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Designing Epigenome Editors: Considerations of Biochemical and Locus Specificities

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Abstract

The advent of locus-specific protein recruitment technologies has enabled a new class of studies in chromatin biology. Epigenome editors enable biochemical modifications of chromatin at almost any specific endogenous locus. Their locus-specificity unlocks unique information including the functional roles of distinct modifications at specific genomic loci. Given the growing interest in using these tools for biological and translational studies, there are many specific design considerations depending on the scientific question or clinical need. Here we present and discuss important design considerations and challenges regarding the biochemical- and locus-specificities of epigenome editors. These include how to: account for the complex biochemical diversity of chromatin; control for potential interdependency of epigenome editors and their resultant modifications; avoid sequestration effects; quantify the locus specificity of epigenome editors; and improve locus-specificity by considering concentration, affinity, avidity, and sequestration effects.

Keywords

Epigenome engineering; DNA-binding domain; Chromatin modifiers; CRISPR; zinc finger proteins; transcription activator like effectors; locus specificity; biochemical specificity

1. Introduction

Over 60 distinct chromatin modifications have been identified in eukaryotic cells [1]. These modifications include post translational histone modifications and DNA methylation, and they provide an additional ‘layer’ of information widely referred to as the epigenome. Given its physical proximity to the genomic material, the epigenome has a strong regulatory influence on genome structure and function. However, although the efforts of many studies yielding genome scale epigenome maps have provided important biological insights [2, 3], the functional understanding of the epigenome in gene and genome regulation has remained largely correlative.

The advent of locus-specific protein recruitment technologies has unlocked a new class of studies in chromatin biology. Epigenome editors (EE) enable modification of chromatin at almost any endogenous locus allowing the dissection of the functional roles of distinct modifications in their specific genomic context. These new experimental tools are based on the fusion of DNA-binding domains (DBDs) with chromatin-modifying enzymes (CMEs), thus as a whole EEs are recruited to a specific genomic locus where they change the

chromatin modification state and potentially alter transcriptional activity (Figure 1). Given the growing interest in using these tools for both biological and translational studies, there are many specific design decisions that should be considered with particular regard to the biochemical- and locus-specificities of EEs.

2. Biochemical-specificity

2.1. Biological Considerations

2.1.1 Epigenome biochemistry exhibits complex natural diversity—The large biochemical diversity of modifications is an important characteristic of chromatin and a big challenge for the design of EEs [4, 5]. The fundamental unit of chromatin, the nucleosome, is composed of genomic DNA wound around an octamer of the four core histone proteins (H2A, H2B, H3 and H4). Numerous modifications decorate the N-terminal tails of histone proteins, including methylation, acetylation, phosphorylation, and ubiquitination [6, 7]. In addition, there are at least 6 different forms of DNA methylation [8–11]. A major goal over the past several decades has been to understand the functional roles of these modifications. Towards this goal, the identification of enzymes that direct these modifications has been the focus of many studies, and many of these enzymes can and have been used in designing EEs [12–15]. However, many CMEs are catalytically promiscuous and/or interact with other endogenous proteins that could have other regulatory properties. This confounds the ability to draw conclusions about the functional roles of specific chromatin modifications at the targeted locus. Improving and confirming the specific perturbations induced by EEs will improve the quality of biological interpretations and discoveries that can be made from this type of experiments [12].

2.1.2 Epigenome modifications exist in combinations—In addition to the numerical complexity of chromatin modifications, histone modifications and DNA methylation coexist in combinations, leading to theoretically very large numbers of chromatin states. Interestingly, several studies suggest only a small fraction (though still large overall number) of all possible combinations occur naturally and that these specific combinations are highly correlated with regulatory elements and their states such as ‘active promoters’ and ‘enhancers’ [12, 16–18]. Combinatorial chromatin states can serve as binding surfaces for other proteins, as several protein domains have been shown to recognize modified histones either alone or in combination [19–23]. Dissection of these complex states require more sophisticated editing tools that can induce multiple changes in chromatin state or respond to the presence of pre-existing modifications.

2.1.3 Epigenome modifications can be interdependent—The presence or absence of one modification can influence the placement of another histone modification. This crosstalk can enhance or reverse existing modifications, as seen in H3K4 methylation [24]. In their study, Sun and colleagues demonstrated that mono ubiquitination of histone H2B by Rad6 is required for H3K4 methylation by COMPASS (Complex of Proteins Associated with Set1, the yeast homolog of the mammalian MLL complex) [24]. A later study revealed that recruitment of Cps35, an essential subunit of COMPASS, to chromatin in the presence of H2B mono ubiquitination facilitates the recruitment and catalytic activity of COMPASS

in vivo [25]. These findings also show that histone modifications can control the order in which biological events, including additional histone modifications, occur. As other examples, association of HP1 with H3K9me3 is abolished with the phosphorylation of the neighboring Ser10 residue [26], while the demethylation of Arg2 or phosphorylation of Thr3 reduces the association of CHD1 with H3K4me3 (two-fold and 25-fold reduction respectively) [12].

A recent study by Cano-Rodriguez and colleagues used a combination of different CMEs to investigate the key events necessary for stable reactivation of silenced genes [27]. Their results showed that if there is H3K79 methylation and no (or low) DNA methylation present at the promoter, targeting the PDRM9 H3K4 methyltransferase is enough to achieve stable gene activation. In contrast, when promoter DNA methylation is present, gene activation induced by H3K4 methylation is only transient. In this situation, stable gene activation is improved when both PRDM9 (H3K4 methylation) and Dot1L (K79 methylation) are co-targeted in combination with DNA demethylation. Results of these studies underlie the importance of knowing the local chromatin environment at the target loci and point out that combinatorial epigenome editing techniques will be important to reveal the true functional interdependency of these modifications as well as their interactions with chromatin proteins and the surrounding DNA sequence [12, 27, 28].

2.2 Design Considerations and Methods to Assess and Improve Biochemical-Specificity

2.2.1 Using Core vs Full Length Proteins to Improve Biochemical-specificity—

Targeting full-length CMEs and transcriptional activators/repressors has been effective in inducing transcriptional and other functional changes. However, these approaches are likely to recruit multiple other proteins through binding interfaces on the CMEs and thus may exert broader changes to chromatin state than expected or desired. CMEs with improved biochemical-specificity are needed to more specifically link chromatin modifying activities with their specific functional roles [12].

One promising approach to improve the functional specificity of a CME is to truncate it to its core catalytic domain. As an example of this approach Hilton and colleagues showed that a nuclease-dead dCas9 fused with the core domain of p300, a histone acetyltransferase (HAT), is able to catalyze the acetylation of H3K27 at target promoter sites and increase gene activity more efficiently compared to a full length p300 fusion to dCas9 [29]. Removal of non-catalytic parts of a protein can also be used to minimize potential non-specific interactions. For example, Snowden and colleagues demonstrated that engineered zinc-finger (ZF) arrays fused to catalytic histone methyltransferase (HMT) domains of Suv39H1 or G9a can efficiently repress an endogenous promoter. In contrast, full-length Suv39H1 or G9a fusions to ZFs did not repress the promoter, potentially due to sequestration of the HMTs at other genomic locations through binding interactions of the full length proteins [30]. Several other related studies developed EEs by coupling catalytic domains of CMEs to ZFs [27, 29, 31–36], to transcription activator-like effectors (TALEs) [29, 37–39] and to dCas9 [27, 29]. However, an important point to note is that it is still unclear if using the minimal catalytic domains of CMEs improves specificity in all cases, and if they do so by removing other binding surfaces, increasing catalytic activity by reducing steric hindrances, or both. In

particular, likely due to cost and throughput limitations, few studies have comprehensively assessed changes in a full panel of chromatin modifications besides those that were originally targeted for editing.

2.2.2 Using Multiple Chromatin Modifying Enzymes to Increase Activity—

Targeting multiple chromatin modifying enzymes that work synergistically may allow self-sustained robust epigenome editing. Amabile and colleagues adopted a combinatorial strategy, in which they targeted the Kruppel-associated box (KRAB) domain, DNMT3A, and DNMT3L to an eGFP-expression cassette and endogenous loci [40]. Time-course flow cytometry analyses of the transduced cells showed that transient co-delivery of these three domains enabled long term silencing at repression levels which were not achievable by targeting individual editors [40].

A similar idea is to couple multiple subunits of CMEs with one DBD to increase chromatin modifying activity. For example, using ZFs or dCas9 to recruit a stable DNMT3A/DNMT3L single-chain fusion protein versus an individual DNMT3A catalytic domain to an endogenous promoter results in two to four fold higher methylation [36, 41]. This boost in chromatin modifying activity may arise from synergism between the domains rather than simply doubling the number of domains, as DNMT3L is known to stimulate the DNA methylation activity of DNMT3A [42].

2.2.3 Advanced Multiplexing Technologies—Earlier studies show that transcriptional output is strongly dependent on the number of transcriptional activators recruited to a promoter [43–45]. For example, gene activation is stronger when dCas9 fusions to transactivators are co-expressed with multiple gRNAs targeting the same promoter [46–50]. However, using multiple gRNAs may also increase the off-target binding activity of dCas9 yielding confounding results. An alternative approach is to use arrays of peptide or RNA sequences that bind multiple modifying protein domains to a single DBD.

The SunTag protein-tagging system enables protein multimerization on a single dCas9 while avoiding the use of multiple gRNAs [51]. The SunTag system is comprised of two components, a peptide repeat containing up to 24 copies of a short peptide epitope (GCN4) and a single-chain antibody (scFv) that recognizes the epitope. The antibody can be conjugated to either a fluorescent protein or a CME for imaging or gene expression studies, respectively. Several groups utilized this technology when designing EEs by coupling the peptide repeat with dCas9 and co-expressing it with an antibody-CME fusion [51–53]. For example, Morita and colleagues used this system to recruit multiple copies of the catalytic domain of ten-eleven translocation methylcytosine dioxygenase (TET1CD) which induces DNA demethylation at the target region. Their results showed increased demethylation activity when SunTag was used compared to direct fusions of TET1CD to dCas9 [52]. It is important to note that the linker length between the GCN4 peptide and the copy number in the peptide repeat should be optimized depending on the specific CME. Morita and colleagues' work also shows improvement in performance with longer linkers which can be explained by the reduction in steric hindrance between the scFV-TET1CD fusion proteins [52].

Another approach to increase transcriptional or chromatin modifying activities involves extended gRNAs to recruit multiple editor domains. This strategy features gRNAs that are modified to include protein-binding RNA hairpin loops such as MS2 and PP7 binding sequences [54, 55]. These hairpins can be bound by editor domains fused to MS2 or PP7 in homogenous or heterogeneous combinations [54, 55]. One example of this system is demonstrated by Konermann and colleagues where engineered RNA loops are used to recruit the RNA-binding protein MS2 fused with the p65-human heat shock factor 1 (HSF1) activator domain [56]. This allowed them to achieve robust single gRNA mediated gene upregulation.

2.2.4 Chromatin Immunoprecipitation to Determine Biochemical-specificity—

To date, most CMEs have not been comprehensively checked for their biochemical-specificity. While expensive and laborious, determination of the substrate specificity of a CME is critical to link specific modifications to functional roles. Methods that could be used to determine biochemical-specificity are discussed below.

Chromatin immunoprecipitation (ChIP) is a powerful technique for measuring the locus occupancy of histone modifications or other proteins that bind either directly or indirectly to DNA [57]. This technique starts with covalent stabilization of protein-DNA complexes achieved by *in situ* cross-linking. This step is followed by cell lysis, DNA fragmentation, and selective immunoprecipitation with specific antibodies. The final step in this assay is purification and analysis of the immunoprecipitated DNA by techniques such as qPCR and next generation sequencing [58].

It is important to note that the specificity and performance of antibodies can affect the validity of ChIP results. A recent study reported the specificity and utility of over 200 commercially provided antibodies against 57 different histone modifications [59]. Their results showed that among specific antibodies, over 20% failed in ChIP experiments. This study showed that there is substantial lot-to-lot differences in antibody performance, and that the epitope's species of origin also affects the performance of antibodies. An antibody validation website (<http://compbio.med.harvard.edu/antibodies/>) can be a useful tool when determining the performance of antibodies [59]. This database provides up-to-date validation information, including tests of batch-to-batch variability, and it allows researchers to post their assay results. One alternative to ChIP antibodies are recombinant epigenetic readers [60, 61]. For example, recombinant TAF3 PHD domain, a motif that reads H3K4me3 modifications [60, 62], may be used in lieu of anti-H3K4me3 antibodies in applications ranging from western blotting and ChIP-qPCR.

ChIP has been instrumental to our current knowledge of chromatin structure and function. However, because it typically measures chromatin states from bulk cell populations, it is unclear whether combinatorial states exist on individual nucleosomes or even single genomic loci. To address this limitation, recently reported combinatorial indexed ChIP (combinatorial-iChIP) maps the genome-wide co-occurrence of histone marks at single-nucleosome resolution [63].

2.2.5 *In vitro* Screening of Biochemical-specificity of CMEs—While economically impractically and laborious, fully checking the biochemical specificity of CMEs would involve repeating ChIP followed by qPCR (ChIP-qPCR) with many different histone antibodies to quantify the full array of modifications made to a specific locus. Due to the throughput and cost limitations of ChIP, a higher throughput alternative to screen for the biochemical specificities of CMEs is to produce CMEs recombinantly and mix them with histones *in vitro*. For example, Fingerman and colleagues developed an *in vitro* assay for HMT activity that uses bacterial cell extracts in which expression of a methyltransferase of interest is induced [64]. As a subsequent step to this protocol one could flow the histones over a glass slide that has multiple different anti-histone antibodies attached to it. One caveat of this method is that it is done outside of the context of a cell, does not take into account the context of different genomic loci, and is thus not representative of the natural chromatin environment.

2.2.6 Mutations to Reveal Catalytically Important Residues—Inactivating the catalytic activities of CMEs through point mutations can be used to reveal their biochemical-specificity. One can mutate the catalytic domain of a CME then check which histone modifications are present or absent using ChIP. If tractable, it would then be desirable to use many different histone antibodies to identify the comprehensive array of differences in histone modifications. For example, a single inactivating amino acid substitution within the p300 core domain abolished its transactivation capacity, demonstrating that the intrinsic p300 core HAT activity is crucial for gene transactivation [29]. Similarly, point mutations in key residues of the catalytic core of HMT Suv39H1 resulted in the loss of its catalytic activity and greatly reduced its ability to drive transcriptional repression of the target gene [30]. A similar approach was also employed by Stepper and colleagues where they used a DNMT3A/DNMT3L single-chain fusion protein to achieve efficient and widespread DNA methylation. To test if the multimerization of DNMT3A has a role in targeted DNA methylation, they used DNMT3A mutants with a catalytic core mutation, a polymerization interface mutation, or both. While the mutants with the catalytic core mutation did not methylate the target loci at all, the mutant with polymerization interface mutation showed decreased DNA methylation activity by ~2-fold *in vitro* which is in agreement with the role of multimerization for DNA methylation activity [41].

2.3 Future Work

Given that many chromatin modifications are structurally similar (i.e. methylation of different lysine residues), engineering CMEs that specifically catalyze only one modification may be challenging. Furthermore, proteins have many binding surfaces and interact with many other proteins and RNAs that could in some cases promote biochemical-specificity of the catalytic enzyme or recruit proteins with other confounding activities. These interactions are lost when only catalytic cores are used. **One approach** to address these challenges could be to rely on natural biological mechanisms by recruiting specific protein domains that provide desired binding surfaces to protein complexes known to specifically induce desired chromatin states. This could potentially decrease the off-target histone catalytic activity of CMEs and increase their biochemical-specificity. However, since additional cellular components also play a role in this strategy, these systems may not work in all cell types and

across species. Another tradeoff is that these proteins could be sequestered to other regions of the genome through their binding surfaces, or recruit other undesired proteins and RNAs. An interface of biochemistry, molecular biology, and bioinformatics expertise will be needed to address these challenges. Ultimately, the characterization, discovery, and re-engineering of new CMEs will expand the tool set available to design advanced EEs with high functional specificity [12, 20, 65–68].

One should also note that it is often difficult to compare the data from *in vitro* vs *in situ* (cellular) experiments. This can be attributed to the lack of control in plasmid concentrations, the presence of other proteins, and differences in chromatin state and salt concentrations in *in situ* compared to *in vitro* studies. Development of cell-free experimental systems that mimic the intracellular chromatin more accurately can address some of these challenges. In addition, future experiments are needed to investigate the effects of CME concentrations on non-specific modifications.

A goal of many researchers in the field is to use EEs in therapeutic applications. Towards this goal, it is important to understand the basic functional roles of numerous chromatin modifications in different contexts and combinations. Most pertinently, how specific is specific enough for safe therapies and clinical applications? Will undesired, non-specific chromatin modifications lead to dangerous signaling events or give rise to other disease states like cancer? [3] In some cases where the benefits outweigh the risks, such as many cancers, non-specific modifications may be acceptable. For example, there are already small molecule drugs that lead to genome-wide changes in chromatin state approved for such diseases. If effective therapeutic delivery systems for EEs can be engineered, it is exciting to speculate how EEs may provide enhanced safety and efficacy over current treatments.

3. Locus-specificity

3.1 Biological Considerations

3.1.1 Off-Target Binding—Given that the target for a DBD is usually a unique site among ~3 billion base pairs (in the human genome), off-target binding is a potential challenge. Furthermore, the CMEs themselves may have interfaces that can bind to other chromatin-bound proteins elsewhere in the epigenome. Off-target binding could lead to pleiotropic effects in biological studies or to toxicity in therapeutic applications, and has been a concern in DNA-editing applications [69–75]. The use of EEs could have similar concerns; however, it is arguable that less binding specificity may be needed for EEs compared to genome editors as EEs are not making irreversible changes to the genome sequence (mutations). Some evidence suggests the potential advantages of using EEs when appropriate. For example, a recent RNA-seq experiment by Polstein and colleagues demonstrated that both TALE-VP64 and dCas9-VP64 transcriptional activators exhibit exceptional levels of specificity of activating only targeted genes [76]. This result does not necessarily indicate the EEs are not binding to off-target sites, but perhaps that off-target binding may not be stable enough or at high enough occupancy to affect the functional outcome of gene activation. In addition, the off-target binding site may be far from any gene or regulatory element. Synergy in gene regulation resulting from the recruitment of multiple EEs could also provide additional specificity, much like split nucleases have enhanced specificity for

genome editors [46, 47, 77]. Despite these promising results, off-target binding should continually be assessed and studied as new EEs are developed. For example, it remains unclear how fusions to different DBDs might influence off-target binding. Previous studies suggest that the addition of a KRAB domain to a ZF can greatly increase the ZF's off-target interactions [70]. This is in contrast to other studies that did not find evidence that addition of the KRAB domain to dCas9 increased off-target binding [78, 79]. Why these differences are observed is both intriguing and important to understand in informing the choice of DBDs in different applications.

3.1.2 Fusion-dependent changes of DBD and CME activities—In addition to changes in off-target binding upon addition of editor domains to DBDs, a number of studies reported different activity levels when the same editor is fused to different DBDs [76, 80]. Polstein and colleagues performed a genome-wide analysis of DNA binding and gene regulation using ChIP-seq and RNA-seq, respectively, for both TALE-VP64 and dCas9-VP64 transcriptional activators [76]. Comparisons between eight TALEs and eight gRNAs-dCas9 pairings suggested that these two DBDs have similar genome-wide specificity. Their results also show that activation with TALE-VP64 was substantially greater than activation with dCas9-VP64 which is consistent with previous studies [46, 47, 80–82]. However, it is important to consider that the expression level, protein stability, and steric interactions between CMEs and different DBDs they are fused to may differ considerably, and it is difficult to make direct conclusions from comparisons of DBD systems. In particular, the cell type, CME, and expression system may all influence EE activities and specificities and are important design criteria to consider.

3.1.3 DNA Accessibility—The positioning of histone proteins, transcription factors and DNA modifications on the genome as well as chromatin compaction mediates the accessibility of DBDs to DNA [83]. Although initial evidence suggests that modifications such as DNA-methylation does not inhibit Cas9 binding [84], recent work shows chromatin modifications influence Cas9 accessibility particularly when CpG containing sites are targeted [27, 85]. One should also note that DNA accessibility changes with cell type [17, 27, 80]. DNA hypersensitivity sites could therefore be consulted when choosing locations to target with DBDs.

Recent work also suggest that local chromatin structure may relax in response to binding of individual DBDs making it more accessible to other DBDs [76, 80]. Garcia-Bloj and colleagues showed that activating silenced genes is possible by recruit EEs with different DBDs in combination. This could be explained by one EE remodeling the local chromatin configuration thus facilitating the binding of the second EE [80]. Future experiments are necessary to understand the precise mechanisms by which different DBDs access chromatin. More detailed analysis of the specificity and impact of chromatin state on the binding and function of DBDs is also critical to their further development and to the interpretation of results obtained with these systems.

3.2 Design Considerations and Methods to Assess and Improve Locus-Specificity

3.2.1 Binding Affinity and Concentration Effects on Locus-specificity—The specificity of an interaction refers to the relative strength of the interactions between one protein and its target ligand compared to alternative ligands (in this case non-specific genomic binding sites). In a highly specific interaction the free energy change upon binding to a target ligand is much greater than that for other ligands [86]. However, in the context of genome binding where there are limited (often just one) target site, concentration effects can have an outsized role on non-specific binding since the target site will become saturated very quickly, and the concentration of unbound protein may be quite high yielding noticeable binding to off-target sites. Thus, reducing the DBD concentration could be applied as a potential strategy to decrease off-target DNA (ligand) binding activities.

Recent studies applying this approach with targeted genome editing nucleases have yielded promising, though in some cases mixed, results. Pattanayak and colleagues demonstrated that decreasing ZF nuclease (ZFN) concentrations resulted in fewer off-target edits [74], and another study by Guilinger and colleagues concluded that high TALE nuclease (TALEN) concentrations increase off-target activity of this domain [73]. Two recent *in vivo* studies used different plasmid concentrations of Cas9 and gRNAs to investigate this strategy. Fu and colleagues found that transfecting lower amounts of gRNA and Cas9-expressing plasmids decreased the mutation rate at the on-target site, but did not appreciably change the relative rates of off-target mutations [87]. In contrast, Hsu and colleagues reported that they can minimize the level of off-target activity by titrating the amount of Cas9 and gRNA delivered [84]. Collectively, the results of these studies show that decreasing the concentration of editors could provide a simple solution to reduce off-target effects. It will therefore be important to consider how DBDs are being delivered, as standard plasmid transfection or lentiviral transduction can result in expression levels beyond those needed for a desired biological effect [88]. Delivering the editor as a ribonucleoprotein complex [89–93] could allow for easier control of its cellular concentrations [88].

An important point to note is the examples discussed in this section are focused on concentration effects when targeting nucleases rather than EEs. A number of recent studies strongly suggest that the binding specificity of dCas9 is different from the nuclease specificity of Cas9 [75, 78, 94, 95] and varies with the amount of dCas9 protein [78]. Concentration effects on the off-target activities of dCas9, TALE and ZF based EEs should be a topic of future studies. In particular, methods for inducible control of EEs enable their titration and may be useful to study concentration effects [81, 96–98].

3.2.2 Using Obligate Pairs of Chromatin Modifying Enzyme Domains to Improve Locus-Specificity—Decreasing the off-target activity of a DBD may also be possible by using obligate pairs of CMEs. In this strategy, CME function depends on co-recruitment of two DBDs fused with catalytically incompetent pairs of CMEs (or two halves of a split CME). Reconstitution and assembly of a full CME is achieved upon binding of both DBDs to adjacent loci. These co-dependent EEs can potentially increase the specificity of CME activity by requiring the binding of two distinct target sequences at the same locus. As examples of this approach, two studies targeted ZF fusions to DNA methyltransferase

fragments [99, 100]. These studies showed that this strategy can yield a methyltransferase capable of significant levels of methylation at the target site with undetectable levels of methylation at non-target sites in *Escherichia coli*. It should be noted that this is a promising but challenging task as it requires complex protein engineering of CMEs, and the number of possible off-target regions is substantially greater in the human compared to bacterial genome. Directed evolution and screening/selection methods may be useful when designing split proteins.

3.2.3 Computational Selection of gRNAs for dCas9 Targeting—Computational algorithms based on guide-sequence similarity are also useful tools to identify potential off-target sites [88]. Recent algorithms incorporate a scoring function in which the position and type of mismatch are used to rank potential off-target sites [101–110]. Guide RNA sequences with low-probability of off-target activity can be determined using these computational techniques. Improving the specificity of the predictions of these algorithms is a subject of future research, and more data on genome-wide off-target activities of DBDs will help inform and refine these computational methods [88].

3.2.4 Altering or Removing the Non-Specific Binding Activity of the CME to Enhance Locus-specificity—In addition to engineering the DBD, the locus-specificity of an EE can also be enhanced by modifying the CME that it is fused to. For example, CMEs may have domains that interact with DNA or other proteins located at off-target loci [70]. This sequestering effect can be mitigated by fusing only the core catalytic domains of CMEs to DBDs, or by mutating residues in CMEs to reduce its intrinsic interactions with other binding partners. In addition, off-target chromatin modifications could be reduced without altering the binding profile of CMEs, by attenuating the CME's catalytic activity. For example, ZF fusions of mutated DNA methyltransferases with reduced catalytic activity significantly reduced off-target methyltransferase activity [111, 112]. This method requires confirmation that sufficient catalytic activity is maintained at the target locus.

3.2.5 Reducing overall DNA-binding energies to Reduce Non-specific Binding—Reducing the overall DNA-binding energies of ZFs and TALEs is shown to improve their locus-specificity. Thermodynamically, this may be due to the fact that there is only one target site while there are usually many copies of the DBD protein. Thus, at equilibrium the target site may remain saturated with the DBD even if the binding strength is reduced, while off-target genomic sites with lower binding affinities to the DBD would exhibit reduced equilibrium occupancies. Lowering the overall DNA-binding energy of a DBD can be achieved by altering the length of its DNA recognition sequence [73, 74]. Guilinger and colleagues constructed TALE nucleases (TALEN) targeting 10, 13, or 16 base pairs, and they subjected them to *in vitro* selection. Their results revealed that shorter TALENs have greater cleavage specificity, whereas longer TALENs have reduced specificity per nucleotide. Longer TALENs tend to cleave sequences with more mismatches. It was hypothesized that these longer TALENs may have a greater overall affinity than is required to bind and cleave the target site and this “extra” affinity can lead to off-target binding. However, a tradeoff arises as the target sequence drops in length since the frequency with which shorter sequences appear in the human genome is greater than for longer sequences.

Thus, TALENs targeting longer sequences are estimated to have higher overall specificity in the context of the human genome than ones that target shorter sites [73]. A similar study done by Pattanayak and colleagues suggest that longer ZF nucleases (ZFN) exhibit a higher tolerance of partially mismatched sequences [74]. These studies show that excess DNA-binding energy results in increased off-target ZFN and TALEN cleavage activity. An important note is that these studies focus on nuclease activity rather than epigenome editing, and further studies are necessary to assess how closely specificity compares when DBDs are fused with CMEs.

3.2.6 Truncated gRNAs Increase Locus-Specificity in Cas9 Applications—Fu and colleagues demonstrated that the off-target nuclease activity of Cas9 can be significantly mitigated by shortening the gRNA [113]. They used gRNAs shortened by 2 or 3 bases (17 or 18 nucleotides of complementarity) at the 5' end of their complementarity regions, and demonstrated that these gRNAs function as efficiently as full-length gRNAs, but showed decreased mutagenic effects at off-target sites. Interestingly, while the use of modified gRNAs with truncated 3' ends also yielded better on- to off-target ratios, the efficiency of on-target cleavage was considerably lower than for 5' truncated gRNAs [114, 115]. This can be attributed to the discovery that the 8–12 bp at the 3' end of the targeting sequence are crucial for target recognition [116, 117], and mismatches are better tolerated at the 5' end of the gRNA targeting region [84, 87, 118]. Using truncated gRNAs is a promising approach to reduce the likelihood of off-target dCas9-binding in epigenome editing proteins. However as mentioned earlier in this chapter, the binding specificity of dCas9 fusion proteins have been shown to be different from the cleavage specificity of Cas9 nuclease [75, 78, 94, 95]. Thus, future experiments are necessary to characterize the off-target binding activities and chromatin-modifying activities of dCas9-CME fusions with truncated gRNAs.

3.2.7 Structure guided alterations to DBDs to Reduce Non-Specific Binding—DBDs have interactions with DNA that are not specific to their target sequence. Structure guided approaches have been applied to reduce the binding energies of these interactions to reduce off-target binding. For example, it has been suggested that the Cas9-gRNA complex may possess more binding energy than is needed for recognition of its intended target DNA site leading it to cleave off-target sites [113]. Thus, a Cas9 nuclease with enhanced locus-specificity (eCas9) was recently developed using structure-guided protein engineering approaches that lowered the binding energy of the Cas9-gRNA complex [119, 120]. These eCas9 domains were engineered by neutralizing mutations of positively charged residues that normally provide binding to the non-target (not gRNA-bound) DNA strand. These mutations weaken eCas9 binding to the non-target strand allowing the non-target and target DNA strands to re-hybridize more easily. The results of these studies demonstrate a significant reduction in genome wide off-target nuclease activity due to the increased dependence on the binding energy provided by the Watson-Crick base pairing between the gRNA and the target strand [119, 121]. In addition, eCas9 is reported to have comparable on-target cleavage activity with wild type Cas9. This feature could make this new system superior to other strategies to improve Cas9 specificity, because it increases specificity without sacrificing efficiency, although any impact on epigenome modifications should be confirmed [121]. Applicability of this elegant approach to dCas9 binding in epigenome

editing studies needs to be investigated by future studies. An interesting note is that use of truncated gRNAs resulted in substantially lower on-target nuclease efficiencies with the eCas9 system which is not surprising given the reduced gRNA length [120, 121].

A similar approach could be useful with other DBDs including ZFs. For example, mutations can be made to reduce the binding of ZFs with the DNA phosphate backbone. This strategy could allow a longer array of ZFs to be created with a long enough sequence to be unique in the mammalian genome but with low enough overall binding energy to minimize off-target binding [122].

3.2.8 ChIP-Seq to Determine Locus-Specificities of EEs—As discussed earlier in this chapter, confirming the locus-specificities of EEs increases confidence in relating EE-induced chromatin modifications at specific loci to their functional roles. The most reliable assessments of the locus-specificities of DBDs use methods that do not presume the off-target site sequence is closely related to the sequence of the target site (unbiased methods) [75, 121]. Chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) is a powerful tool to identify genome-wide binding locations of proteins in an unbiased manner [123]. ChIP-seq results from recent studies suggest that dCas9 can bind a higher number of off-target sites than previously anticipated [71, 94]. Kuscu and colleagues confirmed that depending on the gRNA used gRNA:dCas9 complexes can have tens to thousands of off-target binding sites [71]. While the PAM-proximal sequence was more strongly conserved with the gRNA, some of the dCas9 binding sites differed overall by up to 9 mismatches, more than the 3–5 mismatches that Cas9 catalytic activity has been shown to be tolerant to [87, 114, 118, 124–127]. This indicates that dCas9 binding may be substantially more promiscuous than Cas9 catalytic activity. Furthermore, dCas9 binding was found to be biased towards DNase I hypersensitive sites ('open' chromatin). Thus, these studies suggest that applications based on dCas9 or dCas9-CME fusions, such as transcription modulation, imaging, and epigenome editing, could be complicated by the promiscuity and biased targeting of dCas9 binding.

3.2.9 Mismatch Reporter Assays—One other high-throughput method to detect dCas9 binding sites is based on the dCas9-mediated activation of a reporter [128]. This method is known as the mismatch reporter assay [121]. In this assay, a library of off-target sequences is cloned upstream of a promoter driving expression of correspondingly barcoded RNAs. dCas9 is fused to a transcription activator or repressor. The extent to which potential off-target sites are bound is measured by dCas9-mediated activation or repression of the promoter as determined by RNA sequencing (RNA-seq) [121]. This method can also be applied to other DBDs, such as TALEs and ZFs [128] and has also been used to identify proto-spacer adjacent motif sequences for other CRISPR systems [129].

3.2.10 Future Prospects for Epigenome Editors—Moving forward, advancements in both enhancing and quantifying the locus-specificity of epigenome editors are needed. Common methods applied to determine the specificities of nucleases, such as surveyor assays and whole genome sequencing [130, 131], could also be useful to detect the binding sites of DBDs. However, currently they can only act as a helpful surrogate for DBD-CME fusions, as specificities of EEs are based on the recruitment of non-nuclease, chromatin-

modifying activities to a specific locus. In addition, the field will benefit from more comprehensive data measuring both changes in panels of chromatin modifications and genome-wide DNA-binding profiles of these editors. Furthermore, while ChIP will likely remain the main method to interrogate the binding position of DBDs, alternative methods such as DNA adenine methyltransferase identification assay (DamID) could be used to validate results, especially given some of the problems associated with ChIP reagents, i.e. variable quality of antibodies and bias introduced by fixation and DNA fragmentation [5, 132, 133]. DamID is a method for mapping the distribution of chromatin-associated proteins by fusing a protein of interest with *E. coli* Dam, thus methylating GATC adenines near the binding site of the DBD protein. As adenine modification is absent or present at only very low levels in most eukaryotes [134–136] this approach can assay binding events without the need for specific antibodies [5, 133].

In addition to methods to measure locus-specificity, there are many strategies that could be employed to enhance specificity as well. For example, incorporation of experimental observations with existing computational models could lead to the development of improved algorithms and tools with better predictive power of potential off-target sites [137]. Another subject for future research could be continued investigations into the effects of core vs full-length proteins or split CMEs on locus and biochemical specificity. We discussed two example studies [111, 112] that applied a similar approach with ZF DNA-methyltransferases fusions. Future work should investigate this potential using different DBDs and CMEs.

In this chapter, we discussed design considerations and challenges regarding biochemical- and locus-specificities of EEs. Given the broad importance of chromatin in most cellular processes and many diseases, continued work in enhancing the predictability and precision of controlled chromatin modifications will have a major impact on research and human health.

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Figure 1.

Epigenome editors (EEs) are engineered by fusing chromatin-modifying enzymes (CMEs) to DNA-binding domains (DBDs). They enable biochemical modification of chromatin at almost any specific endogenous locus. **1)** The majority of CMEs are known to have binding domains that allows them to interact with other proteins that may affect its biochemical-specificity. Engineering CMEs that specifically catalyze only one modification and that avoids or tightly controls interactions with other proteins is important to improve biochemical-specificity. **2)** While EEs allow the interrogation of chromatin context and function, binding to off-target loci could raise concerns about pleiotropy in biological studies or about safety in therapeutic applications. Locus-specificity is an important feature that needs to be considered when designing these tools.