through a combination of ChIP at the target site and siRNA knockdown of putative interaction partners. The development of MYB as a new tool for gene expression control in mammalian cells warrants further investigation for its utility in more complex devices and endogenous settings. Future work should focus on incorporating MYB into multi-activator fusions such as VPR or SunTag (Z. Li et al.; Guo et al.; Papikian et al.). The ability of MYB to activate endogenous genes for cell programming should be investigated as well, to understand its transferability to *in situ* applications.

To facilitate our investigation of SPiFs, we needed to develop a robust method to assay their presence in parallel with their functionality. We developed such an assay as described in Chapter 3, allowing us to visualize SPiF protein production in combination with their ability to activate gene expression in induced Polycomb heterochromatin via luciferase expression. Our work in this chapter also indicated

dynamic inconsistencies in beta barrel fluorescent fusion tag signal expression across SPiF types and sampling date. This suggests the need to further investigate individual protein level differences in folding across parameters such as cell cycle, fusion protein localization, and construct configuration. Mitigation of signal variation was achieved here by pooling cells, as opposed to reading signal from individual cells. Fusion protein tag folding dynamics warrants further investigation due to its ubiquitous use in synthetic biology and the common under reporting of negative results.

Being able to visualize SPiFs and having quantified their ability to activate gene expression in closed chromatin, we turned to the other central issue with heterochromatin in engineering mammalian cells. A growing body of literature supports the hypothesis that chromatin prohibits reliable access to DNA by CRISPR/Cas9 (Daer et al.; Hinz et al.; Horlbeck et al.; Isaac et al.). Here, we show this to be the case for the newly discovered FnCas9 as well as canonical SpCas9 (Chen et al.). Development or discovery of new Cas9 species should be accompanied by experimental evaluation of the effects of eukaryotic chromatin on these nucleases. For example, the recently discovered CasX protein is significantly smaller than SpCas9 (J.-J. Liu et al.). Will this smaller protein have a harder time pushing nucleosomes out of the way to access DNA or an easier time slipping in between them? With the use of cell sorting and cheap, effective editing efficiency analysis via Synthego ICE or similar algorithms as we present here, high-throughput pipelines to assay the effects of chromatin on Cas nucleases could easily be implemented for rapid evaluation of new enzymes.

We hypothesized that some of our SPiFs that did not activate gene expression may still be functionally altering chromatin because of their inherent pioneer function. This function may improve access to DNA without directly inducing gene

expression, as many canonical pioneer factors do in creating a poised chromatin state (Bernhart et al.; Iwafuchi-Doi and Zaret). Indeed we identified two SPiFs in our panel, Gal4_KAT2B and Gal4_SMARCA4 that significantly increased Cas9 editing efficiency in closed chromatin without activating gene expression. These "silent" SPiFs are potentially useful for engineering or *in vivo* applications where ramping up target gene expression is undesired. Increasing expression before editing could destabilize metabolic circuits in the context of an engineered cell line, or potentially be highly detrimental in a gene therapy context where the therapy itself aims to remove a mutant gene that produces toxic product. Inducing accessibility without gene expression enhancement could thus be highly valuable.

We also investigated changes to the types and range of indels induced by Cas9 with SPiF co-treatment. Being able to tune these parameters could provide another layer of control for Cas9 in chromatin. Future directions of this work should primarily be focused on characterizing the chromatin that is responsible for these differences in editing efficiency and range. ChIP to evaluate the nature of a poised state based on specific histone modifications and colocalization of repressors/activators could tell us a great deal about how to induce this state and to what degree it mirrors natural poised chromatin. NOMe-seq mapping of nucleosomes before and after SPiF treatment should be completed. Future studies should also test more domains related to KAT2B and SMARCA4 to help elucidate the mechanism behind their ability to increase accessibility. Crystallography to examine their direct interactions with nucleosomes could also be informative.

Over all we conclude that there is a distinct difference between active and accessible chromatin as induced by SPiFs, and most likely by other targeted epigenetic modifiers and activators. Accessibility and gene expression enhancement can both occur in isolation from one another due to a variety of mechanisms. This

difference warrants closer investigations of chromatin before and after treatment with targeted epigenetic modifiers to elucidate the structural differences between these states. This work suggests the development of distinct tool kits in chromatin engineering for inducing both accessible and active states separately from one another, as each has its own unique utility to synthetic biologists. In order to achieve this goal, we must first define what each state looks like based on its basic chromatin structure. To what degree are histone modifications predictive of either condition? What cofactors induce prohibitive crowding or stabilize an accessible state? To what degree are larger forces such as phase separation playing a role in the accessibility to certain loci and the longevity of any induced changes to the chromatin therein? Characterization of induced open chromatin states, rather than just their outcomes will be the key to furthering our ability to engineer such complex biological microenvironments as chromatin.

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APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER 2

Table A1. Detailed description of AAP interaction partners. Interaction partners are grouped by general function: histone acetyltransferases (HATs or HAT complex subunits), histone deacetylases (HDACs), histone methyltransferases (HMTs or HMT complex subunits), coactivators, kinases, ubiquitinase, DNA methyltransferases (DNMTs), and non-catalytic structural proteins. Chromatin modifications (histone post-translational modifications and DNA methylation) are listed next to each enzyme as appropriate. This table exceeds dimensions of this file and can be accessed at https://www.biorxiv.org/content/10.1101/487736v1.supplementary-material.

Table A2. AAP accession numbers and primers. Capitalized nucleotides indicate overhangs for the addition of XbaI and NotI sites for cloning.

AAP	UniProt	Isoform	NCBI RefSeq	Amplification primers (5' ..
VP64 (4xVP16)	n.a.	-	-	F: CCTTATCTAGAgacgctttggacgacttcga R: CTTAGCGGCCGacaacatgtccaagtcgaagt
(NKκB)-p65	Q04206	-	NM_001145138.1	F: CCTTATCTAGAtacctgccagatacagacga R: CCTTAGCGGCCGatctcagccctgct
MYB	P10242	Isoform 1 P10242-1	NM_005375.2	F: GGCCTTATCTAGccagctgccgcagccattca R: AATTAGCGGCCGcccacccggggta

ATF2	P15336	Isoform 1 P15336-1	NM_001256090.1	F: CCTTATCTAGAgagatgacactgaaatttgg R: CCTTAGCGGCCGaggatcttcgtta
p300	Q09472	Q09472-1	NM_001429.3	gBlock from IDT
KAT2B (PCAF)	Q92831	Q92831-1	NM_003884.4	F: CCTTATCTAGActcaaccagaaaccaaacaa R: CCTTAGCGGCCGatttagctcacatc
KMT2A	Q03164	Isoform 1	NM_005933.3	gBlock from IDT
KMT2C	Q8NEZ4	Isoform 1	NM_170606.2	gBlock from IDT
KMT2D	O14686	Isoform 1	NM_003482.3	gBlock from IDT
KMT2E (MLL5)	Q8IZD2	Isoform 1 Q8IZD2-1	NM_182931.2 (variant 1)	F: GCGCTCTAGAaatttggataaagagagggc R: AATGCGGCCGcttcattactaatagg
SETD1A (SET1)	O15047	O15047-1	NM_014712.1	F: CCTTATCTAGAaagaagctccgatttggccg R: CCTTAGCGGCCGagtttagggagcc
SETD1B	Q9UPS6	Isoform 1	No RefSeq number	gBlock from IDT
SETD7	Q8WTS6	Q8WTS6-1	NM_030648.2	gBlock from IDT
PRMT5	O14744	Isoform 1 O14744-1	NM_006109.4	F: ATGCTCTAGAatggcggcgatggcggtcgg R: ATGCGCGGCCGcgaggccaatggt

FOXA1	P55317	P55317-1	NM_004496	F: GGCTCTAGAatgttaggaactgtgaagatgga R: ATTGCGGCCGccaaggaagtgtttaggacgg
SMARCA4 (BRG1)	P51532	Isoform 1 P51532-1	NM_001128844.1 (variant 2)	F: CCTTATCTAGAatgtccactccagaccacc R: CCTTAGCGGCCGgctgctgtccttgt

Table A3. dCas9-MYB sgRNA targeting sequences. The targeting sequences for g46, g31, g32, and g25 have been used in our previous work.

Transgene	**Cell line**	**Target Name**	**Targeting Sequence**
Tk-Luciferase	HEK293 Gal4-EED/luc	g46	5’ cctgcataagcttgccacca 3’
		g31	5’ cgaggtgaacatcacgtacg 3’
		g32	5’ cctctagaggatggaaccgc 3’
		g25	5’ accgtagtgtttgtttccaa 3’
CMV-GFP	HEK293 GFP (Chang Liu, UC Irvine,	L1	5’ ctctgtcacaggactcagcc 3’

	unpublished)	L2	5’ gggcggtaggcgtgtacggt 3’
		L3	5’ cactggtgtcgtccctattc 3’
		L4	5’ cttcttcaagtccgccatgc 3’

Table A4. Publicly accessible plasmids used in this study. Annotated sequences can be viewed online in the Haynes lab Benchling collection “DNA-Binding Fusion Transcriptional Regulators” at https://benchling.com/hayneslab/f_/5wovkOaK-gal4-dna-binding-fusion-transcription-regulators/?sort=name.

Name	**DNASU Accession #**
MV14	-
MV14_VP64	HaCD00812388
MV14_p65	HsCD00812387
MV14_MYB	-
MV14_ATF2	HsCD00833013
MV14_p300	HsCD00833014
MV14_KAT2B	HsCD00833015
MV14_KMT2A	HsCD00833016
MV14_KMT2C	HsCD00833018
MV14_KMT2D	HsCD00833017
MV14_KMT2E	-
MV14_SETD1A	HsCD00833019
MV14_SETD1B	HsCD00833020
MV14_SETD7	HsCD00833021
MV14_PRMT5	-
MV14_FOXA1	-
MV14_SMARCA4	HsCD00833022
pX330g_dCas9_MYB	-

Table A5. Primers for qPCR to quantify Gal4-AAP mRNA

Target	Amplification primers (5'... 3')
mCherry (Gal4-AAP transcripts)	F: gctccaaggcctacgtgaag R: aagttcatcacgcgctccca
TBP (housekeeping gene)	F: caggggttcagtgaggtcg R: ccctgggtcactgcaaagat

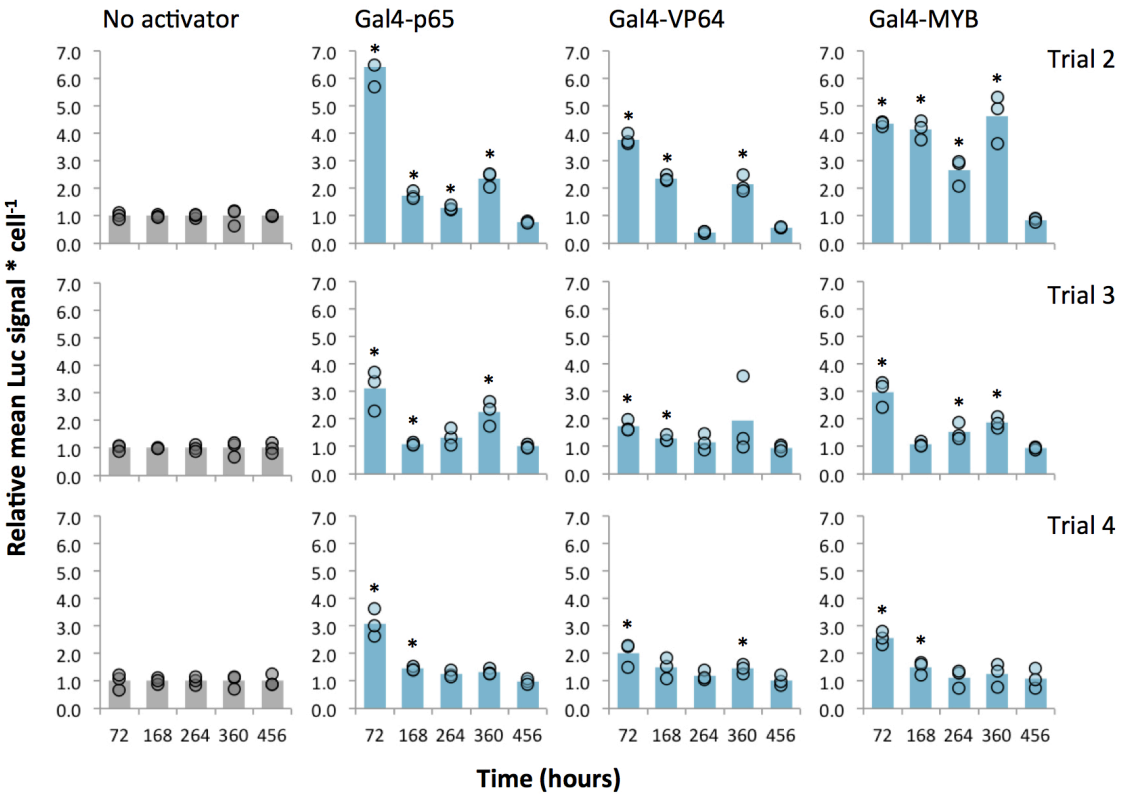

Figure A1. Additional trials of time course experiments with Gal4-AAP-expressing cells. Experiments were performed as described for Trial 1 in Figure 5A. Asterisks (*) = $p < 0.05$ for mean values greater than the mean for the "No activator" negative control sample.

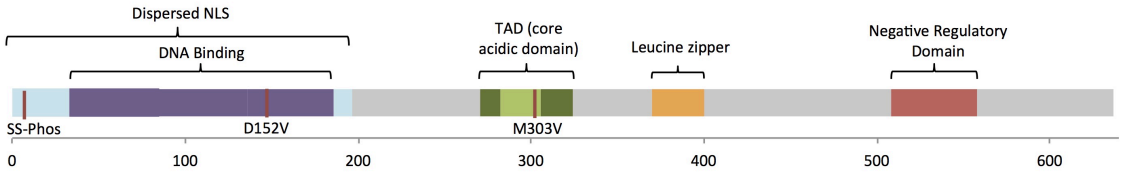

Figure A2. Annotated motifs within the MYB protein. Annotated motifs within the full-length MYB protein (AAA52032.1). From left to right: Dispersed nuclear localization signal (NLS) (M1-Q200; in light blue); Casein kinase II phosphorylation sites that reduce MYB DNA binding when phosphorylated and D152V mutation site that nullifies limited MYB pioneer function by disrupting DNA binding site recognition (S11, S12, D152; in red); Repeat regions (R1-R3) that facilitate MYB binding to its recognition element (G34-L86, N87-L138, N139-M189; in purple); Transcription activation domain (P275-W327; in green); Core acidic domain of TAD that facilitates interaction with CBP/p300 (D286-L309; in bright green); M303V mutation that disrupts p300 recruitment and thus, activation by MYB (M303; in red; Leucine zipper domain that interacts with other cellular proteins (L383-L403; in orange); Negative regulatory domain (V512-P566; in red), removal of which increases MYB-mediated expression increase.

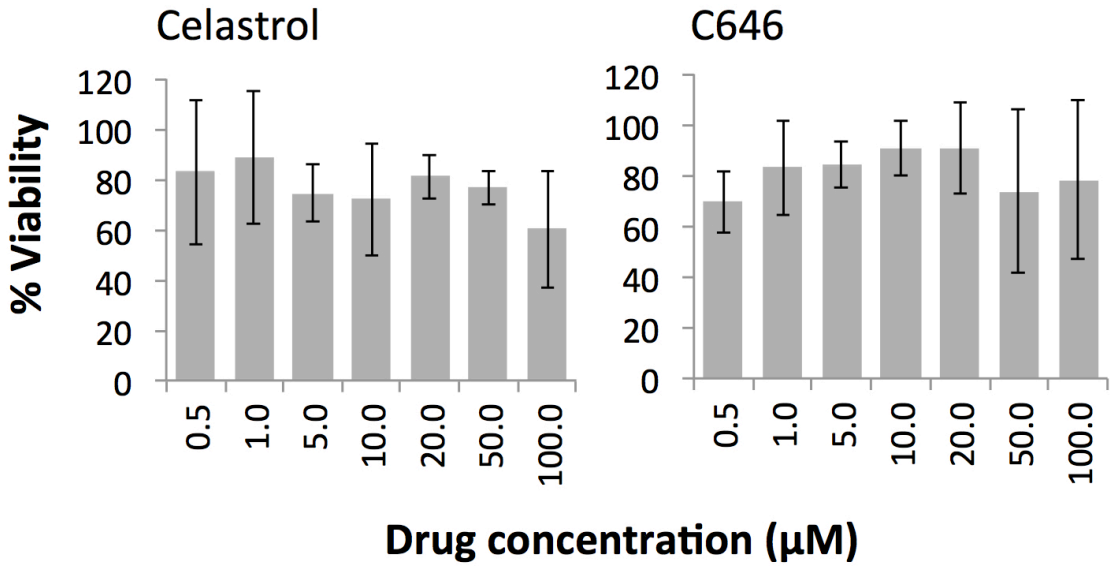

Figure A3. MTT cell viability assays. An MTT ((3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide)) cell viability assay to determine the effects of C646 and Celastrol on cell survival was performed as described previously by Godeshala et al. [S20] In brief, Gal4-EED/luc cells were treated with either C646 or Celastrol diluted at different concentrations in Gibco DMEM high glucose. Cells were incubated with the drugs for six hours before being washed and cultured for 3 hours in drug free medium containing MTT reagent solution. Finally, cells were incubated with methanol:dimethyl sulfoxide (1:1) at room temperature for 30 minutes and mixed thoroughly before absorbance was read at 570 nm. The relative cell viability (%) was calculated from [ab]test/[ab]control × 100%. For each sample, the final absorbance/cell viability was reported as the average of values measured from three

wells in parallel. Bars = standard error. We found no significant impact on cell viability after treatment with celastrol or C646 at any of the concentrations tested.

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

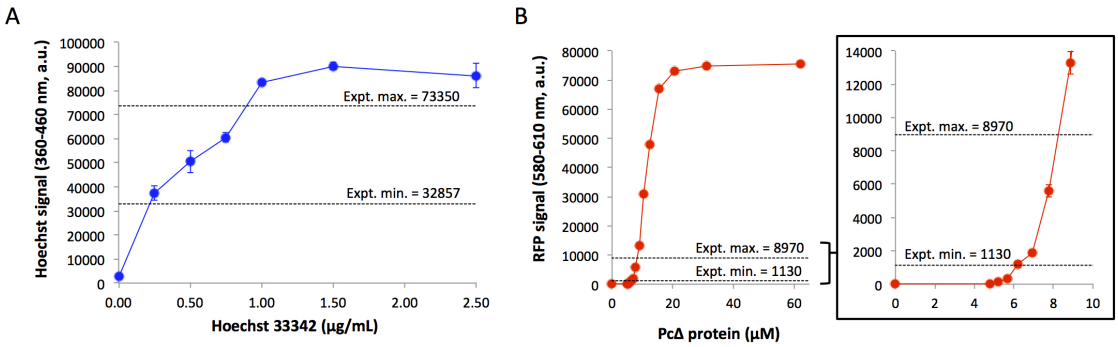

Figure B1. Determination of plate reader detection limits for Hoechst and RFP (mCherry). Samples of varying signal intensity were used to determine the linear range of signal detection for the Biotek Synergy H1 plate reader using the settings described in our report. (A) To prepare samples for the Hoechst channel (360-460 nm), HEK293 cells were grown to 90% confluency in a 12-well plate, collected, stained with varying concentrations of dye. Dots show mean values from triplicate cell samples (bars, standard deviation). The maximum and minimum Hoechst signal values from the plate reader data from Figure 5 are marked with dotted lines. (B) Samples for the RFP channel contained 5.0 to 62.0 µM 6-histidine-tagged recombinant PcΔ protein diluted in 1X PBS (200 µL per well). PcΔ was over-expressed in *E. coli* cultures and purified on a nickel-column as described in detail in Tekel et al. Dots show mean values from triplicate protein samples (error bars, standard deviation). (This figure and its legend are reprinted here with permission from ACS Publications, Copyright © 2018 American Chemical Society Publications)

APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER 4

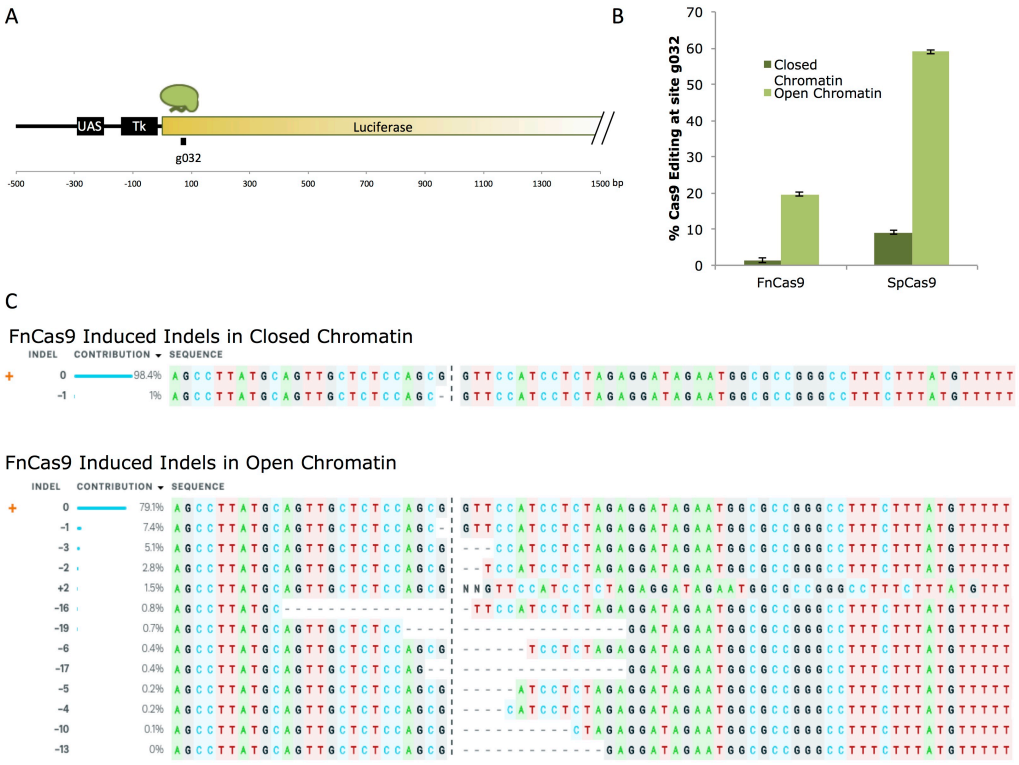

Figure C1: Induced Polycomb heterochromatin inhibits FnCas9. A) We targeted both SpCas9 and FnCas9 (Chen et al.) to site sg032 in our luciferase transgene. B) We compared editing by both Cas9 exonucleases in closed chromatin (silenced Gal4-EED/luc HEK293 cells) and open chromatin (Luc14 HEK293 cells). Both Cas9 exonucleases showed a relative reduction in editing in closed chromatin as compared to open chromatin. SpCas9 is a more robust exonuclease in both chromatin states. C) Variety and spread of indels from NHEJ after FnCas9-mediated cleavage is significantly higher in open chromatin as compared to closed chromatin.

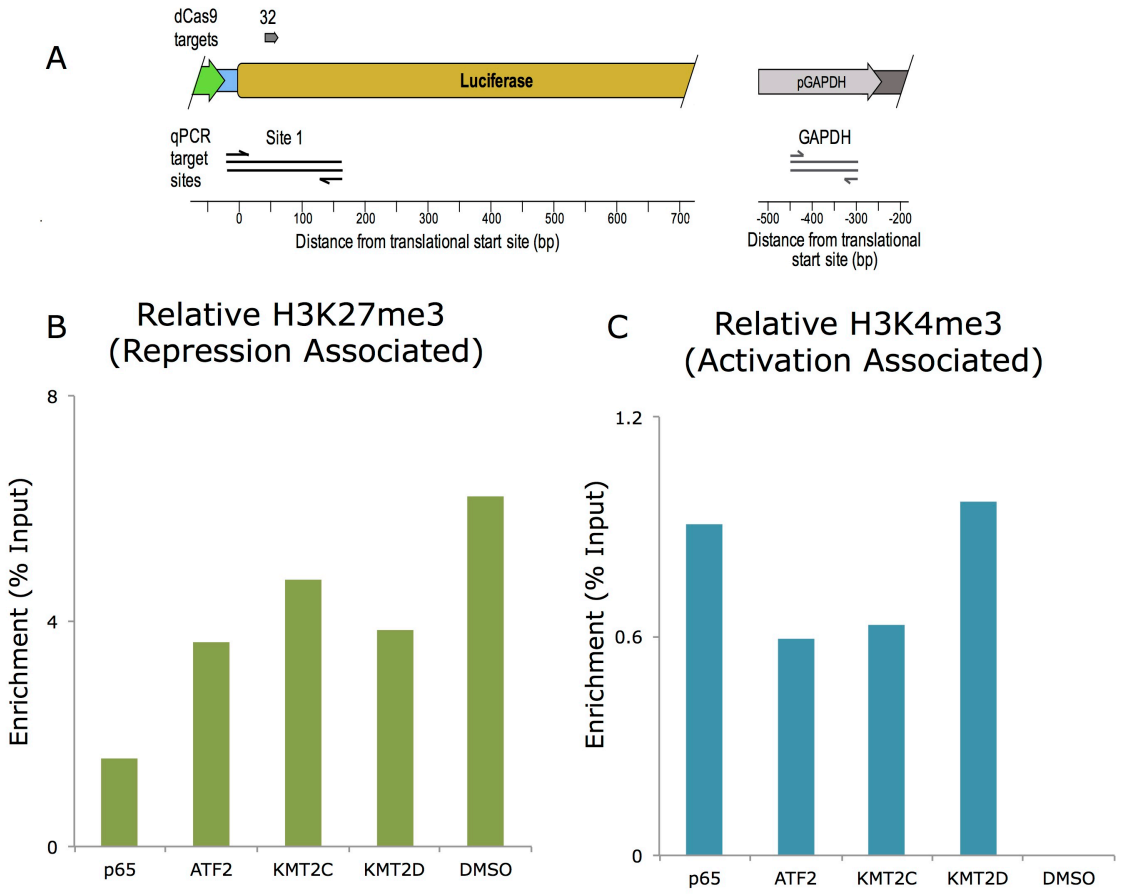

Figure C2: Changes in local histone modifications are not predictive of SPiF function. A) We performed chromatin immunoprecipitation (ChIP) qPCR across the sg032 editing site and GAPDH (control) in Gal4-EED/luc HEK293 cells, to determine changes in two local histone modifications after SPiF treatment. B) For all SPiFs that we treated cells with before ChIP-qPCR there was a reduction in repression-associated H3K27me3, a mark directly deposited by PRC2 in our inducible Polycomb system. DMSO serves as a null-treatment control, indicating baseline levels of H3K27me3 in silenced Gal4-EED/luc HEK293 cells. While p65 induces increased levels of expression (Chapter 2), ATF, KMT2C and KMT2D do not, although they do reduce repression associated histone modifications. C) All four SPiFs increase H3K4me3. KMT2C and KMT2D directly deposit this modification, while p65 has no direct catalytic activity on histones. Again, despite differences in functionality, all SPiFs modify the local histone code as compared to a DMSO control.

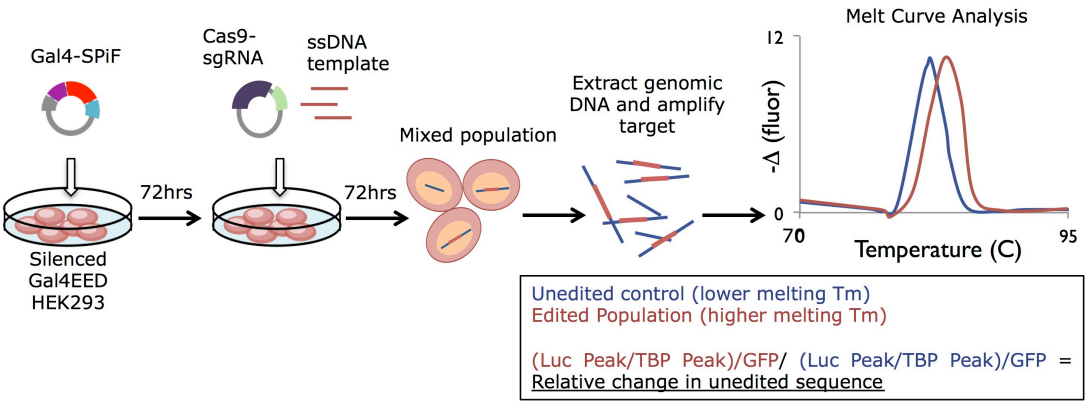

Figure C3: Melt curve analysis method for the detection of homology directed repair (HDR) after Cas9-mediated cleavage. Melt curve analysis allows for low cost detection of ssDNA template insertion into a target site. We transfected silenced Gal4-EED/luc HEK293 cells with SPiF constructs and subsequently Cas9-sgRNA plasmids along with ssDNA target site template DNA. Seventy-two hours post transfection, we harvested cells and extracted genomic DNA. These cells are a mixed population of edited and unedited cells. The target site was amplified with quantitative PCR and the resulting melt curves were compared. Larger sequences melt at higher temperatures allowing us to assess insertion rates of the ssDNA template. TBP was amplified for each sample as a loading control.

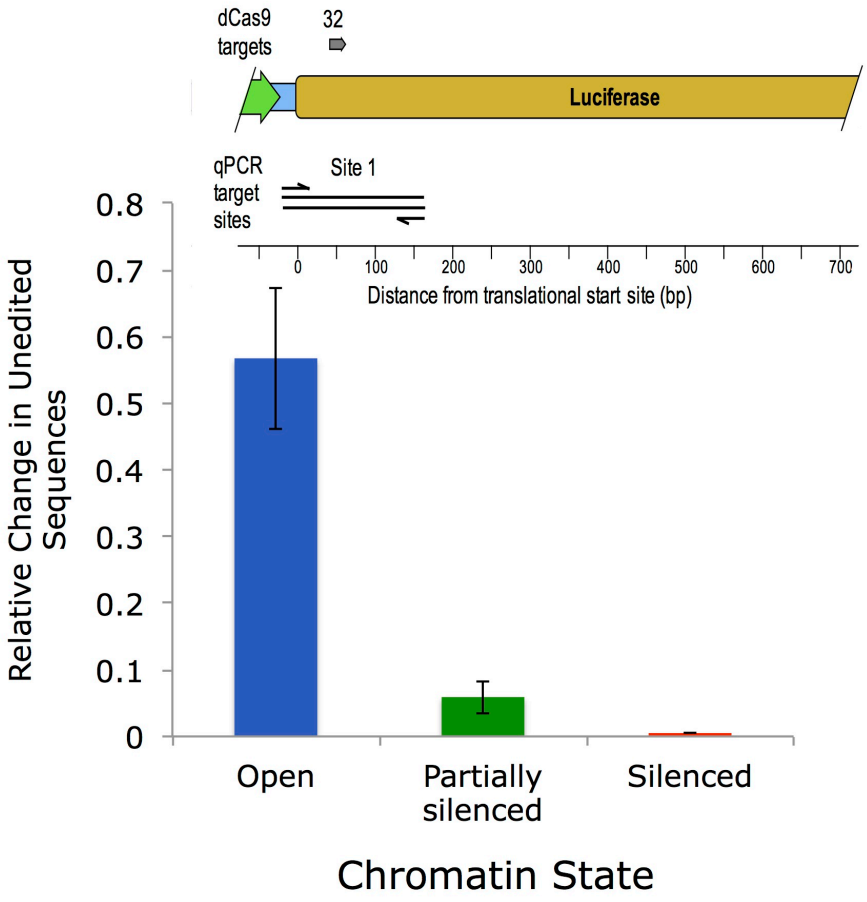

Figure C4: Replicating the results of Daer et al., 2017 with HDR melt curve analysis. In Daer et al, 2017, we found that SpCas9 editing is significantly reduced in closed chromatin and partially silenced chromatin as compared to an open chromatin control. These results were determined via SURVEYOR assay. Here, we attempted to replicate those results using HDR melt curve analysis in order to vet the assay. We targeted site sg032, which previously showed substantial inhibition in closed chromatin, but was still accessible to Cas9 in open chromatin. The figure above shows a recapitulation of these results using HDR melt curve analysis. Bars represent the median of three biological replicates. Error bars indicate standard deviation.

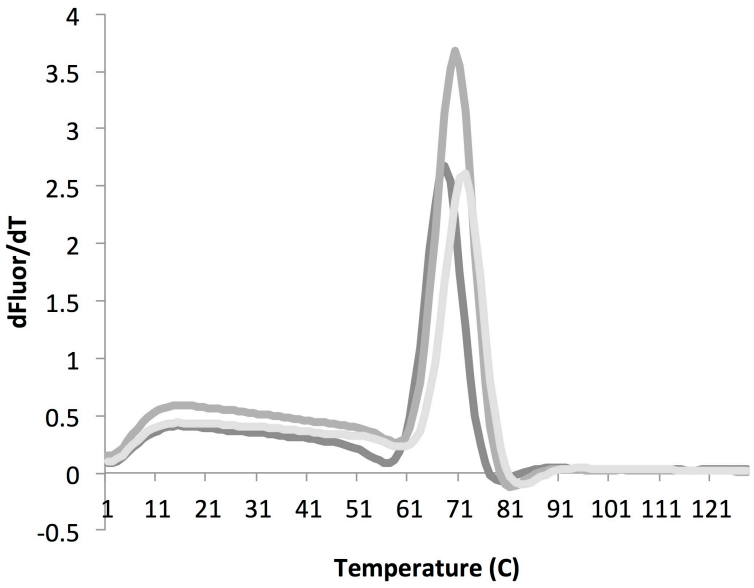

Figure C5: Max target peak heights show variability across biological. The graph above is the first derivative of the melt curve for three replicates of Gal4-EED/luc HEK293 cells treated with Cas9-sg32 and ssDNA template. Significant variability within the same treatment type was common among each treatment, making it difficult to identify small shifts in sequence length from editing. This prohibited effective cross-treatment comparisons. We hypothesize that these differences are due to the low general efficiency of HDR as compared to NHEJ, a competing pathway, which is simultaneously inducing indels and thus changes to the melt curve.

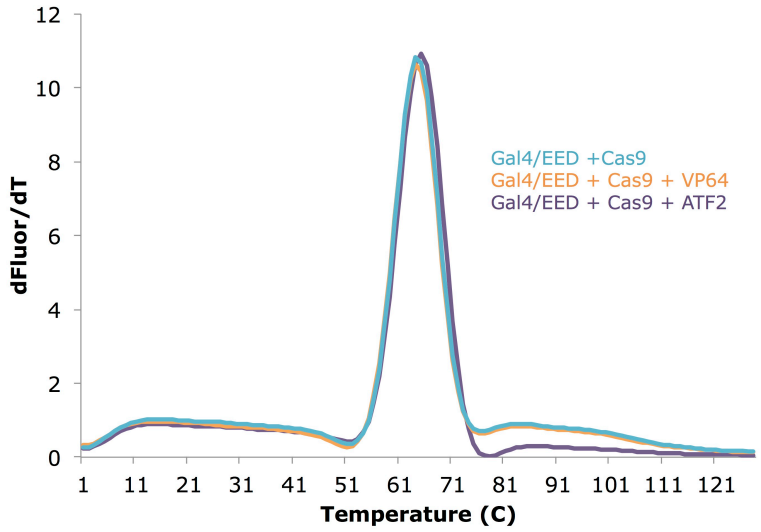

Figure C6: Diverse melting temperatures of differently edited sequences in a single sample creates challenges for cross-treatment comparison. The graph above is the first derivative of the melt curve for three distinct treatments of Gal4-EED/luc HEK293 cells with Cas9-sg32 and ssDNA template. While all three are different treatments, their maximum target peak height is nearly identical, most likely due to the variety of edited sequence types within each sample. Given the difficulty of peak height interpretation, we abandoned this method for cell sorting and Synthego ICE for the detection of Cas9-induced indels from NHEJ as opposed to HDR, which has a notoriously low efficiency.

APPENDIX D

PERMISSIONS AND AUTHORSHIP

D.1 Permission and Authorship for Chapter 1

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Authors: Cassandra M. Barrett and Karmella A. Haynes

D.2 Authorship, Acknowledgements, and Contributions for Chapter 2

Authorship: This chapter is currently under review for publication. Contributing authors to this work include (in order of contribution) Cassandra M. Barrett, Reilly McCracken, Jacob Elmer, and Karmella A. Haynes.

Acknowledgements: I thank C. Liu and T. Loveless for generously providing cells with a silenced CMV-GFP transgene, K. Rege and R. Niti for celastrol and C646, and R. Daer and D. Vargas for early efforts on this work.

Contributions: CMB completed all Gal4- and dCas9-fusion cloning, HEK293 cell culture, luciferase assays, flow cytometry, and PCR related to targeted fusion activators. CMB also carried out experimental design, STRING analysis, statistical analyses, and manuscript writing. JE designed and performed PC-3 transfections with enhancer motif-EF1a-luciferase constructs, and luciferase assays. RM cloned the enhancer motif-EF1a-luciferase constructs. KAH oversaw all work, finalized graphics for the figures, and assisted with manuscript preparation and submission.

D.3 Permissions for Chapter 3

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D.4 Contributions for Chapter 4

Contributions: I would like to acknowledge the contributions of my mentee Fatima Hamna to the statistical analyses and graphical representations of CRISPR edits presented in this chapter.