Trends in Genetics

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Review

Live-Animal Epigenome Editing: Convergence of Novel Techniques

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Epigenome editing refers to the generation of precise chromatin alterations and their effects on gene expression and cell biology. Until recently, much of the efforts in epigenome editing were limited to tissue culture models of disease. However, the convergence of techniques from different fields including mammalian genetics, virology, and CRISPR engineering is advancing epigenome editing into a new era. Researchers are increasingly embracing the use of multicellular model organisms to test the role of specific chromatin alterations in development and disease. The challenge of successful live-animal epigenomic editing will depend on a well-informed foundation of the current methodologies for cell-specific delivery and editing accuracy. Here we review the opportunities for basic research and therapeutic applications.

Highlights

Epigenome editing and preclinical therapies can now be performed in live animals.

Epigenome editing is mediated by effector domains fused to the editing tools to mediate transcriptional activation, repression, and long-range chromatin alterations.

CRISPR technology, zinc fingers, and transcription activator-like effectors (TALEs) each offer unique advantages in live-animal epigenomic editing.

Delivery form (viral, cell implant, transgene, or macromolecular assemblies) and route (intracranial, intravenous, intrathecal, intraperitoneal) are two of the biggest challenges to successful epigenome research in live animals.

The Prospect of Live-Animal Epigenomic Editing

Targeted alteration of chromatin and gene transcription in mammalian cells is a rapidly evolving field of research. Much effort in epigenome editing has focused on understanding the role of chromatin in cell function using tissue culture models. The contribution of this work, in conjunction with human epigenome sequencing studies, has reinforced the functional role of chromatin in normal development and disease. However, the frontier is shifting to live-animal studies. The ability to precisely and accurately edit chromatin structure in multicellular organisms is becoming the system of choice to answer questions in biology and explore new avenues for therapeutic intervention. Such avenues of investigation will greatly advance our understanding of many areas in biology including aging, neurological function, cancer development, circadian biology, and multiorgan-level functions.

This review of the seminal studies in live-animal epigenetic editing focuses on **CRISPR/dCas9** (see Glossary) technology but also touches on the other two well-known genome targeting platforms: **zinc fingers (ZFs)** and **transcription activator-like effectors (TALEs)**. It will also discuss the effector domains that can be fused to these platforms to mediate transcriptional activation, repression, and long-range chromatin alterations. Considering that one of the biggest challenges to successful epigenome research in live animals is the delivery of the therapeutic agent, the choice of delivery form (viral, cell implant, transgene, or macromolecular assemblies) and the delivery route (intracranial, intravenous, intrathecal, intraperitoneal) is a particular focus. In addition, the prospect for basic biology research and therapeutic development is also discussed.

CRISPR-Based Epigenome Editing Technologies: Overview and Design Principles

Unlike gene editing, the goal of epigenome editing is to alter transcription without altering DNA sequences. Nuclease-based gene editing relies on the cellular repair of double-strand breaks, which can lead to unintended consequences such as chromosomal rearrangements [1,2], non-contiguous repair patches [3], integration of normally episomal viral vectors [3,4], apoptosis of

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damage-sensitive cells, and selection of clones with impaired sensitivity to damage [3–6]. By contrast, altering epigenetic information is the primary method used by cells to cause long-term changes in the expression of their genes. To manually perform such alterations at specific genes, ZFs, TALEs, and the rapidly advancing CRISPR/dCas system can be used (Box 1). In epigenome editing, the catalytically dead nuclease (dCas9) has been the major player allowing precise binding without cleavage. Epigenome editors are developed by fusing genome targeting platforms to domains that function as **writers** or **erasers** of epigenetic modifications associated with activation or repression of gene transcription. Of the three available platforms, CRISPR has become the preferred form for genome [7] and epigenome [8,9] editing.

CRISPR is the platform of choice for technology development because of the ease of its two-component, RNA-programmable genome targeting feature [10]. CRISPR/dCas9 epigenome editing technologies fall into four non-mutually exclusive classes based on their primary biochemical action on chromatin (Figure 1). The first class of epigenome editors is those whose primary function is to alter chromatin topology and long-range chromosomal interactions. Members of this class include **artificial proximity dimerizers** fused to dCas9 [11], which can bring together distal sites in a chromosome, facilitate interchromosomal interaction, and localize loci to specific nuclear suborganelles. The second class of epigenome editors is nucleosome remodelers, which

Box 1. Design Parameters for Epigenome Editing

Recruitment Designs and Current Tools

Two major design parameters affect the engineering of CRISPR/dCas9 epigenome platforms: recruitment and source. The recruitment parameter deals with the mode by which dCas9 enables localization of the epigenome editing machinery to the site of interest on DNA. Localization can be mediated either by direct covalent attachment to dCas9 or through noncovalent protein–protein or protein–RNA interactions. For live-animal studies, recruitment by noncovalent attachment may allow the multimerization of epigenomic editing enzymes at specific loci, increased complexity of the epigenome machinery, and packaging into multiple viral vectors. The second design parameter deals with the source of the epigenome machinery: exogenous or endogenous. Exogenous design platforms are those in which a catalytic or functional domain is engineered into the transgene. Endogenous design platforms are those in which cellularly encoded enzymes and chromatin organizers are relocalized to specific loci. The exogenous design platforms engineered into CRISPR include histone methyltransferases and demethylases: EZH2, G9A, SUV39H1 [13], DOT1L, PRDM9 [65], SMYD3 [66], MLL3SET [67] and LSD1 [68], histone acetyltransferase P300 [69], and CBP [70]. Examples of endogenous design platforms by recruiting peptide are KRAB [14], HP1a [12], SID [71], FOG [13], C-terminal-MECP2 [72], VP64 [14], P65 [14], Rta [17], and HSF1 [15]. Together these epigenetic editing tools offer many options to alter the epigenome in live animals for locus-specific biochemistry. The choice of recruitment modality and source of epigenetic machinery has a significant impact on the functionality of the epigenome editing system.

Future Applications of Other Nucleases

Novel type of nuclease were introduced for RNA editing by separate teams led by Doudna [73] and Zhang. The latter team showed that a catalytically inactive Cas13b ortholog from *Prevotella* sp. is the most efficient and specific for mammalian cell applications [74]. The deactivated variant of Cas13b was fused to the ADAR2 deaminase domain, which participates in the conversion of adenosine to inosine. This system was able to recognize a specific target sequence, while the ADAR2 element performed the base conversion. These studies demonstrate an excellent new platform for precise RNA editing with broad applicability, and since then other, smaller orthologs with robust activity in human cells have been characterized [75]. In the case of large proteins like Cas9, smaller orthologs have now been reported to show equal editing efficiency [42,76,77], which is especially useful for AAV packaging.

Maximizing the Packing Size of AAVs

Given that several of the epigenetic tools, including some of the more commonly used nucleases, exceed the 3.5-kB AAV-packaging limit, there have been several studies addressing the limiting packaging size. For the CRISPR/dCas system, smaller dCas9 orthologs [42,77,78], a split-dCas9 system [79], and a dual-AAV system [32,80] have been suggested. Future studies will show whether dual-AAV systems are as efficient in transducing target cells as the generation of cell-specific AAV capsid libraries (for a recent review on AAV dual systems, see [80]).

Glossary

Artificial proximity dimerizers: chemical inducers of dimerization that are designed to bind to two different proteins and bring them into close proximity in the presence of the dimerizer.
CRISPR/Cas: identified in bacteria as an antiviral defense strategy; now utilized as a technology for gene editing, which includes an enzyme called a nuclease and a guide RNA that allows targeting of practically any sequence in the genome for genetic or epigenetic editing.
Erasers (epigenetic): epigenetic marks can be removed by a group of enzymes called 'erasers' that can reverse the influence of an epigenetic mark on gene expression.
HSF1: a potent transactivator peptide known for recruiting endogenous transcriptional activation cellular machinery; derived from a conserved fragment of mammalian heat shock protein 1.
Knock-in mouse strain: a mouse strain in which a transgene has been inserted at a specific site in the genome as a single-copy integration; traditionally constructed by introducing foreign DNA (transgene) into embryonic stem cells by homologous recombination. Edited embryonic stem cells are then injected into a blastocyst and embryos are brought to term. Mosaic founders are tested for germline transmission of the transgene.
Krüppel-associated box (KRAB): the KRAB domain is potent transcriptional repressor. The peptide is highly conserved across species and is known to interact with the KAP1 complex to mediate trimethylation of lysine 9 of histone 3.
P65: a potent transactivator peptide known for interacting with NF-κB and mediating transcriptional activation; derived from a conserved fragment of the mammalian RELA gene.
SunTag system: a repeating peptide array that can recruit multiple copies of an antibody-fusion protein for signal amplification.
Rosa26: a specific site/locus in the mouse genome that is widely used to achieve generalized expression in the mouse and has been used in generating over 100 knock-in cell lines. A human homolog of the mouse Rosa26 locus was identified later and has been used to generate embryonic stem cell lines.

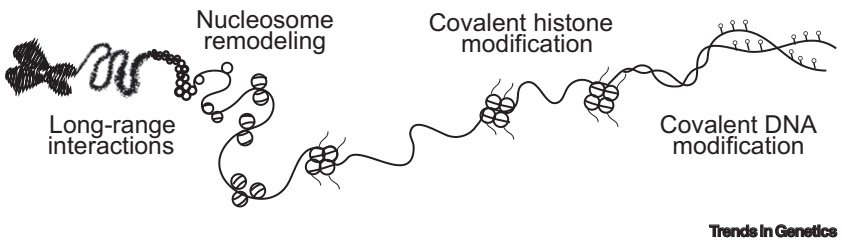

Figure 1. Classes of CRISPR/dCas9 Epigenome Modifications. CRISPR-based epigenome editors mediate four distinct classes of modifications on chromatin based on the domains utilized. Long-range interactions between different loci and nuclear compartments are facilitated by proximity interacting domains. Nucleosome remodeling is regulated by enzymatic domains that mediate the arrangement of nucleosomes on DNA. Covalent histone modifications are catalyzed by enzymatic domains that target amino acids on histones. Covalent DNA modifications are catalyzed by enzymatic domains that add or remove methyl groups from cytosine.

can result in nucleosome repositioning [12]. The third class of editors, and the most documented, result in covalent modifications on histones including changes in methylation and acetylation [13]. This class also includes dCas9 recombinant proteins fused to peptides that recruit cellular histone-targeting enzymes [14,15]. The fourth class of epigenome editors is those that covalently modify DNA through cytosine methylation [16]. Together these classes of epigenome editors can mediate a larger set of biochemical actions on chromatin that results in enhanced transcription or repression of target loci (Table 1).

The effects of epigenome editors on transcription depend largely on the catalytic domains engineered into the CRISPR/dCas9 platform or the intermolecular interactions mediated by specific domains. For gene activation, dCas9 has been used to induce several modifications on histones and DNA associated with RNA polymerase transcription (Figure 2, left panel). For gene repression, several modifications on histones and DNA have also been deposited by dCas9 epigenome editors (Figure 2, right panel). For live-animal epigenome editing studies, several of these editors have been applied, including the use of the **Krüppel-associated box (KRAB)** domain for repression [14,15] and the **VP64** [14], **P65** [14], **Rta** [17], **HSF1** [15] ,and VP64-P65-Rta (VPR) [14,17] domains for activation [18]. These applications also include the use of the **SunTag system**, which is based on an epitope array-linked dCas9 for the recruitment of recombinant proteins fused with an intracellular single-chain antibody (scFv). Important considerations for live-animal studies include whether the target tissues possess the endogenous machinery for epigenetic editing or whether dosage effects will confound experimental or therapeutic goals. Our discussion of the literature on live-animal studies is guided by the mode of delivery: cell implantation, viral delivery, purified ribonucleic complexes, and/or transgenesis.

Live-Animal Epigenome Editing by Cell Implantation

One of the most technically feasible approaches to investigate the effect of epigenetically edited genes in multicellular organisms is through cell implantation models. This approach provides a useful experimental link between tissue culture and live-animal studies. It has the added advantage that human cells can be epigenetically edited and its consequence monitored in more physiologically relevant environments.

Three recent studies illustrate the application of this approach in understanding cancer biology and neuronal function (Table 2). In one of the first studies involving live-animal epigenetic reprogramming, TALEs were used to alter the expression of genes involved in hematopoiesis [19]. Specifically, in this study TALE binding arrays were fused to KRAB to silence c-kit and PU.1 in mouse bone marrow (BM) stem cells. After generating stable cells encoding the TALE-KRAB protein, the researchers introduced the cells into mice and monitored BM repopulation.

Rta: a potent transactivator peptide known for interacting with OCT1 and SP1 in mediating transcription activation; derived from a fragment of the R transactivator protein of Epstein–Barr virus.
Transcription activator-like effectors (TALEs): proteins that can be designed to modulate gene transcription. These proteins recognize and bind to DNA sequences based on a variable number of tandem repeats.
Transgenic mouse strain: a mouse strain in which a transgene has been inserted at random sites in the genome as either single-copy or multicopy integration; traditionally constructed by introducing foreign DNA (transgene) into single-cell fertilized eggs and screening founders for transgene integration.
VP64: a potent transactivator peptide known for recruiting endogenous transcriptional activation cellular machinery; comprises four copies of a fragment from viral protein 16 (VP16), which is derived from herpes simplex virus.
Writers (epigenetic): a group of enzymes that catalyze the addition of chemical groups onto either histone tails or the DNA itself, which affect gene expression and are also known as epigenetic marks.
Zinc fingers (ZFs): engineered proteins that behave like transcription factors with the ability of each finger module to recognize three or four bases of sequence; by mixing and matching those modules, almost any sequence can be targeted.

Table 1. *Ex Vivo* Study Summary of Various Cas Protein Modular Systems for Epigenetic Editing

Cas protein	Modular system	Delivery form and route	Gene targeted	Comment	Refs
dCas9Sp and dCas9Sa	CLOuD9 technology – dCas9 fused to induced proximity system that utilizes the plant phytohormone *S*-(+)-abscisic acid (ABA) and modified components of the plant ABA signaling pathway	Lentiviral transduction of K562 cells	β-Globin gene locus control region which regulates the expression of the distant β-like globin genes through formation of a long-range chromatin loop	Authors developed a new method for chromatin loop reorganization	[11]
dCas9Sa	FIRE-Cas9 system – enhanced dCas9–MS2 with Fkbp/Frb dimerizing fusion proteins	Lentiviral transduction of HEK293 cells and mouse embryonic stem cells	Three loci upstream of the highly expressed *CXCR4* (C-X-C motif chemokine receptor 4) gene and regulatory sequences upstream of *Oct4*	Reversible epigenome editing by endogenous chromatin regulators that is particularly suited to the analysis of endogenous multisubunit chromatin regulator complexes	[12]
dCas9Sp and dCpf1As	Epigenetic repressors fused (G9A, SUV39H1, KRAB, DNMT3A) as well as the first targetable versions of Ezh2 and Friend of GATA-1 (FOG1) to dCas9 or Cpf1	HCT116 cell line transfection	Promoters of *HER2* and *MYC* and *EPCAM* genes	Screened different repressors and highlighted the differences between them in achieving persistent repression Demonstrated that the dCpf1As fusions were not active when used to repress Her2 expression	[13]
cCas9Sp	dCas9-NLS-HA-BFP fused to KRAB, CS, or WRPW for repression or for activation: VP64 or p65 activation domain	Lentiviral transduction of GFP^+ HEK293 cells	GFP, transferrin receptor (CD71), and C-X-C chemokine receptor type 4 (CXCR4)	Showed robust gene knockdown of both reporter and endogenous genes and improved their previously published work on CRISPRi as an alternative method to RNAi for repressing gene expression in mammalian cells	[14]
dCas9Sp	Structure-guided engineering of a CRISPR/Cas9 complex; combination of sgRNA 2.0, NLS-dCas9-VP64, and MS2-p65-HSF1; synergistic activation mediator (SAM)	Transient transfection experiments in Neuro2 cells and HEK293FT cells and lentiviral transduction of A375 cells	12 Genes that were previously found by several groups to be difficult to activate using dCas9-VP64 and individual sgRNA	Showed that SAM stimulated transcription at least twofold for all genes and more than 15-fold for eight of 12 genes Engineered sgRNA to incorporate protein-interacting aptamers for gene upregulation	[15]
dCas9Sp	dCas9-DNMT3A fusion	Transfection of HEK293T cells with lipofectamine and lentiviral transduction	CDKN2A, ARF, and *Cdkn1a* promoter	The developed system allows mechanistic studies of DNA methylation	[16]
dCas9Sp	dCas9-VPR	HEK293T cells and Neuro2A cell transfection	*NGN2* and *NEUROD1*	Showed activation of endogenous coding and noncoding genes, targeted several genes simultaneously and stimulated neuronal differentiation of human induced pluripotent stem cells	[17]
dCas9	dCas9-SunTag-DNMT3A vs dCas9-DNMT3A	Transfection of MCF-7 or HeLa cells	CTCF and NRF1	Showed that the dCas9-SunTag-DNMT3A system can recruit multiple DNMT3A catalytic domains to a target site for editing DNA methylation at a much higher induction rate	[18]
dCas9	dCas9-Dnmt or Tet	Lentiviral transduction of multiple FXS patient-derived iPSCs and as *in vitro*-derived FXS neurons	FMR1 reactivation: reverse the hypermethylation of CGG repeats at the *FMR1* locus	Demonstrated evidence that demethylation of the CGG repeats is sufficient to reactivate *FMR1*	[21]

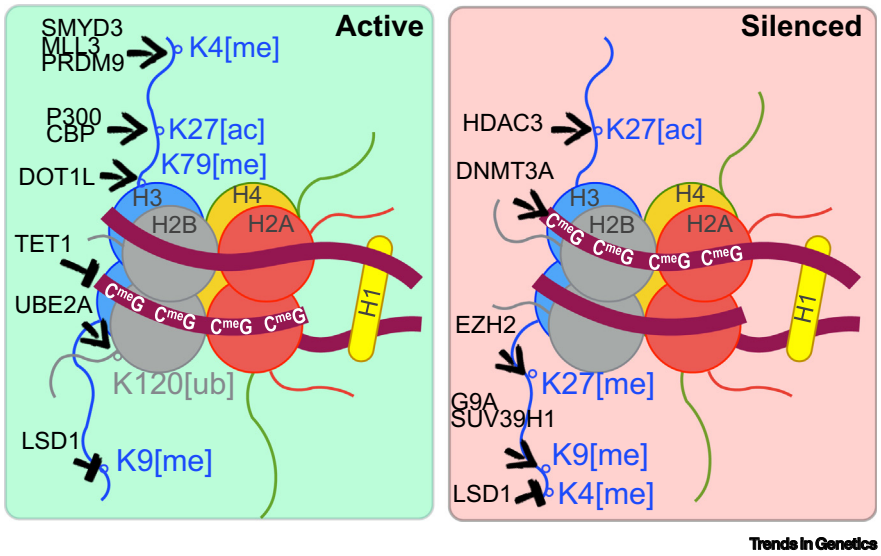

Figure 2. Enzymatic Domains for Covalent Histone and DNA Modifications. Several catalytic domains associated with gene activation (left) or silencing (right) have been paired with CRISPR-based epigenome editing. The enzymes and their DNA or amino acid targets on a nucleosome are shown in each panel. Open circles denote the targeted lysine and their position on the histone protein. The induced post-translational modification is denoted by brackets: me, methylation; ac, acetylation; ub, ubiquitination. Arrows indicate the deposition of the modification and blunt-ended arrows indicate removal.

To monitor the fate of the edited cells once inside the mouse the team included fluorescent markers in the transgene design. This allowed the researchers to measure cell proliferation and monitor repopulation of the BM. Although this study did not track the edited cells during differentiation of lymphoid and myeloid lineages, it was successful in providing a rational basis to study the role of precise epigenetic editing in hematopoiesis.

A similar study has used CRISPR-mediated epigenetic editing to targeting immune cells for the purpose of investigating the role of oncogenes and tumor suppressors in a mouse model of acute lymphoblastic leukemia [20]. The focus of this study was to epigenetically edit B lymphoma cell lines by inhibiting or activating genes involved in cancer growth, implant the cells into mice intravenously, and monitor B cell replication. Specifically, the team used lentivirus and retrovirus transduction to construct stable B lymphoma cell lines expressing dCas9-gRNA pairs for either suppression of transformation-related tumor protein 53 (*Trp53*) by KRAB or activation of O^6-methylguanine–DNA methyltransferase (*Mgmt*) by VP64. Edited cells were delivered by tail-vein injection. Through this approach, the researchers were able to monitor several physiologically relevant phenotypes including accumulation of edited cells in lymph nodes, growth relative to nontargeting gRNA controls, resistance to cisplatin and temozolomide (TMZ), and survival of mice. The team also conducted the first application of epigenetic activation screens in live animals by coinfecting a population of fluorescently labeled lymphoma cells each carrying a gRNA targeting one of 25 putative genes involved in TMZ resistance. That Chek2 was de-enriched in TMZ-treated mice relative to untreated controls suggested that this gene increased sensitivity to drug treatment, which was validated in subsequent experiments. This study is quite successful in illustrating the versatility of CRISPR epigenetic editing in live animals and the potential to conduct gain-of-function screens via CRISPR/dCas9.

A more recent study explored the role of epigenetic editing in live animals by using a cell-implantation model in the mouse brain [21]. The goal was to reactivate the *Fragile X Mental Retardation 1*

Table 2. Summary of *In Vivo* Epigenetic Editing Studies: The Epigenome Editor Used, Genes Modified, Target Tissues/Cells, Disease, and Delivery Mechanisms Are Listed

Epigenome editor	Gene	Target	Disease or condition	Delivery form	Delivery route	Comment	Refs
TALE-KRAB	*c-Kit*, *PU.1 (Spi1)*	BM	BM transplantation	Cell implantation	Intravenous injection	Combined BM transplantation with a multicolor TALE-KRAB expression vector to knock down two target genes in hematopoietic compartment *in vivo*; a technology for investigating functional roles of multiple gene targets	[19]
dCas9-eGFP, dCas9-VP64	*Trp53*, *Mgmt*	B cell lymphoma, B-ALL leukemia	Lymphoma	Cell implantation	Intravenous injection	Showed dCas9-mediated gene-level perturbation for modeling cancer progression and therapeutic relapse both *in vitro* and in mouse models	[20]
dCas9-Tet1	*FMR1*	iPSC-derived neurons	Fragile X	Cell implantation	Intracranial injection/neonatal engrafting	Showed sustained reactivation of *FMR1* in the methylation-edited FXS cells when injected into the P1 mouse brain for subsequent analysis 1 or 3 months post-transplantation	[21]
ZF-VP64	*GDNF*	Glial cells	Parkinson's disease	AAV2	Striatum injection with convection-enhanced delivery	Demonstrated that the activation of endogenous GDNF is sufficient to protect against 6-OHDA lesion in rat and showed that ZF-vp64 activates the endogenous human GDNF gene	[31]
dCas9-expressing mice with MS2-P65-HSF1	*Fst*, *Il10*, *klotho*, *Pdx1*, *Utrn*	Liver, muscle and kidney	Diabetes, muscular dystrophy, and acute kidney disease	AAV2/9	Intravenous, intramuscular, intracranial	The authors showed that this system can activate genes by modulating histone marks rather than editing DNA sequences and claimed that it can be used to express genes to compensate for disease-associated genetic mutations or to overexpress long noncoding RNAs and GC-rich genes, which has been difficult until now	[32]

Table 2. (continued)

Epigenome editor	Gene	Target	Disease or condition	Delivery form	Delivery route	Comment	Refs
dSaCas9-KRAB	*Pcsk9*	Liver	High-cholesterol diseases	AAV8	Intravenous	Authors discuss that one potential improvement of dSaCas9-KRAB system is that they need to deliver two components (the Cas9 and gRNA), which minimizes additional recruitment and potential immune response	[33]
TALE-VP64 or TALE-SunTag VP64	*FXN*	Liver, heart, muscle, brain	Friedreich's ataxia	AAV9	Intraperitoneal injection	Authors discuss that the delivery of AAV9 could be improved with intracranial and intravenous injections in future studies; the delivery of AAV9 intraperitoneally is suboptimal if the target organ is also the brain	[34,35]
dCas9-KRAB, dCas9-VP64-Rta	*Cd81*, *Afp*, *Nrl*	Liver, retina	Retinitis pigmentosa	AAV8, AAV2-Y444F	Intravenous, subretinal	Utilized split-Cas9 system and showed up to 80% transcriptional repression and up to sixfold transcriptional activation and showed efficacy of using AAV-KRAB-Cas9 in the context of gene therapy in a mouse model of retinitis pigmentosa	[36]
ZF-KRAB	*Ube3a*	Brain	Angelman syndrome	Protein	Intraperitoneal, subcutaneous	Transient activation of the Ube3a gene in the brain by using a ZF protein that when injected subcutaneously crosses the blood–brain barrier	[46]
dCas9	*cmyc*	T cells	N.A.	Transgenesis, retrovirus	N.A.	The generated transgenic animals represent a useful tool for enChIP analysis in primary mouse cells	[47]
dCas9-eGFP KRAB	Telomeres *Trf1*	Liver	N.A.	Transgenesis, DNA	Microinjection for the generation of the animals in mouse zygotes and hydrodynamic tail-vein (intravenous) injection	The generated dCas9-EGFP knock-in mouse provides a useful tool to dissect genome functions, including chromatin dynamics in live animals	[48]

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Table 2. (continued)

Epigenome editor	Gene	Target	Disease or condition	Delivery form	Delivery route	Comment	Refs
dCas9-SunTag-P65-HSF1	*Ascl1*, *Neurog2*, *Neurod1*, *Dkk1*, *Hbb*, many	Brain, astrocytes, fibroblasts	N.A.	Transgenesis, AAV8, and lentivirus	Intracranial, intravenous	The generated transgenic mouse model allows flexible screening for studying complex gene networks, including long noncoding RNAs and gain-of-function phenotypes in the nervous system	[49]
dCas9-SunTag VP64	Myc, Tnfrsf1a, Slc7a11, Tp53	Liver	Liver injury and tumorigenesis	Transgenesis and AAV8	Intravenous	The authors envision that their CRISPRa system will be useful for performing additional tissue-specific genetic screens as dCas9 can be activated in any tissue of interest; they showed hepatocyte specificity with an AAV-Cre virus, but viruses with other cell type-specific tropisms could be used to target other tissues	[50]

(*FMR1*), the causative gene of fragile X syndrome (FXS). In most cases, the disease allele is silenced through epigenetic mechanisms that include DNA methylation of the promoter region [22]. To reactivate *FMR1* in FXS neurons, a transgene encoding the components necessary for CRISPR-mediated recruitment of Tet methylcytosine deoxygenase 1 (Tet1) to the *FMR1* gene was first introduced into iPSCs by lentivirus transduction. After verifying that Tet1 could lead to reactivation of FMR1 in iPSCs, the research team then implanted the cells into brains of recipient mice. Once in the brain, these cells were monitored for neuronal differentiation and continued FMR1 expression by immunohistochemistry assays. Although several questions remain about the specificity of dCas9-Tet1, this study successfully demonstrates how it is possible to study the role of epigenetics in neuronal diseases through cell-implantation assays.

There are several valuable advantages to live-animal epigenomic studies by cell implantation. First, cell-implantation studies enable testing of the consequences of epigenetic editing in more relevant anatomical environments. Second, by encoding the implanted cells with fluorescent markers, the researchers were able to monitor the longevity of the epigenetic changes over the lifespan of the mouse. The potential for using the mouse as a 'tissue culture vessel' by cell implantation of epigenetically edited cells offers a first step for researchers to translate experimental observations from the tissue culture hood to the mouse.

Live-Animal Epigenome Editing by Adeno-Associated Virus (AAV) Delivery

Of all the delivery methods explored to date, viral delivery has been the most extensively used to target a broad range of cells including blood cells, liver cells, and neurons. In this regard, even the most precise epigenome therapy is destined to fail if the delivery of the therapeutic is not achieved in the desired cell types. The brain remains the least therapeutically accessible organ in mammals because of the physiological barrier of specialized endothelial cells, also known as the blood–

brain barrier. From all of the available viral vectors for genetic therapy, recombinant AAVs (rAAVs) are most commonly used for CNS disorders [23]. Transduction efficiency for different cell types varies between AAV serotypes and the route of administration [24]. AAV9 is particularly effective for transducing cortical neurons, whereas AAV8 is more efficient for transduction in astrocytes [25]. In many brain regions, AAVrh.10 is at least as efficient as rAAV9 [26].

One common application of viral vector delivery has been for TALE and ZF epigenome editing in the brain. This is partially due to the fact that these proteins match the coding capacity of most viral vectors and to the ability of these viral vectors to transduce many types in the brain [27–30]. One of the earliest applications of this method for live-animal epigenome editing focused on activating expression of the *glial cell line-derived neurotrophic factor* (*GDNF*) gene in a rat model of Parkinson's disease [31]. Noting that loss of GDNF expression is common feature in Parkinson's disease, this study focused on characterizing a ZF-VP64 peptide that could specifically target the promoter of both the rat and the human *GDNF* gene. The use of genome-wide expression analysis showed conclusively the specificity of the ZF-VP64 peptide. Furthermore, the use of the AAV2 capsid variant and injection into the brain striatum of rats was sufficient to lead to rescue of motor function phenotypes. Although the study did not test the long-term of effect of this therapeutic approach, the potential of create long-lasting changes after repeated injection remains a possibility.

Outside the brain, several proof-of-concept studies were recently published, which highlight how the combination of delivery route and choice of rAAV capsid mutant can be used to reach different target cells. In one study CRISPR/dCas9 was used for activation of target genes by a dual-AAV system: coinjection of AAV9-dCas9 and AAV9-gRNA [32]. In the study, P65-HSF1 transcriptional activation peptides were used. The researchers tested several routes of delivery including intramuscular, intracranial, tail vein, and facial vein. Optimizing their system for each delivery route allowed them to target genes whose functions affect a broad range of physiological phenotypes, including muscle mass and contraction by activation of *Follistatin* or *Utrophin*, kidney regeneration by activation of *Il10* or *Klotho*, and liver cell reprogramming by activation of *Pancreatic and duodenal homeobox 1*. For transcriptional repression, another study used a dual-AAV8 (a serotype that transduces preferably the liver) vector system to deliver dCas9-KRAB and a gRNA targeting *Pcsk9*. *Pcsk9* is a regulator of LDL cholesterol levels in the liver and the epigenomic editing strategy showed 24-week repression of the targeted gene in adult mice [33]. To target the human *Frataxin* (*FXN*) gene, a different study used TALE-VP64 [34]. Here, rAAV9 and intraperitoneal injection were used as the delivery route to reach several tissues, including the liver, muscle, and heart, of a **transgenic mouse strain** expressing human *FXN*. Presence of the TALE-VP64 in these tissues correlated with increased expression of *FXN*. More recently, a study combined the effectiveness of the TALE-mediated *FXN* targeting with the multimerization capability of the SunTag system to potently activate *FXN* in muscle and heart [35]. From these studies, two key advantages of live-animal epigenomic editing by TALEs can be appreciated compared with CRISPR/dCas9. First, TALE-mediated genome targeting is a one-component system, which facilitates delivery compared with the two-component CRISPR systems. Second, TALEs are much smaller than dCas9. This feature facilitates packaging and the addition of effector domains, like the SunTag array.

To circumvent the packing limits of the rAAV when using CRISPR epigenome editing, a recent study used a split-dCas9 system. In this study, split components were separately packaged into two AAV particles and a pair of intein peptides between the dCas9 fragments mediated reassembly in cells transduced with both viral particles [36]. The goal of this study was to correct retinal dystrophy disease in retinitis pigmentosa, a disease whose manifestation begins with degradation of rod photoreceptor cells in the eye. To circumvent disease development, the team focused on cellular reprogramming of disease-susceptible rod cells into disease-resistant cone photoreceptor cells in mice. This goal was achieved by repression of neural retina leucine zipper

(Nrl), a master regulator of rod cell identity. Here, an AAV2-Y444F variant that displayed tropism for photoreceptor cells was delivered by subocular injections. Tissue histology and gene expression from injected mice displayed a thicker retinal cell layer and a higher percentage of cone photoreceptor cells, as well as the rescue of photosensitivity and behavioral phenotypes associated with visual acuity. This study in particular highlights the elegance of epigenomic therapeutic approaches in which a disease associated with a specific cell type (rod photoreceptor cells) can be circumvented by reprogramming into resistant cells (cone photoreceptor cells) of similar function. In this case, the knowledge of Nrl as a master regulator between rod and cone cell differentiation made this approach feasible.

Another approach to expanding AAV delivery-cell specificity is by using cell-specific promoters to preferentially drive transgene expression. For neurons, the synapsin promoter has been used efficaciously [37,38]. An additional approach to deliver AAVs to the brain noninvasively is by generating BBB-penetrating capsids. This advance has been made possible by the generation of highly diverse capsid libraries through peptide insertion, homology-based recombination, and error-prone PCR [39,40]. An unprecedented ability to transfer genes to the CNS in the adult mouse showed >40-fold enhancement with the AAV-PHP.eB version over the previous standard AAV9 [39,40]. Although it opened an exciting field for capsid improvement for BBB crossing, it has been shown that the AAV-PHP.eB capsid transduction potency is limited to C57Bl/6J mice [41]. These studies illustrate several important lessons for epigenetic editing in live animals. First, picking the AAV serotype with the desired tropism for the organ of interest and a cell-specific promoter for the target cells is an important first step in the success of the study. Second, the AAV capsid may require extra modifications for transduction efficiency and passing through anatomical barriers, which may lower the AAV titer. Third, the coding capacity needs to be considered ahead in case the use of a dual-AAV approach for delivery is required. In the case of large proteins like Cas9, smaller orthologs have now been reported [42]. Finally, for brain-wide distribution, consideration of delivery methods like intrathecal slow infusion may provide a strong advantage [43].

Live-Animal Epigenome Editing by Macromolecule Delivery

Injection of purified macromolecules including ZFs, TALEs, or dCas9-gRNA ribonucleic complexes is a promising alternative to AAV-based delivery. This approach offers more control over dosage and rapid pharmacodynamics. Several attempts to find the most efficient nanoparticle composition for nucleic acid and protein delivery to the specific target tissue has been explored through screens of complex libraries of lipid nanoparticles (see [44] for an example) and the use of stable metal–protein complexes like CRISPR-Gold (see [45] for an example). While work in this area has clearly gained interest in genome editing, the approach has not been widely adopted for epigenome editing. This could be explained by the fact that the preparation of injection-quality macromolecules is more laborious and time consuming. However, a recent study demonstrated the potential of this approach for a neurodevelopmental disease [46]. Using a purified ZF-KRAB peptide, this study silenced a repressor of the gene *Ube3a* whose absence causes Angelman syndrome. The macromolecule was engineered with an RFP and a cell-penetrating peptide, which allowed BBB passage and tracking of the ZF in live animals. Interestingly, the macromolecule displayed a large distribution over the brain and induced widespread *Ube3a* activation in the CNS. This study exemplifies the power of macromolecule-based epigenetic editing in live animals. It also offers a possible path for biological therapeutic interventions.

Transgenic Mouse Strains to Facilitate CRISPR Epigenome Editing

Investigators studying epigenome editing in live animals will benefit from the recently reported knock-in and transgenic mouse lines carrying dCas9 alone or paired with epigenomic editing domains. Since these strains lack gRNA sequences, researchers can deliver gRNA against their genes of interest for various targeting experiments. These strains will be of great use for basic

biology and therapeutic development studies. The choice of strain will depend on the specific question and the characteristics sought by the researchers. Currently, there are four strains available for CRISPR epigenetic studies with different CRISPR components (Figure 3).

Two mouse strains are particularly useful to monitor chromatin in live mice when paired with AAV-delivered gRNAs (Figure 3A,B). These strains are **Rosa26** knock-ins in which the transgene is driven by a CAG promoter system for high transcription in all tissues [47]. With the presence of a loxP-stop-loxP sequence between the promoter and coding sequence, Cre-mediated activation of the transgene in specific cell types or developmental time points can be obtained. This strain has been used for chromatin immunoprecipitation by isolating primary immune cells and transfecting them in tissue culture with plasmids expressing specific gRNAs. A similar strain was recently reported with the main difference being that dCas9 was fused to eGFP [48]. This allowed the researchers to conduct cell imaging of specific loci in liver cells when AAV-packaged gRNAs were introduced by tail-vein injection. Although imaging is limited to repetitive loci in which multiple dCas9-eGFP proteins can bind, these mouse strains offer the possibility to monitor specific loci by fluorescence-based assays in live animals to understand their changes in response to development, again, and cancer.

Two additional strains were recently reported that offer the potential to perturb transcriptional activity. Both of these strains are based on the CRISPR activation paradigm in which dCas9-gRNA complexes are used to tether specific transcriptional activation domains to sites of interest. One strain was constructed by introducing a transgene that included dCas9-SunTag fusion followed by an scFv antibody fragment fused to transactivators P65 and HSF1, and a P2A-eGFP element (Figure 3C) [49]. The strain has been used for cell-specific reprogramming and multigene editing. Researchers interested in activating multiple genes simultaneously in the same cells will greatly benefit from this mouse strain since all of the components except the gRNA are already encoded in the genome. A similar strain for CRISPR activation was also recently reported [50]. This mouse

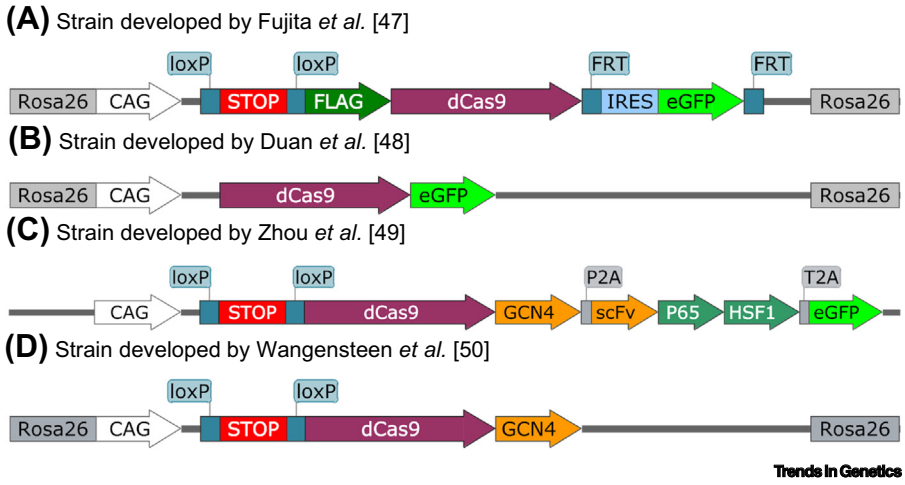

Figure 3. Sequences Used to Construct Transgenic and Knock-In Mouse Strains. (A) Strain with Cre-inducible expression of a FLAG-tagged dCas9 protein with FLP-controlled eGFP expression. (B) Strain with constitutive expression of a GFP-tagged dCas9 protein. (C) Strain with Cre-inducible expression of a dCas9-GCN4-epitope recombinant protein, followed by a detached single-chain antibody fused to P65 and HSF1 domains and a detached eGFP protein. (D) Strain with Cre-inducible expression of a dCas9-GCN4-epitope recombinant protein. Rosa26 denotes the locus on mouse chromosome 6. White arrow indicates a chimeric CAG promoter system. loxP-STOP-loxP denotes the presence of stop codons and a polyadenylation sequence flanked by loxP sites. P2A and T2A denote the location of exclusive intramolecular cleaving proteases for processing of the polypeptide. All mouse strains were constructed with *Streptococcus pyogenes*-derived Cas9.

strain was generated by homologous recombination into the Rosa26 locus and does not include the scFv portion. To validate this strain, the researchers examined whether they could conduct an epigenetic selection screen in live mice by selecting for genes whose activation would lead to the proliferation of liver cells. This mouse strain offers two key advantages. First, since this is a **knock-in mouse strain**, tracking alleles and crossing to other mouse strains is much easier. Thus, researchers focused on genetically defined diseases will benefit greatly from this mouse strain. Second, the scFv-activator coding sequence is not part of the transgene, allowing this mouse strain to be injected with AAVs encoding scFv repressors allowing CRISPR repression experiments.

Taking these findings together, these mouse strains offer the potential to track and alter the transcriptional trajectory of specific genes across development and in physiological environments that are difficult to recreate in tissue culture. In these systems, viral transduction was the choice for gRNA delivery. The selection of injection route and the increasingly large number of capsid variants allows cell type-specific delivery of gRNAs in mouse models of disease and development.

Concluding Remarks and Future Perspectives

The study of epigenomic editing in live animals is about to enter a rapid growth phase thanks to the convergence of the technologies discussed. These technological advances include the optimized AAV capsid variants for tissue- and cell-specific tropism, the large catalog of CRISPR/dCas9 epigenome effector domains, and the growing number of transgenic and knock-in mice carrying epigenomic editing machinery. Along with cell implantation models and direct macromolecule injection, researchers have at least four modes to transition their studies from tissue culture to live rodent models (Figure 4, Key Figure).

Live-animal epigenomic editing has several applications. One of those is modeling and treating brain disorders. Almost all diseases have a genetic component as well as an epigenetic contribution in which DNA methylation, histone modifications, chromatin accessibility, miRNA expression, pre-mRNA splicing, or long noncoding RNAs are affected. Many of these epigenetic components play an important role in CNS disorders, either in neurodegenerative diseases like Alzheimer's disease [51] and Parkinson's disease [52] or in neurodevelopmental diseases such as FXS [22], Rett syndrome [53], Angelman syndrome [54], and Prader–Willi syndrome [55]. The latter group of listed neurodevelopmental disorders is also part of the autism spectrum disorders with symptoms varying in severity affecting cognitive plasticity, presenting with broad impairments in communication and restricted, repetitive behaviors [56]. All of these disorders are monogenic, and disease manifestation and severity arise from dysregulation of downstream epigenetic pathways, making them excellent candidates for genetic and epigenetic therapies [57].

In cancer, the role of epigenetics continues to draw much attention. These efforts are informed, and motivated, by recent patient epigenome sequencing studies, which have uncovered profound changes in DNA methylation, genome-wide accessibility, and the localization of histone post-translational modifications in patients' cancer tissues (recently reviewed in [58]). In a few well-studied cases, causal relations between oncogenesis and epigenomic deregulation have been established. These include the role of DNMT3A in acute myeloid leukemia [59] and PBMR1 chromatin remodeling in clear-cell renal cell carcinoma [60], among others. Although tissue culture models have helped to elucidate key events in cell division, many aspects of oncogenesis that cannot be modeled in a Petri dish are still unknown. Live-animal epigenome editing studies offer the potential to dissect the temporal dynamics from initial cell cycle deregulation to metastasis. Future live-animal epigenome editing research will help to elucidate how epigenomic dysregulation helps cancer cells overcome anatomical and immunological barriers at each stage of cancer growth.

Outstanding Questions

Which parameters need to be considered when designing an epigenetic editing tool?

Which packaging system will show the most promising results for CNS delivery in humans?

Which classes of epigenomic modification remain across mitotic cell division?

For disease treatment, will transient epigenomic editing changes lead to stable, long-lasting effects in terminally differentiated cells?

Which of the several approaches addressing AAV limited packaging is the most efficient?

Will AAV mutants with optimized cell specificity in mouse models display similar tropism in humans?

What is the most effective approach to suppress a human immune response to CRISPR components?

Key Figure

Summary of Methods for Live-Animal Epigenome Editing

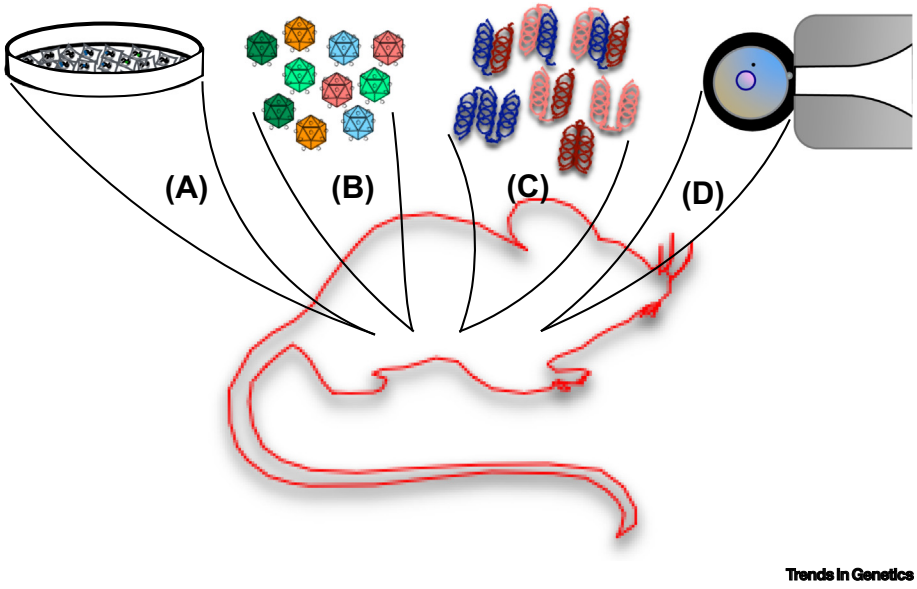

Figure 4. (A) Epigenomically edited human or murine cells can be implanted into model organisms and their differentiation, growth, and localization monitored over time. (B) To alter endogenous mouse cells, the epigenomic editing machinery can be packaged into adeno-associated virus (AAV) with mutant capsid variants for specific tropism. (C) Alternatively, the epigenomically edited machinery can be purified and ribonucleoprotein complexes can be delivered via different routes for targeted organ distribution. (D) Genetically encoding dCas9 and epigenomic editing domains in the mouse genome by transgenesis can greatly facilitate the study of epigenomic editing in live animals.

The promise of epigenetics-based therapies is the ability to alter the expression of disease-associated gene networks without creating permanent mutations in the human genome. However, one of the clinical translation obstacles that CRISPR gene and epigenome editing approaches face is immunogenicity [61–63]. The most commonly used sources of Cas9 come from bacteria known to cause infections in humans [64]. Thus, work is needed to resolve this issue. This and many questions remain on the applications of epigenomic editing in live animals. The studies highlighted inspire the imagination on the potential of this technology but do not address the breath and optimization landscape still needed for reproducible and translatable applications (see Outstanding Questions). Live-animal epigenetic editing studies will be a necessary catalyst to advance the development of this class of therapies into useful clinical treatments.

Author Contributions

J.A.G., U.B., and D.J.S. all participated in the writing and editing of the manuscript.

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