CRISPR-tools for physiology & cell state changes - potential of transcriptional engineering and epigenome editing

Christopher T. Breunig[1,2], Anna Köferle[2], Andrea M. Neuner[1], Maximilian F. Wiesbeck[2], Valentin Baumann[1], Stefan H. Stricker[1,2]

[1]MCN Junior Research Group, Munich Center for Neurosciences, Ludwig-Maximilian-Universität, BioMedical Center, Grosshaderner Strasse 9, Planegg-Martinsried, 82152, Germany

[2]Epigenetic Engineering, Institute of Stem Cell Research, Helmholtz Zentrum, German Research Center for Environmental Health, BioMedical Center, Grosshaderner Strasse 9, Planegg-Martinsried, 82152, Germany

Abbreviated Title: Targeting tools for cell state changes

Correspondence to Stefan H. Stricker, Epigenetic Engineering, Institute of Stem Cell Research, Helmholtz Zentrum, German Research Center for Environmental Health, BioMedical Center, Grosshaderner Strasse 9, Planegg-Martinsried, 82152, Germany, Stefan.stricker@helmholtz-muenchen.de

TABLE OF CONTENT:

ABSTRACT

Given the large amount of genome-wide data that has been collected during the last decades a good understanding of how and why cells change during development, homeostasis and disease might be expected. Unfortunately, the opposite is true; Triggers that cause cellular state changes remain elusive and the underlying molecular mechanisms are poorly understood. Although genes with the potential to influence cell states are known, the historic dependency on methods that manipulate gene expression outside the endogenous chromatin context has prevented us from understanding how cells organize, interpret and protect cellular programs. Fortunately, recent methodological innovations are now providing options to answer these outstanding questions, by allowing to target and manipulate individual genomic and epigenomic loci. In particular, three experimental approaches are now feasible due to DNA targeting tools: namely, activation and/or repression of master transcription factors in their endogenous chromatin context, targeting transcription factors to endogenous, alternative or inaccessible sites; and finally, functional manipulation of the chromatin context. In this article, we discuss the molecular basis of DNA targeting tools and review the potential of these new technologies before we summarize how these have already been used for the manipulation of cellular states and hypothesize about future applications.

I. Introduction:

A. Summary:

In this review, we focus on how physiological cell states are controlled by gene activities during development and disease. We will address several aspects of gene regulation, but concentrate on the role of transcription factors and chromatin modifications in this context. We will review available methods allowing the manipulation of these processes, in particular transcriptional engineering and epigenome editing (Figure 1). The experimental approaches we introduce are applicable to most, if not all, aspects of physiology and have already successfully been used for manipulating insulin response (61), regulate neuronal physiology (42), reduce synuclein levels (124) and increase levels of disease relevant genes, such as CFTR (271). We will give a detailed overview of studies that have already applied this technology to alter cellular states implicated in human diseases and pay special attention to a particular state, the cell type or identity, as it is well defined and therapeutically relevant. Since a large number of excellent reviews exist that summarize the molecular basis of genome editing ("Recent advances in the CRISPR genome editing tool set")(14), we will mostly omit this topic.

B. Cell states and cell identities in physiology

Physiology is one of the oldest branches of the life sciences. It deals with the biological mechanisms that functionally enable life in all its forms. For centuries, physiology has mostly been restricted to understanding organs and their function. Until the early 19th century, organs were seen as functional units acting in the human body like specialized workers would do in a factory. Since then, physiology has extended its scope to incorporate insights about underlying microscopic structures. The notion that the minimal physiological unit is the individual cell rather than the organ, put forward by the French scientist Henri Dutrochet, is of particular importance in this context (93). Although animal and plant cells had been discovered more than 150 years earlier, they had initially been interpreted as merely structural units providing scaffolding for the organs.

Today, we know that all biological processes are implemented by individual cells, of which the human body contains a staggering 10^13 (20)). Each of these individual cells can adopt a number of physiological states that are a necessary pre-requisite to fulfil different cellular functions. While some states are transitory and/or only present during embryonic development, others are stable for a (cellular) life time. Cell states change not only during development, but also during disease. These changes can be classified into the following (somewhat overlapping) categories: (A) adoption of un-physiological cellular states that do not

normally occur (e.g. the progressive and potentially additive changes accumulating during neurodegeneration (64)); (B) adoption of “normal” cellular programs in a “wrong”, i.e. a disease, context (e.g. the acquisition of the epithelial-to-mesenchymal transition during cancer progression (2)), and (C) shifts in the quantitative ratios of physiological states within an organ (e.g. the pathological imbalance of immune cells during the severe combined immunodeficiency syndrome (30)). Importantly, cellular states can change acutely, and revert as soon as the trigger is gone, but they can also persist for a life-time and even be inherited. Typical examples of the first case are acute signaling events; cell types (or cell identities) fall in the latter category.

Cell identity is currently the most commonly studied cellular state. It is estimated that approximately 400 distinct cell types exist in the human body (270), however their classification is unsystematic. Most cell type classifiers were chosen a long time ago and are either based on a morphological feature (astrocyte, pyramidal neuron), their functional role (natural killer T cell, glutamatergic neuron), their potency (neural progenitor cell, intestinal stem cell) or their discoverer (merkel cell, purkinje cell) (7). Furthermore, classification relies on cell type-specific markers, which are often only specific in a relative manner (at a certain expression level, in a specific tissue or developmental stage). Recent advances in single cell RNA-Sequencing technology promises a revolution in classification by providing comprehensive data sets (220). However, an important caveat should also be mentioned: we currently lack the ability to reliably demarcate reversible cell states from irrevocable cell identity alterations by single cell transcriptomes (263). To make most of the molecular data currently available, we have to find reliable rules informing us how much difference justifies the separation of two cellular states into separate categories and how much similarity legitimates grouping; whether it is more expedient to determine these from global transcriptomic differences, a smaller informative subset of genes or a completely different criterion (functional characterization, developmental lineages or evolutionary heritage). It will be helpful in this context to combine single cell RNA-Seq with functional assays and/or genetic perturbations (as summarized by (257)) .

A spectacular variety of cell states is present in each multi-cellar organism. This entails a paradox that was already recognized a long time ago. Since every human life begins with one single cell (the zygote), and thus with only one genome, all cells that make up the human body are born from genetic uniformity. Since the vast majority of cells in the body retain the original genome throughout their life, they do carry all the genetic information they need to be in any other cellular state. How genetic uniformity can lead to phenotypic diversity is still a big question in many branches of life science. With regard to cell identity, Conrad Hal Waddington made the most important conceptual contribution. In his 1942 publication “the epigenotype” (275) he found a compelling visual metaphor for the changes cells go through during development (276). In his image of the “epigenetic landscape”, the totipotent zygote is

depicted as a marble sitting on top of a hill. The only option for it is to roll downhill; this symbolizes development, which is largely driven by cellular differentiation. There are several paths a cell can take; those are however dictated by the activity of specific genes, which underpin the landscape. A communality all cellular fates share is that they result in continuously fewer available options. In Waddington's landscape every cell eventually ends up in a dead-end valley, representing a terminally differentiated cell type (Figure 2). Waddington coined the term "epigenetics", as the branch of biology dealing with all the non-genetic processes "which bring the phenotype into being" (277). The Waddington landscape highlights a key feature of epigenetic changes; once cells have undergone an epigenetic change (e.g. differentiation), they (and their progeny) have undergone a lasting change that will not reverse once the initial stimulus is gone. Formulated only few years after Watson and Crick had discovered the structure of DNA, Waddington's model has persisting influence on current concepts. Cell identity (what a cell is), plasticity (the sum of states a cell or its progeny could under certain constraints adopt), potency (the sum of identities a cell or its progeny take on naturally) and cellular lineages (a developmental path that may be chosen during development) are already part of the visual metaphor (Figure 2). Unfortunately, one interesting aspect of Waddington's model is omitted in most reprints. He predicted that the epigenetic landscapers, e.g. the entities that shape cellular fate, are embodied by a limited number of genes; more specifically by their disparate activity.

Today we know that a certain class of genes, transcription factors, is especially relevant for defining cellular states. Transcription factors are proteins that bind to DNA. Through this interaction they have the potential to influence gene expression of a large number of genes (for the operating principles of transcription factors see (243)). Meta-analysis of human transcriptomes suggests that roughly a quarter of transcription factors are ubiquitously expressed, 50% in several (often developmentally related) tissues, while the last quarter is tissue- or cell type-specific (267). Several cis-elements that can serve as transcription factor binding platforms have been described. In the context of cell identity, however, the enhancer element likely has the most crucial role (285). Discovered almost 40 years ago (10), enhancers are transcription activating, modular DNA elements, that are each characterized by a high density of transcription factor binding sites. Interestingly, transcription factors are known for having cooperative and additive effects for both, DNA binding and/or their consequences on transcription of nearby genes. Taken together, their specific properties are thought to constitute a transcription factor "grammar" that might explain the often highly cell type-specific activity of enhancer elements (243).

Some of cell type-specific transcription factors can (through their absence or robust activity) influence cellular lineage choices during development (and/or differentiation) and thus fulfill the criteria of master transcription factors (285). Indeed, all cell identities and lineage choices

are thought to be directed or protected by one, or a combination of master transcription factors (185). An especially potent subset of master transcription factors is represented by the so-called reprogramming factors. Reprogramming factors are able to influence identities of differentiated cells beyond their natural potency. Although the experimental procedure of reprogramming usually is based on a genetic manipulation, the delivery of exogenous gene copies, cellular reprogramming is inherently epigenetic. Consequently, newly acquired states are stable and heritable, even if the added reprogramming factors are silenced or removed (288). Interestingly, not all reprogramming factors are equally potent. Instead they can be classified according to whether they revert development (de-differentiation and induced pluripotency), enable alternative options for differentiation (trans-differentiation or trans-determination) or enforce alternative differentiated identities in already differentiated cells (direct conversion, Figure 2). In principle, neither developmental master nor reprogramming factors are required once a new cell identity has been adopted. This is in contrast to terminal selector genes; their loss directly leads to a disintegration of an existing identity, and a undirected acquisition of a new cellular identity (103).

II. Genomic targeting by programmable DNA binding proteins

A. Genomic targeting proteins

The successful cloning of the first genes was only reported in the 1970s (69, 172, 173). Since then, three main approaches have been used to manipulate gene activities: gene knockout, knockdown and ectopic expression. The gold standard for functional gene analysis is the conditional knockout. This approach is dependent on the availability of transgenic mouse lines and, even when applicable, some caution has to be exercised when phenotypes are attributed to gene function, since genetic background can have considerable influence on the phenotype of mouse mutations (156, 182). The discovery of the molecular mechanism driving RNA interference twenty years ago opened up a new option for functional analysis, namely temporary knockdown of transcripts using short, hairpin or double-stranded RNAs (149). Although remarkably fast and simple, knockdown strategies are not without flaws, as residual gene expression levels are often relatively high while off-target and/or unspecific effects are common (149). Ectopic expression through the use of plasmid vectors equipped with viral or other strong promoters, selection markers or reporter cassettes is still an integral part of functional gene analysis today, although examples of un-physiological consequences of gene over-expression have been evident since early days (as summarized in (215)).

A large number of additional experimental options to manipulate gene expression and to study gene function have recently emerged. All of these are built upon genome targeting systems

that allow molecular effectors to specifically find and bind individual DNA sequences. The first comprehensive genomic targeting system was based on a widespread class of transcription factors, known as zinc finger proteins. Zinc finger proteins are common in mammalian genomes and account for almost half of all human transcription factors (267). Members of this group share DNA binding modules that have affinity to short DNA motives, usually triplets (287). Synthetic combination of different zinc finger motifs (e.g. 10) allows the design of artificial proteins that recognize a stretch of DNA (e.g. 30bp) with a specific sequence (133). This elegant approach was quickly superseded by TALEs (Transcription Activator-like Effectors), synthetic proteins derived from DNA binding domains of a bacterial transcription factor. Each TALE domain is similar in length to a zinc finger (33-35 amino acids), but only recognizes a single DNA base, simplifying assembly (186). By combining repeats with different base specificities it is possible to target any DNA sequence of choice. TALE and ZF design depends on the assembly and/or synthesis of long sequences. If, for example, a 20 base pair target sequence is desired, TALE repeats spreading over roughly 600 amino acids have to be assembled. Moreover, given that the DNA target specificity of TALEs and ZFs is encoded in the amino acid sequence of the assembled repeats, a new TALE or ZF protein has to be generated for each new target site. This is in sharp contrast to RNA-guided endonucleases (RGEN), which constitute a more recent addition to the genome editing toolbox. In these systems, DNA target recognition is not based on interactions between protein and DNA, but rather on RNA-DNA hybridization. The most commonly used RGENs are derived from CRISPR (clustered regularly interspaced short palindromic repeats) systems. In bacteria and archaea, CRISPR systems have evolved as a form of adaptive immunity against invading phages, recognizing and digesting foreign DNA (14).

The CRISPR system from *S.pyogenes* is the basis of the most frequently used genome engineering tool today. The most critical pre-requisite for this success was the demonstration that the complex endogenous bacterial RNA components can be replaced by a single synthetic RNA molecule. This single guide RNA (sgRNA or gRNA) comprises two regions: a 20 nucleotide long targeting sequence and a 42 nucleotide long scaffold sequence which forms a hairpin loop (118), which has been further improved (28, 88, 109) (Figure 1). The second CRISPR component, the CRISPR-associated protein (Cas9), is an endonuclease able to recognize the gRNA scaffold structure. The sequence preceding the scaffold sequences - known as the spacer- determines the binding specificity of the gRNA-protein complex to DNA (14). This is how one invariant Cas9 protein can be targeted to different DNA target loci simply by exchanging the 17-20 bp long spacer sequence of the gRNA.

Importantly, the experimental use of Cas9 extends beyond genome editing. A number of different protein domains have been attached to the nuclease dead-version of Cas9, commonly referred to as dCas9 (216), allowing the manipulation of gene activities (see Section III, VI and

VII below). The ease of use of the CRISPR/Cas9 platform is reflected by the rise in the number of publications using this method in recent years. Moreover, RNA molecules can also be targeted by CRISPR proteins. In vitro studies have shown that *S. pyogenes* Cas9 can act on single stranded RNA as well (197), although the efficiency is quite low. The Cas9 protein of *Neisseria meningitides* (223) and the recently described family of Cas13 CRISPR proteins are better suited for RNA targeting (1, 53). Cas13 acts as an endoRNAse when targeted by a 64-66 bp CRISPR RNA (crRNA) that recognizes a 28-30 bp target site on the RNA molecule. In conclusion, although several molecular strategies for site-specific targeting exist, which each have their own advantages and disadvantages, CRISPR systems are standing out through their versatility, their adaptability and their widespread use. In summary, the gRNAs currently experimentally used came a long way from their bacterial precursor transcripts. Whether *S.pyogenes* Cas9 will remain the gold standard, or new CRISPR proteins or targeting systems from other species will prove to be more applicable (32), only time can tell.

B. Limitations of Cas9 binding

The crucial determinant for successful binding of Cas9 to its target sequence is the presence of the so-called protospacer adjacent motif (PAM). This is a short region immediately downstream of the target binding site that is not part of the gRNA sequence itself. When present immediately downstream the target site, the PAM sequence stabilizes binding of the Cas9-gRNA complex to DNA (5). For *S. pyogene*s Cas9 it consists of “NGG” (or “NAG” (106, 247)). The requirement for the presence of a particular 3’ PAM sequence limits the number of sites in the genome that can be targeted with *S. pyogenes* Cas9 (especially in G-poor regions). Several approaches have been taken to overcome this bottleneck and provide options to target sites lacking a *S. pyogene*s PAM. The first approach makes use of the large variety of Cas9 proteins in the bacterial kingdom, since Cas9 proteins from different organisms have different PAM requirements. *Neisseria meningitidis* Cas9 (NmCas9), for example, requires a NNNNGATT PAM sequence (108), Cas9 from *Staphylococcus aureus* NNGRRT (and to a lesser extent also accepts NNGRRN) (217). The second approach is to alter PAM requirements of *S. pyogenes* Cas9 through selection-based directed evolution. In this way, variants that recognize different PAM sequences such as NGAN or NGNG (the VQR variant) or have very loose requirements (either NG, GAA, or GAT; xCas9) have been generated (111, 137). Finally, there is also the option to turn to an alternative CRISPR system. The most commonly used alternative CRISPR systems is Cas12a (also named Cpf1), which requires a 5’TTN PAM (314). Together, these proteins greatly expand the CRISPR toolbox as they allow the targeting of sites that inaccessible for wild-type *S.pyogenes* Cas9.

III. gRNAs encode the targeting information of the CRISPR complex

A. gRNA targeting specificity, design and delivery

It has been shown that Cas9 does not always exclusively cut at the desired target site. Instead, mutations also occur at off-target sites, because mismatches between the spacer and the genomic DNA are tolerated to some degree (48). The sensitivity towards mismatches in the target sequence is gRNA specific; however, the 5' end of the gRNA is in general less sensitive to mismatches than the 3'end. Consequently, the PAM-proximal 3' end has been named "seed sequence" in analogy to miRNAs which behave similarly (70) (Figure 1). To reduce off-target cleavage, different measures have been tested. Fu et al. (2014) showed that truncating the spacer length by two to three nucleotides reduces off-target cleavage while maintaining on-target efficiency (71). Ran and colleagues hypothesized that lengthening the target sequence from 20 nucleotides to 30 nucleotides could increase the specificity since a longer sequence is more unique within the genome, however observed that longer gRNAs were processed to 20 bp spacer by endogenous RNAses (218).

Several chemical modifications have been introduced on gRNA molecules to increase gRNA stability leading to higher editing efficiency and reduced immune-stimulatory effects (reviewed in (127)). gRNAs can be modified at the 5' and the 3' end, the backbone, the base, or the sugar. Furthermore, some nucleotides of the gRNA can be replaced with DNA nucleotides, which apparently results in improved targeting specificities (308). For chemical modifications gRNAs have to be synthesized, whereas in most cases expression vectors, viral particles or in vitro transcribed RNA molecules are used. Typical expression and viral vectors employ the RNA polymerase III U6 promoter, which demands the presence of a G at the 5' end of the gRNA (119, 170). For in vitro transcription the T7 system is often used, which shares this requirement (227). Both, in vitro transcription and oligo-nucleotide synthesis enable a DNA-free approach when Cas9 is delivered as either mRNA or protein. The DNA-free approach is more transient than continuous expression from a plasmid or viral copy, which may lead to fewer off-target events (127).

Several recent studies have used adeno-associated virus (AAV) to deliver gRNAs (and/or other CRISPR components) (253, 290, 302), which brings with it the advantage of tissue-/cell type-specificities due to tissue tropisms of different AAV serotypes (244), a prerequisite of therapeutic approaches (209). An alternative approach for targeted organ delivery is to complex recombinant baculoviral vectors to magnetic nanoparticles. In this way locally applied magnetic fields can be used to direct the virus into specific body parts (323). To facilitate the design of highly specific and efficient gRNAs, different algorithms have been developed. These are available on several online platforms (e.g. E-CRISP, sgRNA designer, and CCTop; for more

details and information see the excellent reviews from Wilson (286) or Cui (55) and colleagues). In addition to different underlying algorithms for the assessment of efficiency and specificity, these platforms offer options for designing gRNAs for CRISPR-based gene activation and suppression (see Section IV below).

B. gRNAs facilitate multiplexing of gene targeting

Many CRISPR approaches (especially those for manipulating cell states) require simultaneous targeting of more than one site and therefore depend on more than one gRNA. The simplest solution is to mix CRISPR vectors or components before transfection or transduction (Figure 3) (174, 261, 282). Thereby however, it cannot be controlled which cells receive which gRNAs, i.e. whether the cells got gRNAs targeting all different loci of interest, or only a subset. Moreover, it is unlikely that cells receive the gRNAs in stoichiometric ratios. To overcome these issues single vectors containing all gRNAs of interest have been developed. The number of possible gRNAs is limited by the packaging limit of the vector used, but since gRNA sequences are short, many gRNA cassettes can be combined on one single vector (currently up to 14 individual gRNA cassettes have been reported to be expressed in plant cells (206)). Multiplexing vectors can be generated by DNA synthesis of large oligonucleotides containing the complete expression construct (Figure 3). To offer faster and cheaper options several combinatorial cloning methods have been developed in different species as an alternative. These either depend on classical restriction and ligation based cloning (63, 151, 170, 206, 305), isocaudomer based cloning (278), Golden Gate based cloning approaches (123, 226), the Golden Braid variant (268) or Gibson assembly based cloning approaches (27, 295) Figure 3).

Besides assembling multiple gRNA expression cassettes into one vector, all gRNAs together can be transcribed as a single precursor transcript, which is then processed to individual gRNAs by different measures (Figure 3). In this way, tissue-specific Pol II promoters can be used to drive the transcription. This has been pioneered in so called crRNA arrays, which resemble the natural CRISPR type II-A (52, 54) or Cpf1 (Cas12a) crRNA arrays (12, 315). In addition to methods imitating the natural CRISPR system, several strategies have been developed to cut mature gRNAs out of precursor RNAs. The first makes use of ribozymes, either the hepatitis delta virus type (224), or hammerhead type ribozyme (116, 258), or both (298, 311). The second system exploits the ability of endogenous RNases to recognize and excise tRNA sequences. When tRNAs and gRNAs are arrayed in tandem, the excision of the tRNAs leads to the simultaneous excision of the gRNAs (293). Applying this system Xie and colleagues (2015) achieved the expression of eight gRNAs from a single vector. Similar systems employ the endogenous ribonuclease DROSHA to cut at shRNA or miRNA instead of tRNA sequences (292, 305). A third system for the expression of multiple gRNAs from a single transcript is based on

Csy4, which is an endoribonuclease from *Pseudomonas aeruginosa*. Here, the gRNAs are cloned in an array separated by Csy4 excision sites. Cermak and colleagues (2017) used the Csy4 system to deliver up to twelve gRNAs from a single transcript driven by a single promoter. Comparing the Csy4 system to tRNA- and ribozyme-based systems, Cermak et al reported that Csy4 has the highest efficiency, at least in plant cells (36). In summary, gRNA multiplexing facilitates strategies that other gene targeting systems were unable to achieve, namely the simultaneous manipulation of multiple genes in single cells.

C. Strategies for CRISPR activity control

In controllable CRISPR systems, Cas9 activity can be induced and/or discontinued (Figure 4). One approach is to express Cas9, gRNAs or both from inducible promoters, which rely on the presence or absence of light, chemicals or a specific temperature. Thermal control depends on heat-shock promoters (Figure 4A) or temperature-sensitive (d)Cas9 variants (Figure 4B) and has been applied in *E.Coli*, *C.elegans* and *Danio rerio* (221, 238, 274, 309). Optogenetic approaches employ a light activatable Cas9 protein (achieved through incorporation of a caged lysine amino acid (99), Figure 4G), light inducible promoters (235) (Figure 4F), or light inducible nuclear localization (189), while chemical control of CRISPR components has been achieved through the use of promoters controlled by small molecules (e.g. doxycycline, tetracycline or 2,4-diacetylphloroglucinol (8, 19, 59, 63, 67, 85, 134, 171, 190), Figure 4C).

On top of controlling the expression of CRISPR components, regulated dimerization or degradation has been exploited as well (Figure 4). Several strategies have been published, for example the fusion of small molecule controlled destabilization (168) or effector domains (78) with Cas9; but most rely rather on fragments of the Cas9 protein. When these split Cas9 parts are fused to certain domains, its assembly can be regulated chemically or optogenetically. Examples of dimerization domains that have been used with split Cas9 are: the rapamycin regulated domains FKBP and FRB (192, 316), the abscisic acid regulated domains ABI and PYL1 (192), the glucocorticoid receptor α (GR)(188), as well as engineered intein domains (58). Split Cas9 proteins (as well as the full length version (157)) have also been combined with 4-hydroxytamoxifen regulated ERT domains of the estrogen receptor to achieve high temporal control by regulating nuclear localisation (188). A photoactivatable split Cas9 (paCas9) has also been reported, in which blue light is necessary for dimerization (191). A special case is represented by “CRISPR ChaCha” and “CRISPR Tango” systems, both in which Cas9 proteins are coupled to extracellular signals via G-protein-coupled receptors (135).

Next to controlling expression, degradation or dimerization, Cas9 activity can also be efficiently inhibited (Figure 4E). This has become possible through the discovery of a large number of

highly specific anti-CRISPR proteins. These proteins have evolved in phages to overcome the bacterial CRISPR-defense. Anti-CRISPR proteins can be differentiated into different categories regarding their mode of action. Dong et al. (2018) created a comprehensive anti-CRISPR protein database (http://cefg.uestc.cn/anti-CRISPRdb) (62). Anti-CRISPR proteins have already been successfully employed experimentally for tight repression of Cas9 activity (203, 219, 240). Thus, it is likely that they will become an important component of the CRISPR toolbox (160). Moreover, variants of anti-CRISPR proteins which can be controlled optically (29) or are only expressed in specific cell types have been generated already (104). In conclusion, a large number of strategies exist that enable temporal control of Cas9 activity; however, a side-by-side comparison of their performance in the diverse CRISPR applications is still missing.

IV. Transcriptional engineering enables targeted manipulation of gene expression

A. CRISPR-mediated gene activation

Pioneering work using Gal4 binding-, Zinc finger- and TALE-proteins has shown that individual transcription factor domains can have a significant effect on gene expression, when bound close to transcription start sites (17, 110, 225). Similarly, modifications to CRISPR proteins can expand their application spectrum beyond genetic approaches (Figure 5). Several studies have compared the performance of TALE- and CRISPR-based artificial transcription factors side-by-side and found that it is generally comparable (77, 79, 110, 196, 208, 304). Although individual TALE constructs can outperform CRISPR constructs, this is not always the case, and variabilities within classes (e.g. by comparing multiple neighboring target sites) are usually larger than between (77, 79, 110, 196, 208, 304). Since CRISPR approaches are however significant more versatile, several “transcriptional engineering” strategies have been developed with the aim to manipulate endogenous gene activities using dCas9. The predominant approach is the generation of direct fusion proteins between dCas9 and putative effector proteins. Many tools contain variations of one particular transcription factor domain, VP16, that has been derived from a herpes simplex transcription factor. In the virus, VP16 initiates a cascade of transcription programs through its strong trans-activator domain that directly interacts with mammalian transcription factors and RNA PolII (161). Moreover, the small modular structure of VP16 makes it easy to generate potent multimers of VP16, which enhances its effect (225, 231). dCas9-VP64 (a quadruple of the transactivation domain) or “CRISPRa” or “CRISPR-on” is the most commonly used CRISPR activator, but others have been reported as well (Table 1). Similarly, VP64 has been combined with other known transactivation domains, either in a direct fusion protein (e.g. VPR), via protein tags (e.g. SAM, SPH) or through RNA binding proteins (and inclusion of binding domains on the gRNA sequence (e.g. MPH, “Casilio”, Table 1, Table 2). Strategies to control dCas9 loading optogenetically (78, 154, 191, 212) or chemically using abscisic acid (78),

gibberellin (11, 78) rapamycin or a rapamycin analogue (11, 78, 255) are however available as well. Overall, a large number of tools have been developed in only few years, many of which have been proven powerful for targeted gene activation. It is nevertheless also evident, that responsiveness to these tools is highly variable (Table 1, Table 2). Indeed, reported activation rates vary by more than five orders of magnitudes for individual genes. Master transcription factor genes reside often, but not always on the lower end of this scale. This can of course have multiple reasons. The most obvious one is that although many dCas9 tools are available, almost all of these are based on the same functional domains (e.g. VP16), exploiting the same gene regulatory mechanisms. It seems not farfetched to assume that some gene promoters (e.g. cell fate determinants) require different (or more) gene regulatory signals to adequately respond (16).

B. Applications of transcriptional engineering to achieve cell state changes

A series of recent high profile publications have nevertheless shown that employing transcriptional engineering can be used to influence the expression of master transcription factors, which is in some cases enough to affect cell identity (Table 2). Targeting of an engineered transactivator (VP192) for example enabled the activation of a series of master transcription (FOXA2, SOX17, GATA4, PDX1, NKX6.1) and pluripotency factors (Oct4, Sox2, LIN28, KLF4) in hESCs and fibroblasts respectively, although to varying degree (9). Cellular state changes were triggered in this experimental system, the most noteworthy ones include the directed endoderm differentiation of human pluripotent cells by induction of FOXA2 and SOX17 (9). More examples of employing transcriptional engineering to direct differentiation of pluri- or multipotent stem cells include adipogenesis in mesenchymal stem cells by targeted activation of PPARG, CEBPA and KLF5 using dCas9-SAM (73), neural differentiation of iPSCs triggered by targeted activation of NGN2 and NEUROD1 using dCas9-VPR (40) or a light inducible transactivator (235) as well as activation of Sox9 to promote stem cell chondrogenesis (264).

In some cases transcriptional engineering is sufficient to reprogram cellular identities beyond the natural potency of the target cell. Examples are the induction of extra-embryonic lineages by inducing Cdx2 and Gata6 with dCas9-VP64 (283), the direct conversion of fibroblasts to neurons by inducing the so-called BAM factors Brn2, Ascl1, and Myt1l (22) and to muscle cells by activating MyoD1 (37). Further examples include the regeneration of human corneal endothelial cells through activation of Sox2 (38), the induction of pluripotency through targeting of Oct4 and Sox2 with SunTag-VP64 (158) and the de-differentiation of neural progenitor cells through targeted activation of Sox1 (16). In a special example, neuronal conversion by transcriptional engineering has even been achieved in vivo (321).

Moreover, the potential of transcriptional engineering goes beyond the induction of mRNAs. Non-coding transcripts can be targeted as well. A good example are long non-coding (lnc) RNAs, which can have important roles in gene regulation, their overall contribution to cellular phenotypes is still under debate (15). This is mainly because their size (up to 100kb) and their gene-regulatory function (often in cis) made it challenging to use classical approaches to manipulate their expression (89). Consequently, several publications have used CRIPSR transactivators to activate lncRNAs in vitro and in vivo already (321), some confirming their roles in particular cellular processes (e.g. NEAT1 lncRNA in paraspeckle formation (303) and linc00513 in interferon signaling (301)), while others did not (86). In conclusion, there are a number of different options for targeted gene activation that when applied appropriately can be powerful tools to manipulate cellular states.

C. CRISPR-mediated gene silencing

dCas9 has also already been used with the aim of suppressing ongoing transcriptional activity (Figure 6). The first publications reported that targeting dCas9 downstream of a transcriptional start site can interfere with active transcription, leading to a reduction of the respective transcript (146). This approach was named CRISPRi (interference). It has, however, been a topic of an ongoing discussion how strong and how generally applicable this effect is (21, 82, 260). To generate more potent silencing tools, several putative repressive domains have been fused to dCas9 (Table 3), of which the most frequently used one is the Krüppel associated box (KRAB) domain. KRABs are transcriptional repression domains that can be found on a large number of zinc finger transcription factors and have been extensively used with engineered zinc finger and TALEN proteins (175). By fusing these domains to dCas9, the transcriptional output of many genes can be reduced by up to 80% (82). Similar to the situation with CRISPR activators, it is however unclear up to now whether the variability in its effects are due to the locus, the targeted gene or cell state. Additional examples of repressive domains that have been tested include the CS (Chromo Shadow) domain of HP1α, and the WRPW domain of Hes1, Mxi (82), the mSin3 interaction domain (SID)(237), MeCP2 and MBD2 ((307), which all can elicit strong, but varying silencing effects (Table 3).

Targeted gene silencing has also been used in a number of ways to influence cell states. Targeting of Oct4 with dCas9-KRAB, for example, has been reported to stimulate pluripotent cells to enter differentiation programs (125), while the targeted silencing of an insulin induced lncRNA (ASIR) in adipocytes demonstrated its role in insulin response (61). KRAB-mediated transcriptional engineering has also been successfully applied in vivo. In this way, it has been shown that targeted (and significantly down-regulated) Tp53 aggravates tumor phenotypes of genetic mouse models of lymphoma (25), while neuronal subtype specific silencing of

synaptotagmin I (Syt1) in dentate gyrus altered the excitatory to inhibitory balance in the mouse hippocampus (320). Ultimately, targeted gene repression has not yet been used to the extent gene activation has, and will likely gain more importance as a tool to manipulate cellular states soon.

D. Transcriptional engineering of orthogonal systems and complex circuits

When striving to engineer cell state changes, manipulating the expression of single target genes might not always be enough. On the contrary, it is probably often necessary to regulate complex gene regulatory networks. For this purpose, several genes have to be altered in their expression, sometimes in different directions, at different time points and to different degrees, in the same cells. While the introduction of multiple gRNAs simply enables the simultaneous targeting of multiple genes, imposing different effects on each of these targets is more difficult and demands the use of orthogonal systems.

Orthogonality can be achieved either by the combinatory use of different Cas9 proteins, the introduction of RNA binding proteins, or the differential control of gRNA expression. Esvelt and colleagues (2013) first employed the combinatorial use of Cas9 proteins derived from different species (*Streptococcus pyogenes*, *Streptococcus thermophiles, Neisseria meningitides, Treponema denticola* (TD)) (68), which all depend on different gRNA scaffolds (24). Combination of Cas9 proteins and dCas9 fusion proteins of these species (as well as Cpf1) provides a platform of orthogonal gene manipulation for yeast and human cells (142, 153) and even orthogonal screens (23). Instead of using different Cas variants to induce orthogonality, gRNAs extended with distinct protein recruiting RNA sequences - MS2 (140, 169), PP7 (39) or com (312) -, can be employed, as those recruit different binding proteins – MCP, PCP, or Com – which can be fused to diverse effector proteins. By this means, Zalatan and colleagues were now able to activate or repress multiple genes seperately, creating synthetic expression programs in yeast and human cells (312). When combining similar systems with orthogonal molecule–degron pairs (168) or chemically-induced dimerization proteins (11), temporal control can be achieved.

Rather than using dCas9 as shuttle for different effectors, Dahlman et al. (2015) used wild-type Cas9 together with either functional gRNAs or gRNAs whose target sequence has been shortened from 20 nucleotides to 14-15 nucleotides. These truncated gRNAs prevent the cutting activity of Cas9, i.e. they cannot be used for gene disruption by mutation. However, the truncated gRNAs are able to form a complex with Cas9 and bind their target. Attaching MS2 aptamers to the gRNAs the truncated gRNAs can be used for gene activation. Using both forms of gRNAs together enables orthogonal gene activation and mutation based on only a single

Cas9 protein (56). Similarly, Kiani and colleagues used Cas9(wt)-VPR fusions to perform gene activation and mutation depending on gRNA lengths (131). Combination of these and other orthologous CRISPR systems with inducible systems has facilitated the creation of transcriptional circuits. The most elementary form of a circuit - an “AND gate”- was built by Liu and colleagues who expressed Cas9 and the gRNA from two different promoters (163): one cancer- and the other bladder-specific. As a consequence, the CRISPR system is only fully active in bladder cancer cells. More complex circuits simultaneously exert genetic activation, repression, and cleavage (130, 131). Meanwhile, even more complex gene expression circuits have been generated in *E.Coli* (190) and in HEK293T cells (78, 130, 193) employing not only “AND”, but also “OR”, “NOT” and “NOR” gates, respectively.

E. Application of complex transcriptional engineering tools to physiology

Non-genetic CRISPR approaches, with and without inducible and multiplexed components, offer a large number of experimental options for basic and applied research. In particular, these tools provide a molecular toolset to determine how genes are regulated and to affect cellular behavior. The options go far beyond simple activation (or silencing) of a specific gene. On the contrary, a recent publication shows that transcriptional engineering is particularly suitable for eliciting subtle gene expression changes (121). In this way dCas9 might be the key to solve a long lasting issue, that is to understand, which expression level (change) is relevant and which not. This alone will have lasting effects on judging molecular data collected from medical samples. In addition, orthologous CRSPR systems, temporal regulation and gRNA multiplexing (as described above) promise to exercise almost unmitigated control over cellular gene expression. This new option could not be any more valuable today, as we have entered an age in which gene regulatory networks can be monitored and modelled routinely, even from a small number of cells (sometimes single cells). Multiplexed CRISPR approaches now lay the groundwork for testing these networks for functionality and causality. In the near future, these approaches will inform us which regulatory networks are critical during homeostasis and disease. Moreover, applications of dCas9 go even beyond that, as they can be used to investigate how cells endogenously control the expression of critical genes. There are multiple approaches towards this goal. One is the use of dCas9 (or wtCas9) to displace TFs bound to a motive in a gene regulatory element (or to mutate the motive directly, Figure 7). This strategy can be used to test, whether an individual TF binding event is necessary for a specific gene expression, and although there are only few examples so far, it has been recently exemplified for a number of Oct4 binding sites (236). A complementary approach to this is the generation of artificial TFs, engineered fusion proteins of dCas9, that unlike VP64 for example, contain domains of TFs naturally binding the gene regulatory region (Figure 8). In this way, it can easily

be tested, whether an individual TF binding event is sufficient for a specific gene expression change, and whether the exact binding location is relevant. A recent example has employed this strategy to show that the targeted binding of the chromatin protein C11orf46 to one of its targets (Sema6a) is sufficient to normalize neurodevelopmental phenotypes (transcallosal connectivity) associated to its loss in vivo (205).

V. Epigenome Editing

A. Limits and barriers to cellular changes

Although a large number of engineered dCas9 transactivators and some repressors have been developed (Table 1-3), we are still lacking a comprehensive understanding of their performance. It does emerge however, that different genes might respond very differently to the same effectors. Some of the disparities might be technical. Others could be misconceptions based on calculating transcriptional activation as relative changes (fold induction); in this way, magnitudes are often rather defined by the detectable background than the obtained level. Unfortunately, it is still completely unclear whether these are irrevocable features of certain genes or whether they depend on specific gRNA sequences or on employed effector proteins. It is also unknown how much influence the underlying cellular state has. To gain insights, comprehensive approaches are needed, as pioneered recently by the Church lab (41).

On the other hand, much variability might be caused by underlying biological mechanisms. After all, it is not unlikely that some genes are intrinsically more resilient against manipulation from outside than others are. If so, master transcription factors, cell identity and reprogramming factors, which are especially detrimental for cellular integrity, are likely part of this group. Therefore, it might be worthwhile to recall which processes affect the consequences of endogenous transcription factor expression. Indeed, a wide range of features are known (e.g. culture conditions, expression level of other genes, active signaling pathways, metabolic conditions…) of which many are thought to act through influencing transcription factor binding. Only the so called pioneering factors, a small subset of master transcription factors are thought to exert their binding autonomously, the binding of other transcription factors depend either on more transcription factors in the vicinity or on further molecular prerequisites (313). Although, these can be quite diverse (reviewed in (147)), many can be grouped under the term “chromatin”, describing the complex aggregate of unmodified and modified DNA, other nucleic acids and proteins that is found in eukaryotic nuclei.

Chromatin features (or “epigenomic” features, an equivalent term) have in common that they are pervasive, that they are occurring in the nucleus and that they usually add at least one additional layer to the duality of DNA and protein. Although chromatin features likely play

various functional roles in epigenetics, it is very important to keep in mind that the terms "epigenetic" (heritable states that have their origin not in the DNA sequence) and "epigenomic" (reversible marks, modifications and features of chromatin implicated in epigenetics) are far from equivalent (249). For the large majority of known types of epigenomic features no functional role has been shown so far, and even for those that can clearly be functionally relevant, this does not have to hold true for the majority of its occurrences (31).

Since it is the case for many transcription factors, it seems plausible that Cas proteins are obstructed by closed chromatin. How much Cas9-mediated targeting is affected by this is however an ongoing debate. In the test-tube, nucleosomes reduce Cas9-mediated cleavage, while addition of chromatin remodeling enzymes improve access to DNA target sites (102, 105, 113). There is also evidence that nucleosomes affect Cas9 binding in cells. Two studies investigating the off-target binding events for 4 and 12 different gRNAs respectively found that off-target binding occurs more frequently at sites that overlap with DNAseI hypersensitive sites (141, 291). This implies that the CRISPR system prefers binding to unobstructed chromatin. An anti-correlation of gRNA activity with nucleosome occupancy has also been observed in the meta-analysis of 30 different CRISPR screens (105). However, it is important to note that while gRNA activity is statistically reduced in the presence of nucleosomes and heterochromatin, it is not absent. Instead, it is clear that genes within heterochromatic chromatin and lacking DNAseI hypersensitive sites can also be efficiently targeted (204). It has even been suggested that binding of Cas9 or dCas9 may locally relax chromatin and induce detectable DNase hypersensitivity (213). In conclusion, manipulation of epigenomic marks has significant potential, be it to enhance CRISPR targeting, to affect TF binding or to influence transcription.

B. Targeted editing of DNA modifications

The first epigenomic mark to be discovered was a chemical modification of DNA, more specifically a methylation of the nucleobase cytosine (5mC) (107). Today we know that adenine can be methylated as well (m6A) and that cytosines may carry residues other than methylation (e.g. 5hmC, 5fC, 5caC). Moreover, many of the enzymes responsible for setting and removing these marks have been discovered, first and foremost, DNA methyltransferases (DNMTs) that are necessary for setting DNA modifications. The *de novo* methyltransferases Dnmt3a and Dnmt3b are responsible for converting unmodified cytosines to 5mC (201), while the maintenance methyltransferase Dnmt1 guarantees the inheritance of DNA methylation during replication (150). Other known DNA modifications are dependent on the presence of methylation and members of the ten-eleven translocation family of enzymes (Tet1, Tet2 and Tet3) (114). Restoration of unmodified residues occurs through the base excision and other repair pathways (96). DNA methylation has been associated time after time to gene silencing,

641 although it transpires that the relation between DNA modifications and gene expression might
642 be a bit more complex. On the one hand a number of functional DNA methylation marks are
643 known (e.g. genomic imprints (13)), on the other functional data indicates that those DNA
644 methylation switches could be rare (reviewed in (248)).

645 The advance of CRISPR opens up a multitude of options employing dCas9 as a targeting shuttle.
646 One strategy named “epigenome editing” aims to edit chromatin features. This approach
647 promises improvement for the targeted manipulation of gene expression and cell states.
648 Furthermore, it enables the distinction of functional chromatin marks, with causal effects on
649 gene expression and cellular states, from non-functional ones without. This issue is relevant for
650 all known epigenomic features, but most pressing for DNA methylation, the most frequently
651 mapped epigenomic modification in human patient samples. It is unclear how many causal
652 marks can be found by EWAS (epigenome-wide association studies), although this question is of
653 high clinical relevance (31). Consequently, several groups have generated dCas9 tools to
654 manipulate DNA methylation (Figure 9, Table 4). To induce methylation on unmethylated CpGs
655 either direct fusions or tagged systems of full length Dnmt3A or its catalytic domain have been
656 generated (4, 6, 75, 112, 159, 207, 246, 250, 273, 324). These examples also indicate that
657 recruiting multiple copies of the DNA methylase (e.g. by the SunTag system) can cause a more
658 efficient and widespread methylation. To improve the efficiency of these constructs, Dnmt3a
659 has also been combined with Dnmt3L (a non-enzymatic enhancer of the endogenous enzyme
660 (246)) or with KRAB domains (4). While all these tools have proven to be powerful in
661 methylating target sites, there are significant differences concerning their efficiencies. For
662 DNMT3A highly efficient editing of DNA methylation have been reported (159), although
663 inherent features, like the used cell line or the targeted genetic loci, seem to matter, causing
664 disparate levels of induced DNA methylation in comparable approaches (75, 273). Combination
665 of DNA methylases with transcriptional inhibitors or co-factors can result in full methylation on
666 the targeted locus (4, 112, 207), although among target genes this varies as well (from 2% up to
667 80% (112, 207)). As an alternative for DNMT3a, the prokaryotic DNA methyltransferase Sss1
668 and a further engineered version, named MQ1 have been fused to dCas9. These epigenome
669 editing tools have been shown to provide comparable efficiency for targeted DNA methylation
670 in vitro, as well as in vivo in mouse zygotes (148, 296, 304).

671 Although the molecular mechanisms of DNA methyltransferases are well understood, and the
672 first attempts engineering this mark are highly promising, a uniform picture about the intended
673 and unwanted consequences has not yet emerged. It is under debate, how precisely
674 methylation marks can be engineered with these tools. Off-target methylation has been
675 reported, but reports disagree about the extent. While Vojta and colleagues report no
676 significant off-target changes in DNA methylation, neither globally, as a consequence of dCas9-
677 DNMT3A expression, nor at predicted off-target sites (273), Stepper and colleagues observed

around 10% increase at remote sequences (compared to 25-35% at the actual targeting sites (246)) and Xiong and colleagues report increased off-target methylation in proximity to the target sites (296). To overcome off-target methylation Pflueger and colleagues make use of a dCas9 SunTag system to recruit DNMT3A and report more specificity compared to direct DNMT3A fusions (207). Interestingly, they also find that an enzymatically dead version of DNMT3A, featuring four alanine substitutions in the catalytic center (F39A, E63A, E155A, R284A) can cause some new DNA methylation marks, suggesting an unwanted recruitment of endogenous DNA methyltransferases to the enzymatically dead enzyme. In contrast to this however, Vojta et al. detected no effects on DNA methylation when using an enzymatically dead version of DNMT3A (273). These discordances are likely explained by the use of different CRISPR constructs, cellular models, expression systems and/or targeting sites. But as recent comprehensive analysis have further consolidated doubts in the specificity of dCas9 DNA methyltransferase fusion proteins (75), the importance of considering non-specific consequences when using these strategies is self-evident.

Nevertheless, transcriptional effects of targeted DNA methylation have been reported, that vary from strong (but heterogeneous) silencing (of an engineered imprinting reporter (159)) to moderate down regulation (of the CpG island genes VEGF, KLF4 and DACH1 (4, 112, 207) or WNT5B (262)), to mild up-regulation (on the gene body of the homeobox gene DLX1 (250)). It has also been shown that engineered DNA methylation marks can affect cellular states, although examples for this are still rare. Noteworthy are the direct downregulation of synuclein alpha protein (SCNA) in Parkinson's patient-derived iPSCs (124) and the indirect expression changes of several genes through local elimination of CTCF binding (159). Another particularly interesting example reports that simultaneous editing of several methylation marks can prevent the onset of senescence in primary breast tissue cells (228).

Similarly to engineered DNA methylation marks, some experimental options for targeted DNA de-methylation are already available (Table 4). Both, direct fusion proteins as well as MCP and SunTag systems, have been developed; almost all of them built on the catalytic domain of mammalian TET1 (6, 16, 49, 74, 159, 176, 184, 200, 300). Moreover, these tools have already been used in oocytes (to manipulate imprints (284)) and in vivo, in embryonic as well as in adult mice (159, 184). All these studies have reported reduced methylation levels on candidate sites following epigenome editing. However, reports about targeted de-methylation using TET1 fusion proteins disagree a lot in their scale. In most cases, the reported reduction of DNA methylation is between 10 and 60% (49, 176, 200, 207, 300), in others cases it is almost complete (16).

The transcriptional consequences of targeted de-methylation are in most cases moderate (49, 184) or even dependent on the additional tethering of trans-activating domains (16), while in

some instances strong gene activation has been reported (e.g. FMR1 (158, 159)). Several publications also report phenotypic effects. Choudhury as well as Xu and colleagues show proliferation phenotypes after the in vitro de-methylation of tumor suppressor and other tumor-relevant genes (BRAC1, RANKL, MAGEB2 and MMP2) (49, 300). In vivo approaches indicate that mouse corticogenesis can be manipulated when TET1 is introduced into the ventricular zone of the mouse fetal brain and GFAP or Dchs1 are de-methylated respectively (184, 195). Further examples of the potential consequence of DNA de-methylation stem from the Jaenisch lab showing that targeting Tet1 to an enhancer of MyoD can de-repress the gene, although this is on its own not sufficient to induce myotube formation in the absence of 5-Aza treatment (159). Similarly, it has been shown that targeted de-methylation of FMR1 in vitro and in in vivo models of fragile X syndrome rescues disease associated phenotypes of affected neurons (160).

Concerning the frequency and complexity of functional DNA methylation marks however, two recent publications deserve attention (269, 324). Ziller and colleagues employ human DNMT3A K.O. (3AKO) ESCs, Verma and colleagues human ESCs lacking the three TET enzymes (TKO). Both cell lines are containing DNA methylation changes on several thousand genomic regions and are affected in their neural differentiation potential. The 3AKO cells produce fewer motor neurons, while the TKO cells fail to undergo neural differentiation altogether. Although in both cell lines a lack of Pax6 expression during differentiation has been found causative, this master transcription factor gene is hypo-methylated in the 3AKO and hyper-methylated in the TKO cells. Targeted DNA methylation using dCas9-DNMT3A in the 3AKO and targeted DNA de-methylation using dCas9-TET1 in the TKO cells both resulted in restored PAX6 expression and an attenuation of the cellular phenotype. This indicates that although (1) only few DNA methylation marks are critical in a certain cellular context, (2) these might have contradicting roles even on a single genomic locus.

C. Editing histone modifications at specific loci

DNA is not present in the nucleus on its own, but incorporated into chromatin. Most complexity in chromatin is represented by the large number of histones and their chemical modifications (117). Histones are structural proteins that bind DNA either as a defined octamer, the nucleosome, or separately in the case of the linker histone H1. The nucleosome consists of two copies each of the histone proteins H2A, H2B, H3 and H4 and 147bp of wrapped around DNA. For each histone, several genetic variants exist, which, although structurally very similar, can fulfill different roles (129). Histones have a common structure, consisting of a globular core and an N-terminal tail (259). Amino acids in both, the core and the tails (but predominantly in the tails) can be post-translationally modified. Currently, 12 chemical modifications on more than

130 sites are known and even more might be discovered in the future (256). Although we understand very little about the vast potential combinatorial complexity, it is already clear that these histone marks are precisely regulated (322). Histone modifying enzymes (e.g. histone methyltransferases, histone de-methylases, histone acetyltransferases, histone de-acetylase) are responsible for catalyzing the reactions (117). Often overlooked is however the fact that many (if not all) of them possess non-histone targets too (83, 318). A large number of chromatin modifying enzymes have been described already (e.g. histone methyltransferase G9a (254), histone acetyltransferase p300 (199), histone de-acetylase HDAC1 (95), histone de-methylase LSD1 (239)). Although we still lack a full understanding of their specificities and interactions, some epigenomic marks have been singled out, since they correlate to gene activities. This holds true for the acetylation of several sites and methylation of H3K4 at active enhancers and promoters (sometimes termed “euchromatin”) and the methylation of H3K27 and H3K9 on repressed regions (such as heterochromatin, reviewed in more detail in (322)).

A series of first examples of epigenome editing to experimentally manipulate histone modifications have been reported (Figure 10); particularly manifold are those dealing with histone methylation marks. Therefore, enzymatic domains of methyltransferases and de-methylases have been directly fused to dCas9 (Table 5) with the aim to engineer the canonical methylation marks on H3K4, H3K27 and H3K9. Examples of effector domains that have been used already include the H3K9 methyltransferases G9a and Suv39h1 (198), the H3K4 methyltransferases PRDM9 and SMYD3 (33, 132) and the SET domain of the polycomb protein EZH2 (methylating H3K27 (44, 72, 198)). The ability of these constructs to generate histone methylation marks differs strongly in the reports, which might be attributed to the different effectors used, the diverse genomic targets, as well as the mark itself. For H3K4 increases in methylation of up to 5-fold have been reported, for H3K9 up to 15-fold and for H3K27 up to 1.4-fold (26, 33, 132, 198).

A special example has been provided by Cano-Rodriguez and colleagues who targeted the catalytic domain of PRDM9 to several inactive promoter regions. In this way they were able to increase H3K4 methylation levels by up to 60% in human cancer cell lines, causing an increase of the according mRNA level of up to 8-fold, although this depended much on the targeted promoter. By analyzing the chromatin state of these promoters, they identified methylation of H3K79 as a predictor for long-term gene induction. Combining dCas9-PRDM9 and dCas9-Dot1 (a H3K79 methyltransferase) significantly prolonged the period of active transcription. Likewise, targeted methylation of H3K9 has also been shown to be expedient for manipulating gene expression. Braun and colleagues developed a chemically inducible dCas9 system to recruit Suv39h1/HP1c complexes to active promoter regions, resulting in an increase of H3K9me3 level of up to 150-fold in HEK293T cells, subsequently resulting in a decrease of mRNA levels of up to 80% (26). O’Geen and colleagues tested SET domains from G9A and Suv39H1 for their ability to

induce H3K9me3 with results ranging from no detectable changes in H3K9me3 (in the case of SUV39H1), to up to 13-fold (in the case of G9A (198)). Similarly, dCas9-Lsd1 has been successfully used for de-methylation of H3K4 (126), resulting in up to 85% loss of H3K4 methylation from active targeting sites.

Among the most frequently used epigenome editing constructs are those that exploit p300, an ubiquitously expressed histone acetylase implicated in enhancer activation (202). The fusion construct dCas9-p300 has been introduced by Hu and colleagues (110), but more recently also been used by a series of other labs (43, 101, 136, 200, 241). Newer versions have been customized to allow chemical control of epigenome editing (76, 241). Taken together, these publications consistently report that targeting of p300 results in local acetylation of K27 on histone H3. Such edits can be very efficient (10-fold over a catalytic inactive dCas9-p300) and result in strong transcriptional gene induction (up to 10000-fold), both on proximal promoter elements as well as on distal enhancers (101). Other studies report that transcriptional activation is usually rather in the range of 10- to 100-fold (43, 110, 200, 241). Recently, Zhang and colleagues reported the use of a different histone acetylase, EIN2. Using dCas9-EIN2 the authors were able to induce acetylation of H3K14, as well as H3K23 in *Arabidopsis thaliana*, causing a 3-fold upregulation of the gene (317). Similarly, targeted histone de-acetylation has been reported by using dCas9-HDAC3 and dCas9-HDAC8 (42, 143).

Examples of cellular state changes through engineered histone marks are still sparse, but they do exist (Table 5). One of the earliest examples was provided by Kearns and colleagues, targeting the histone (H3K4) de-methylase LSD1 to candidate enhancers of Oct4 in pluripotent stem cells (126). On some sites, this resulted in loss of Oct4 expression and pre-mature stem cell differentiation. Conversely, engineering histone acetylation on the Oct4 promoter can facilitate induction of pluripotency in fibroblasts, although with increased latency compared to transcriptional engineering with VP64 (158). An especially interesting cellular feature has been investigated with epigenome editing by Chen and colleagues recently, namely neuronal activity-inducible gene transcription (42). Synaptic activity in neurons results in some immediate transcriptional changes on genes like Fos and Npas4, but how this is molecularly controlled was unknown. The authors found that these expression changes are mainly achieved by regulating the time period these genes are actively transcribed (the length of transcriptional bursts, not their frequency). Through the use of dCas9-p300 and dCas9-HDAC8 the authors showed that histone actetylation is critical in defining the length of transcriptional bursts at these genes, which in turn have significant consequences for the neuronal physiology (42).

Although a plethora of histone modifications and many enzymes responsible for their deposition are known, only a limited number of enzymatic activities, mostly related to acetylation or methylation, have been tested in combination with dCas9 to date. One exception

is again the aforementioned study by Cano-Rodriguez and colleagues, in which the authors report a fusion protein between dCas9 and the histone ubiquitin conjugating enzyme UBE2A. Although this particular construct had little consequences on tested gene activities, it indicates that epigenome editing is applicable to non-canonical epigenomic marks as well (33).

D. Engineering DNA topology

Besides manipulating DNA modifications and histone marks, CRISPR approaches can also be used to alter the three dimensional arrangement of DNA. Clearly, all cells possess regions of high and low DNA compaction, something that can be revealed with unspecific DNA dyes like DAPI. Co-staining with antibodies, RNA or DNA probes indicate that nuclear domains (or compartments) exist, which can be intensely regulated during development (35). Consequently 3D- (or topological) architecture has been suspected to play multiple roles in gene expression for decades (115). Initially, macroscopic nuclear processes were in the focus (nuclear matrix attachment, perinuclear and perinucleolar positions (81, 84, 100, 144, 211)), but more recently novel molecular techniques (3C, 4C, HiC (60)) enable the mapping and quantification of topological differences on different scales. This resulted in the elevation of DNA topology to a canonical epigenomic feature. Today, it is clear that two ubiquitous proteins, CTCF and Cohesin, play key roles in maintaining nuclear architecture, both locally and globally, for example by regulating the activity of insulator elements (18, 92) or the boarders of topologically associated domains (TADs) (180)). While much still has to be clarified about the relationship between topological processes and gene regulation (245), it appears clear that chromatin architecture can play crucial roles in the expression of genes (164), however these might be rare (266).

Approaches employing CRISPR constructs enabling manipulation of nuclear architecture are equally sparse and promising. An orthologous system based on *S.pyogenes* and *S.aureus* dCas9, each fused to a different part of an inducible dimerization system, named CLOuD9, has attracted much attention due to its efficiency and reversibility (183). Addition of the plant hormone ABA is triggering the two protein compartments to join, since those are bound to the dCas9 proteins, these come into proximity. By targeting the orthologous dCas9 proteins to different DNA regions DNA loops are created, although the reported consequences on transcriptional activity were rather minor (1,5x of background levels at the Oct4 gene). These results were in agreement with a comparable system developed at the same time (dCas9_Zip (91)). All in all this indicates, that dCas9 approaches are a unique option to test causality for individual topological features and potentially will help to clarify under which conditions those have an effect on gene activity.

E. Modifying specific RNA sequences

Proteins and DNA are not the only chromatin components that can be chemically modified, also ribonucleic acids can carry a large number of chemical residues (194). First noticed as a common feature of tRNAs, it is now accepted that all sorts of RNAs can harbor modified bases (152). More than 100 types of RNA modifications are known (34, 166) and at least 12 have been observed at eukaryotic mRNAs (152). Although we are far from a complete characterization, technologies for mapping specific features are on the rise (98). A variation of bisulfite sequencing for example enables calling of 5mC residues in RNA (229). Likewise a number of techniques have been published, either based on small molecules or antibodies, that facilitate detection of specific RNA modifications (e.g. pseudouridine (230), inosine (252) or 5mA (155)). These pioneer studies indicate that RNA modifications are undergoing dynamic changes during development. Moreover, although we are far from a comprehensive understanding, indirect evidence suggests roles for some RNA modifications, for example m6A methylation in regulating RNA stability (179, 222). Although targeted manipulation of RNA modifications has only been applied a few times until now, it has already been coined “epitranscriptome editing”. The utilization of constructs based on dCas13 for targeting RNA molecules and its application for site-specific de-amination of adenosines (1, 53) promises to become the blueprint for future experiments testing the significance of RNA modifications (194).

VI. Generation, availability and use of CRISPR libraries

A clear distinction of CRISPR to all other gene targeting options is the straight forward use for genomic, transcriptomic and epigenomic screens. The reason is that Cas9 (and dCas9) targeting sites are solely defined by the 20bp sequence of the gRNA spacer and gRNAs are so short, that they are exceptionally suitable to be arranged in libraries. Screens have been already performed to disrupt, repress, or activate different genes using wtCas9, or dCas9 fused with repression or activation systems (234). To enable stable integration of the gRNA into the host’s genome, the gRNAs are usually cloned into lentiviral vectors (94, 233, 234). Cells that have obtained a virus particle can be selected by drug resistance or fluorescence (3). Lentiviral vectors either also contain a cassette for Cas9/dCas9 expression (233), or the protein is already stably expressed in the host cells (281). Although lentiviral vectors are by far the most frequently used system, adeno-associated viral (AAV) vectors (50, 253), synthetic CRISPR reagents (47), and piggyBAC transposons (297) have also been used already.

In general, three types of gRNA libraries are available: (A) arrayed libraries, in which each expression vector or viral particle is provided in separate reaction vessel (usually arrayed in 96- or 384-well plates), (B) pooled libraries in which all gRNAs targeting the genomic loci to be

screened as well as respective positive, negative, and non-targeting controls are combined in one reaction mix (often as lentiviral particles), and (C) non-synthetic libraries, in which the source of gRNA spacer is less defined (234). The advantage of the arrayed approach is that for every tissue culture well, the targeted sequence is known. This simplifies the analysis, since arrayed screens are not dependent on a readout allowing to physically separate cells with a phenotypic response from the rest. Thus, arrayed libraries allow to screen for complex phenotypes and minor effects, which can be assayed by imaging or gene expression analysis for example (3). Furthermore, transduction can be performed with high multiplicity of infection (MOI) since all gRNAs in one well are identical. Arrayed screens, however, have the disadvantage that each vector and each viral particle has to be produced separately; therefore, arrayed libraries are usually not very comprehensive, but nevertheless expensive. An alternative are pooled libraries, in which mixtures of vectors containing expression cassettes for 10^3-10^5 gRNAs are used to generate one single virus batch. In the pooled approach a low MOI is necessary, e.g. 0.3 (234), ensuring that most cells obtain not more than one gRNA. Since it cannot be controlled which cells receive which gRNAs, cells showing a phenotype of interest have to be physically separated, e.g. by antibiotic selection or FACS. After this selection, gRNA sequences have to be recovered to determine the gRNAs that might have caused the phenotype. Recently, the first strategies to screen pools for combinatorial events have been developed, e.g. CombiGEN-CRISPR (289) and CRISPR-based double knockout (90). Pooled libraries are cheaper and usually contain more gRNAs than arrayed libraries, their complexity is however greatly exceeded by non-synthetic libraries, in which the spacers are not derived from synthesized oligonucleotides, but rather from a more complex source, e.g. fragmented genomic DNA (47, 138, 139, 145). Through these methods comprehensive library pools can be produced very cheaply. Such libraries have the undeniable dis-advantage that they contain many nonfunctional gRNAs and require larger cell numbers for virus production and screening. They are, however, the only available option for entirely unbiased discovery of truly unknown mechanisms so far.

Non-genetic CRISPR screens have already successfully identified genes driving or blocking cellular states. Indeed, almost all reported screens employing dCas9 so far are based on CRISPRa or CRISPRi. Combining dCas9-VP64, VPR or SAM with TSS gRNA libraries enables to attribute new roles to coding and non-coding transcripts. Among the processes investigated (and genes found) are gene activities protecting from Influenza (B4GALNT2 (97)) and Zika virus infection (IFNL2, IFI6 (66)), BRAF inhibitor resistance in melanoma cells (e.g. EGFR (140)/ the lncRNA EMICERI (122)) and mediators of Rigosertib drug sensitivity (TACC3 and KIF2C (120)). Although these cases deal with a rather strong readout (e.g. cell death), this is not a prerequisite. It has been impressively demonstrated recently that CRISPRa screens can also be applied to mediators of cellular transitions (162). Establishing an activator screening platform in ESCs allowed the authors to investigate factors driving neuronal differentiation. Moreover,

some of the found candidates (Ngn1, Foxo1 and Ezh2) were able to reprogram fibroblasts to neuronal fate when simultaneously expressed. In a similar approach factors with the potency to affect different pluripotent stem cell states have been discovered in a CRISPRa screen as well (e.g. Sal1 (306)).

The success of CRISPRa screens indicate that other applications of dCas9 might also be exploited for genome-wide screens. For example, the discovery, functional annotation and molecular classification of cis-regulatory elements seem not far fetched. It has been shown already that gene-regulatory elements can be activated (e.g. using dCas9-p300 (42, 101) or dCas9-VP64 (177) or silenced (e.g. using dCas9-LSD1 (126), dCas9-HDAC8 (42), dCas9-KRAB (242, 260) or dCas9-SIN3A (181)) by transcriptional engineering and/or epigenome editing. Therefore, the extension of these strategies to less biased approaches, to regulatory or epigenomic screens, is promising. Using gRNA libraries targeting non-coding regions surrounding one, several or many gene(s) and combining it with one, several or many dCas9 constructs offers the unique potential to put functional layers on genomic and epigenomic maps. First steps in this direction have been already successful. dCas9-KRAB has been combined with barcoded gRNAs (80, 294). In this way the functional relevance of 71 individual enhancer elements in 15 super-enhancers (e.g. close to HBG2, Pim1, …) has been tested by single cell RNA-Seq with the intriguing result that likely only a small number of annotated gene regulatory elements are relevant effectors of gene expression (294). A similar study used dCas9-KRAB and 5920 barcoded gRNAs to determine ca 500 candidate gene-enhancer pairs without strong a priori assumptions (80). Although screens using chromatin modifiers are not yet on the same level, first published results are promising. Targeting dCas9-LSD1 to nine candidate pluripotency enhancer sites (126), or dCas9-p300 to a series of DNAse Hypersensitive sites (DHS) at one locus using small gRNA libraries (136) was sufficient to identify functional gene regulatory elements. Some care has to be taken in the interpretation of these experiments though, since dCas9 alone has the power to disrupt interactions between transcription factors and DNA as well (see above and (232, 236)). Nevertheless, in future these technologies will allow to address a number of fundamental questions: (1) Which genes are critical for defining cell states? (2) Which chromatin features are critical for defining expression of these genes and where in the regulatory landscape are they found/have to be? (3) Do different promoters require different stimuli for targeted transcriptional changes and if yes, is there an underlying gene regulatory grammar? (4) Is there a clear functional hierarchy of chromatin features? Expanding these approaches, will not only reveal which modifications, where in the genome have gene-regulatory potential, it would also inform, whether these have to coincide with those naturally occurring, and finally, where the molecular switches are that define cellular states in health and disease.

VII. Therapeutic strategies harnessing transcriptional engineering and epigenome editing

Unlike genome editing, transcriptional engineering and epigenome editing are not fully recognized for their potential in future therapeutic strategies yet. This is surprising, since they share with their genetic counterpart the target specificity that would be necessary for personalized and precision medicine approaches. Both can also, and in contrast to pharmacological approaches, be applied in a cell-type specific manner (see above), allowing to target only disease relevant cell populations. dCas9 approaches hold, however, the additional advantage that they are not resulting in DNA mutations.

Multiple proof-of-principle studies have already laid the groundwork for future therapeutic strategies of transcriptional engineering and epigenome editing. In this context they have been used with three quite different objectives in mind, namely (1) to change cell types, (2) to alter cell behavior and (3) to manipulate disease associated gene expression (Figure 11). The first strategy aims to provide cells lost (through injury, disease or aging) for cell replacement therapies. This can be achieved by directed differentiation of stem cells or by cellular reprogramming, either in vitro (requiring subsequent transplantation) or in vivo (in the affected organ) (87). Although in theory, several approaches could be employed, transcriptional engineering has particular appeal. After all, it does not result in genetic mutations, it affects the endogenous master regulator genes directly and the cellular outcome can be precisely defined through gRNA multiplexing. Directed differentiation of stem cells have been amongst the most common applications of transcriptional engineering already, including neurogenesis (40), adipogenesis (73), or chondrogenesis (264). dCas9 approaches have also successfully been employed to bypass the limited regeneration potential of the brain converting non-neuronal cells into neurons, both in vitro (22) as well as in vivo (321). Similarly, transcriptional engineering has been applied to regenerate corneal endothelial cells in the rat (38) and to generate extra-embryonic tissue (283), muscle (37) sweat gland cells (251), and oligodendrocyte progenitor cells (178).

Manipulation of cellular behavior to ameliorate disease is the aim of the second strategy (Figure 11). Cells targeted could be those affected by the disease (e.g. cancer cells), but also different populations (e.g. immune cells). In theory, any cellular behavior (e.g. proliferation, migration, and maturation) should be accessible through targeted manipulation of gene expression; however, studies following this strategy are still sparse. One rare example is the targeted downregulation of the extracellular matrix protein granulin in liver cancer cells, decreasing their invasive potential (280). Others include the targeted upregulation of a non-affected laminin gene in a mouse model of congenital muscular dystrophy type 1A (128) or the activation of TRAIL signaling in human cancer cells, leading to a decrease in proliferation and commencing apoptosis (214).

The third strategy is to reverse changes causing the disease directly (Figure 11). Here, a large number of studies have already provided a solid base for transcriptional engineering and epigenome editing alike. Overall, dCas9 approaches have been used to upregulate silenced transcripts, to downregulate mutated genes and to revert disease relevant epigenetic changes. Typical examples of targeted gene activation of disease relevant transcripts include the normalization of a haploinsuficient mouse models of obesity (177) and Dravet syndrome (51), the activation of the tumor repressor PTEN in human cancer cells (187) and the targeted induction of factor 8, whose reduced expression or mutation can cause hemophilia A (208). Similarly, cystic fibrosis is caused by mutations in the CFTR gene resulting in fewer functional chloride channels at the cellular membrane, which, as recently shown, can be counteracted by transcriptional engineering in human patient cells (271). One example of targeted gene repression deals with transcripts containing expanded microsatellite repeats, a hallmark of a number of human diseases including Huntington`s, ALS, myotonic and corneal dystrophy (210); another the dCas9-mediated down-regulation of the squamous cell carcinoma candidate oncogene DeltaNp63, resulting in reduced cell proliferation and tumorigenicity of human SCC cell lines and xenograft models (310).

Collectively, transcriptional engineering has been used in the context of many medical conditions already, two fields, however, have been especially prolific. The first are the many dCas9 based approaches engineered to purge or repress retrovirus activity (summarized in (279)); the other is cancer research. Here, targeted gene activation has been used in a large variety of ways, e.g. to generate (more realistic) mouse models of cancer (25, 272), to unravel gene interactions during EMT (265), to investigate the effect of cancer-specific overexpressed genes (25, 299), to sensitize cancer cells to apoptotic signals (214) and even to propose a therapeutic strategy based on telomerase activity (57).

Although fewer examples exist, epigenome editing has been applied with therapeutic objectives as well. Outstanding are two studies dealing with different causes of neurological disorders. The first reports the correction of fragile X syndrome through targeted DNA de-methylation (160). Fragile X is caused by a CGG repeat expansion in the promoter of the FMR1 gene that results in gene silencing and intellectual disabilities. Interestingly, epigenome editing not only rescues FMR1 expression, but also electrophysiological abnormalities of neurons derived from fragile X patient derived iPSCs. Moreover, targeted DNA de-methylation rescues FMR1 expression even in post-mitotic neurons (160). The second study shows that epigenome editing can also be applied to normalize misregulated neuropsychiatric risk genes. In particular, the authors show that loss of the haploinsufficient risk gene C11orf46 results in upregulation of multiple axonal genes and loss of transcallosal connectivity in the mouse. Silencing a key target of C11orf46, namely SEMA6A, via epigenome editing was sufficient to rescue this complex brain

phenotype (205). These recent studies indicate that transcriptional engineering and epigenome editing have strong potential for future therapies of a large number of disorders.

VIII. Conclusions and outlook

The rise of CRISPR as a molecular tool promises soon to enable manipulation of cellular states at will. Already now, genome targeting, transcriptional engineering and epigenome editing approaches can be used to mutate, activate and repress individual genes, as well as to manipulate their epigenomic environment by editing DNA modifications, histone marks and other chromatin features. Moreover, these technologies are applicable to in vivo approaches and can be multiplexed and combined with orthologous approaches to regulate complex transcriptional network programs. Importantly, dCas9 approaches also allow addressing unresolved key questions without a priori assumptions through CRISPR screens. Despite promising examples of epigenome editing and transcriptional engineering, there is sustained demand for more refined targeting tools. These should take into account the large number of known and potentially relevant epigenomic features and mechanisms. Once refined these non-gene CRISPR approaches have strong potential for applications in therapeutic settings, be it to change and replace lost cells after injury, to alter cellular behavior to alleviate disease states or to manipulate disease associated gene expression.

GRANTS:

This work was supported by Deutsche Forschungsgemeinschaft DFG (STR 1385/1-1).

DISCLOSURES:

No conflicts of interest, financial or otherwise, are declared by the authors.

FIGURE LEGENDS:

Figure 1: New molecular CRISPR tools to manipulate cellular states. The nucleus is the steering console of the cell. Molecular instructions for all possible cellular states are encoded in DNA. DNA is embedded in chromatin. CRISPR strategies include the activation and repression of critical genes, the interference with TF binding, the use of artificial transcription factors and the manipulation of chromatin features.

Figure 2: Updated annotated alternatives to the iconic epigenetic landscape metaphor (276). (A) Cellular properties (plasticity, potency), natural state changes (fate choice, lineages, cell types, differentiation) and molecular foundations (genes, gene expression) can all be added to the original concept. (B) Cellular reprogramming is a process of state changes beyond the natural potency. Several types can be distinguished. (C) Transcription factors are powerful switches controlling cellular states. In the context of cell identity, critical transcription factors can be classified according to their potency. Master transcription factors influence cell fate decisions during development. Reprogramming factors can alter cell identities beyond the natural potency of the cell.

Figure 3: gRNA multiplexing strategies. (A) Plasmids comprising different gRNAs can be pooled and used for simultaneous transfection of cells. (B) Multiple gRNAs, each with individual promoters and termination signals, can be combined on one plasmid. (C) Cas proteins and gRNAs can be assembled to ribonucleoproteins and pooled before electroporation. (D-G) gRNAs can be expressed from one precursor transcript and separated by Csy4 sites, self-cleaving ribozymes sites, short RNAs (shRNAs/miRNAs) or tRNAs. (H) In a crRNA array gRNAs are expressed under one promoter separated by direct repeats; Cas12a (Cpf1) uses its RNase activity to cut the crRNAs from the precursor transcript.

Figure 4: Different measures to control CRISPR activity. (A) Heat-shock inducible expression of gRNA. (B) Temperature-sensitive Cas9 variant, which is active at 29°C and inactive at 37°C. (C) Ligand dependent expression of gRNA or Cas9 protein. (D) Dimerization domains fused to split Cas9 or dCas9 leading to ligand dependent dimerization and thus activation of Cas9 or effector binding to dCas9. (E) (Inducible) Anti-CRISPR proteins inhibit CRISPR activities. (F) Inducible expression of gRNA upon exposure to light of a specific wavelength. (G) Light-sensitive Cas9 variant, which is active when exposed to light of a specific wavelength. (H) Light-sensitive dimerization domains fused to split Cas9 or dCas9.

Figure 5: Examples of targeted gene activation strategies. Transactivator domains can be directly fused to dCas9 (upper left), bound by a protein (SunTag, upper right) or RNA tag (lower right) or combinations thereof (SAM, lower left). Proximity of transactivation domains to gene promoters results in gene activation.

Figure 6: Examples of targeted gene repression strategies. Several repressor domains (KRAB, CS, WRPW, Mxi, SID) have been fused to dCas9. Proximity of repressor domains to gene promoters results in downregulation of transcription.

Figure 7: Example of targeted interference with TF binding. dCas9 (left) or wtCas9 (right) can be used to block TF binding or mutate individual TF motives. In this way, it can be tested, whether TF binding is necessary for gene expression or a certain cellular states.

Figure 8: Example of an artificial transcription factor. Endogenous TF domains can be fused to dCas9 and targeted to a natural TF motive or an alternative site. In this way, it can be tested, whether TF binding is sufficient for gene expression and a particular cell states, and whether the exact position of TF binding is relevant.

Figure 9: Targeted manipulation of DNA modifications. Combination of dCas9 with enzymatic activities setting (DNMT3a) or removing (TET1) DNA modifications enables epigenome editing.

Figure 10: Targeted manipulation of histone marks. Combination of dCas9 with enzymatic domains from histone modifiers enables epigenome editing. Shown are examples for histone acetylation (p300) and histone methylation (G9a).

Figure 11: Transcriptional engineering und epigenome editing are suitable for complex experimental and therapeutic strategies. (Upper part:) dCas9 approaches enable non-genetic screens, the manipulation of complex transcriptional networks and in vivo approaches. (Lower part:) These experiments will inform, set up and enable therapeutic strategies aiming to replace cell types, to alter cellular behavior and to manipulate disease associated gene expression.

Table 1: Examples of transcriptional engineering employing targeted gene activation.

Load	Cell(s)	Target gene(s)	Transciptional changes (up to)	Publication
dCas9-VP48, dCas9-VP160	HeLa, early mouse embryos	IL1RN, Sox2, Oct4	10x	(46)
dCas9-VP64	HEK293T	NTF3	>500x	(167)
dCas9-VP64	HEK293T	IL1RN, ASCL1, NANOG, HBG1/2, MYOD1, VEGFA, TERT, IL1B, IL1R2	IL1RN (>2000x), ASCL1 (250x), NANOG (13x), HBG1/2 (>100x), MYOD1 (50x), VEGFA (2x), TERT (2x), IL1B (10x), IL1R2 (20x)	(204)
dCas9-VP64	B-cell lymphoblastic leukemia cells	Mgmt	30x	(25)
dCas9-VP64	In vivo	Sim1a	3x	(177)

	(mouse kidney)			
dCas9-VP64, dCas9-VP160, dCas9-VPR	NSC34, HEK293T	TIMP 1/2/3	TIMP 1/2/3 (30x)	(65)
dCas9-VP64, SAM	HEK293T	Rex1, Oct4	Rex1 (30x)	(169)
dCas9-VP64 MPH	HeLa	DR5	5x	(214)
dCas9-VP160	NCI-H460	NOP14-AS1	10x	(86)
dCas9-VPR, SAM	in vivo (Drosophila m.)	Luciferase	1000x	(319)
SAM	NSCLC	FGFR1	50x	(265)
SAM	HAP1, U2OS	NEAT1	3-4x	(303)
SAM, MPH	HEK293T	ASCL1 and various other factors	40x-600x	(140)
SAM	HeLa	Linc00513	15x	(301)
SAM	Fibroblasts	Oct4, Sox2	15x-100x	(158)
SPH	in vivo (mouse brain)	Ascl1, Neurog2, NeurdoD1, Acta1, Dkk1, Slc6a4, Rnf43, Bcl2, Znf3, Prdm16, Miat, Halgr, Fendrr, Lncpint	1-100x	(321)
MPH	HEK293T	IL1B, HBG1, ZFP42	20- 6000x	(56)
Butterfly, dCas9-VP64, dCas9-VPR, SAM, SunTag, dCas9-P300, dCas9-VP160,	HEK293T	Ascl1, NeuroD1, TTN, HBG1, RHOXF2, ACTC1	10x to 10000x	(41)
Casilio	HEK293T	Oct4	6000x	(45)
Cpf1-VPR, Cpf1-P65	HEK293T	HBB, AR, NPY1R	80x	(255)
dCas9(SP) dCas9(NM) with chemically dimerizing VPR	HEK293T	ASCL1, TTN, RHOXF,IL1RN	ASCL1 (90x), TTN (25x), RHOXF (180x), IL1RN (750x)	(11)
mini-dCas9-VTR	HEK293T	eBFP2 Reporter	1200x	(165)
MCP-VP64	HEK293T	CXCR4	5x	(312)

Table 2: Examples of targeted gene activation inducing cell state changes.

Load	Cell(s)	Target gene(s)	Transciptional changes (up to)	Publication
dCas9-VP64	mESCs	Cdx2, Gata6	Cdx2 (>100x)	(283)
dCas9-VP48 dCas9-VP96 dCas9-VP192	Fibroblasts, hESC	Oct4, Sox2, Nanog, LIN28, KLF4, CDH1	Oct4 (100x), Sox2(30x), Nanog(25x), LIN28(5x), KLF4(3x), CDH1 (70x)	(9)

dCas9-VPR	iPSCs	MIAT, NeuroD1, Ascl1, RhoxF2, TTN, ACTC1	MIAT (280x), NeuroD1 (87x), Ascl1 (4600x), RhoxF2 (18000x), TTN (20000x), ACTC1 (330x)	(40)
SAM	Mesenchymal stem cells	PPARG, CEBPA, KLF5	PPARG (15x), CEBPA (10000x), KLF5 (90x)	(73)
Butterfly	Fibroblasts	MyoD1	80x	(37)
Butterfly	HEK293T, Fibroblasts	Ascl1, Myt1l, Brn2	Ascl1 (6000x)	(22)

Table 3: Examples of transcriptional engineering employing targeted gene repression.

Load	Cell(s)	Target gene(s)	Transciptional changes (up to)	Publication
dCAS9	HEK293T	mRFP Reporter	300x repression	(216)
dCas9-KRAB	K562	HBE1	10x repression	(260)
dCas9-KRAB–MeCP2	HEK293T, SH-SY5Y	YFP Reporter, CANX, CXCR4, CHK1, SEL1L, ARPC2, MAPK3, BRCA1, BLM, GZMM, MAPK3, RHOA	10x repression	(307)
PP7-SID	HEK293T	LUC reporter	5x repression	(237)
dCas9-KRAB, dCas9-Mxi1	S. cerevisae, HEK293T, HeLa	Tef1, GFP, CD71,CXCR4	3x - 53x repression	(82)

Table 4: Examples of epigenome editing targeting DNA modifications.

dCas9-	Cell(s)	Target genes	5mC (abs.incr.)	mRNA / Protein	Phenotype	Publication
DNMT3A (cat.dom.)	hESC (DNMT3A K.O.)	PAX6, ARX (RE)	Partial rescue (↑20-40%)	Rescue (↑)	Rescue of motor neurogenesis and repression of floor plate induction	(324)
DNMT3A (cat.dom.)	mESCs (Dnmt3a/b D.K.O. + transient repression of DNMT1)	Dazl, Foxa2, Id3, Foxb1, H19, Fboxo40 (P)	(↑10-40%)	No sign. change	n.s.	(75)
DNMT3A (cat.dom.)	Gastric cell cancer line (AGS)	WNT5B, SOX9, FGFR1, KLF9, HDAC11, APOC3, APOA1…	(↑)	20-70% (↓)	Cell-migration	(262)
Tet1 (cat.dom.)			(↓)	1.5-5-fold (↑)		

		(RE)				
DNMT3A (602-912)	HEK293T	IL6ST, BACH2 (P)	(↑30-60%)	40-50% (↓)	n.s.	(273)
DNMT3A (IF A1 IF A2 cat.dom. SunTag)	HEK293T	BACH2, HOXA5, KLF4 (P)	(↑2-80%)	~80% (↓) DNMT3A1-SunTag (HOXA5)	n.s.	(112)
DNMT3A (cat.dom. E756A SunTag)	HEK293T	DLX1	(↑~60%)	2-fold (↑)	n.s.	(250)
DNMT3A (cat.dom.)	Hep3B Hek 293T	GRN (P)	(↑~50%)	~30% (↓)	Proliferation (↓)	(280)
Dnmt3A (cat.dom.) Dnmt3L	HEK293T	EpCAM, CXCR4, TFRC (↓)	(↑20-40%)	~40-50% (↓)	n.s.	(246)
Dnmt3A (cat.dom.) Dnmt3L	Myo-epithelial donor cells	p16, RASSF, HIC1, PTEN …	(↑20-60%)	10-60% (↓)	Senescence (↓)	(228)
DNMT3A (cat.dom.) SunTag	MCF-7 HeLa	UNC5C, BCL3, DACH1 (P)	(↑20-50%)	n.s.	n.s.	(207)
DNMT3A (cat.dom.)	hIPSC	SNCA	(↑10-60%)	~20% (↓)	Cell viability (↑)	(124)
DNMT3A (f.length)	mESC	SNRNP-GFP reporter	(↑35-70%)	GFP-loss in up to 70% of cells	n.s.	(159)
DNMT3A, DNMT3L KRAB	K562 HEK293T	IFNAR1, VEGFA, B2M	(↑ up to 100 %)	Up to 500-fold (↓)	n.s.	(4)
MQ1 (q147l)	HEK293T, K562 in vivo	HOXA5, HOXA6, Igf2/H19 DMR …	(↑35-70%) HOXA5	40% (↓) HOXA5	n.s.	(148)
SSS1 (cat.dom.)	Embryos (2-cell stage)	Major satellites	(↑20%)	n.s.	Heterochromatin index (↑)	(304)
SSS1 (cat.dom.) Split	HEK293T E.Coli (reporter plasmids)	SALL2 P2 E1a (P)	(↑5-20%) SALL2 P2	n.s.	n.s.	(296)
Tet1	mEmbryo	Dchs1	(↓40-70%)	2.5-fold (↑)	Proliferation (↓)	(195)
Tet1	Tet TKO hESCs	Pax6	n.s.	400-fold (↑)	Neural differentiation (↑)	(269)
Tet1 (cat.dom.)	iPSCs neurons (FX52)	FMR1	(↓60-100%)	481-fold (↑)	Rescue of FXS Phenotype	(160)

Tet1 (cat.dom.)	NPC	Sox1	(10-50% ↓)	n.s.	Neural stem cell reprogramming (↑) (+dCas9-VP64)	(16)
Tet1 (cat.dom.)	HeLa MCF7	BRCA1	(↓10-50%)	2.5-fold (↑)	Proliferation (↓)	(49)
Tet1 (cat.dom.)	mESCs, neurons C3H10T1/2 MEFs, …	Bdnf, MyoD	(↓35-60%)	3-fold (↑)	fibroblast to myoblast conversion (↑)	(159)
Tet1 (cat.dom.) SunTag	ESCs E14 VZ	GFAP, H19	(↓60-90%)	1.7-fold (↑)	n.s.	(184)
Tet1 (cat.dom.) SunTag	Arabidop.	FWA	(↓50%)	8-fold (↑)	Changes in flowering time and leaf number	(74)
Tet1, Dnmt	mouse oocytes	Agouti Snrp	(↑80%; ↓10%)	n.s.	n.s.	(284)
TET1, (cat.dom.) DNMT3A (cat.dom.) SunTag	CHO	ST6GAL1	n.s.	>30-fold (↑) ~80% (↓)	Changes in glycan structure	(176)

1130

1131 **Table 5:** Examples of epigenome editing targeting histone marks.

dCas9-	Cell(s)	Target genes	Histone modifications	mRNA / Protein	Phenotype	Publication
P300 (cat.dom.)	HEK293T	IL1RN, MYOD, Oct4, β-globin	H3K27Ac (↑10-fold)	10-10000-fold (↑)	n.s.	(101)
P300 (cat.dom.)	T-cells (68-41, Primary)	Foxp3	H3K27Ac ↑	3-fold (↑)	Treg signature (↑)	(200)
EIN2-C (C-ter. dom.)	Arabidop.	EBF2	H3K14Ac (↑10-fold)	2-3-fold (↑)	Plant size (↓)	(317)
P300 (cat.dom.)	HEK293T	Oct4	H3K27Ac ↑	5-20-fold (↑)	n.s.	(110)
P300 (cat.dom.)	HEK293T	IL1RN	H3K27Ac (↑~25%)	30-fold (↑)	n.s.	(43)
P300 (cat.dom.)	K562 HEK293T	β-globin Her2	H3K27ac (↑4-fold)	n.s.	n.s.	(136)
P300 (cat.dom.)	HEK293T	IL1RN, RHOXF2, TTN	n.s.	100-10000-fold (↑)	n.s.	(241)
P300, HDAC8	neurons	Fos, Npas4	H3Ac (↑50% P300 ↓40% HDAC	1.5-fold (↑)	neuronal spiking frequency	(42)

HDAC3	N2a	Smn1, Mecp2, Isl1	n.s.	80% (↓)	n.s.	(143)
P300	MEFs	Oct4	H3Ac (↑4-fold)	10^5-fold (↑)	Pluripotency (↑)	(158)
LSD1	mESCs	Oct4, Tbx3	H3K4me2 (↓ > 90%)	90% (↓)	Cell morphology	(126)
PRDM9 (cat.dom.)	HEK293T A549	ICAM1, RASSIF1A, EPCAM, PLOD2	H3K4me3 (↑60%)	8-fold (↑)	n.s.	(33)
Suv39h1/ HP1c	HEK293T mESCs	CXCR4, Nkx2.9	H3K9me3 (↑150-fold)	50-80% (↓)	n.s.	(26)
SUV39H1, G9A, Ezh2, Fog1	HCT116	Her2, MYC, EpCAM	H3K9me3 (↑30-fold), H3K27me3 (↓20%)	80% (↓)	n.s.	(198)
EZH2 (cat.dom.)	Hep3B HEK293T	GRN	H3K27me3 (↑1.5-fold)	↓	Proliferation (↓)	(280)
Ezh2	In vivo (Medaka)	Arhgap35, Pfkfb4a, …	H3K27me3 (↑10-fold)	25% (↓)	n.s.	(72)
SMYD3	HEK293T	FNP1	H3K4me3 (↑2-fold)	4-fold (↑)	n.s.	(132)

REFERENCES:

1. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT, Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY, Zhang F. RNA targeting with CRISPR-Cas13. *Nature* 550: 280-284, 2017.
2. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest* 119: 1438-1449, 2009.
3. Agrotis A and Ketteler R. A new age in functional genomics using CRISPR/Cas9 in arrayed library screening. *Front Genet* 6: 300, 2015.
4. Amabile A, Migliara A, Capasso P, Biffi M, Cittaro D, Naldini L, Lombardo A. Inheritable Silencing of Endogenous Genes by Hit-and-Run Targeted Epigenetic Editing. *Cell* 167: 219-232 e214, 2016.
5. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 513: 569-573, 2014.
6. Anton T and Bultmann S. Site-specific recruitment of epigenetic factors with a modular CRISPR/Cas system. *Nucleus* 8: 279-286, 2017.
7. Arendt D, Musser JM, Baker CVH, Bergman A, Cepko C, Erwin DH, Pavlicev M, Schlosser G, Widder S, Laubichler MD, Wagner GP. The origin and evolution of cell types. *Nat Rev Genet* 17: 744-757, 2016.
8. Aubrey BJ, Kelly GL, Kueh AJ, Brennan MS, O'Connor L, Milla L, Wilcox S, Tai L, Strasser A, Herold MJ. An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo. *Cell Rep* 10: 1422-1432, 2015.
9. Balboa D, Weltner J, Eurola S, Trokovic R, Wartiovaara K, Otonkoski T. Conditionally Stabilized dCas9 Activator for Controlling Gene Expression in Human Cell Reprogramming and Differentiation. *Stem Cell Reports* 5: 448-459, 2015.
10. Banerji J, Rusconi S and Schaffner W. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. *Cell* 27: 299-308, 1981.
11. Bao Z, Jain S, Jaroenpuntaruk V, Zhao H. Orthogonal Genetic Regulation in Human Cells Using Chemically Induced CRISPR/Cas9 Activators. *ACS Synth Biol* 6: 686-693, 2017.
12. Bao Z, Xiao H, Liang J, Zhang L, Xiong X, Sun N, Si T, Zhao H. Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in Saccharomyces cerevisiae. *ACS Synth Biol* 4: 585-594, 2015.
13. Barlow DP and Bartolomei MS. Genomic Imprinting in Mammals. In: *Epigenetics*, edited by Allis CD, Jenuwein T and Reinberg D. Cold Spring Harbor, New York: Cold Spring Harbor Labroratory Press, 2006, p. 357-377.
14. Barrangou R and Horvath P. A decade of discovery: CRISPR functions and applications. *Nat Microbiol* 2: 17092, 2017.
15. Bassett AR, Akhtar A, Barlow DP, Bird AP, Brockdorff N, Duboule D, Ephrussi A, Ferguson-Smith AC, Gingeras TR, Haerty W, Higgs DR, Miska EA, Ponting CP. Considerations when investigating lncRNA function in vivo. *eLife* 3: e03058, 2014.
16. Baumann V, Wiesbeck M, Breunig CT, Braun JM, Koferle A, Ninkovic J, Gotz M, Stricker SH. Targeted removal of epigenetic barriers during transcriptional reprogramming. *Nature communications* 10: 2119, 2019.
17. Beerli RR, Segal DJ, Dreier B, Barbas CF, 3rd. Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc Natl Acad Sci U S A* 95: 14628-14633, 1998.
18. Bell AC and Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 405: 482-485, 2000.

19. Bertero A, Pawlowski M, Ortmann D, Snijders K, Yiangou L, Cardoso de Brito M, Brown S, Bernard WG, Cooper JD, Giacomelli E, Gambardella L, Hannan NR, Iyer D, Sampaziotis F, Serrano F, Zonneveld MC, Sinha S, Kotter M, Vallier L. Optimized inducible shRNA and CRISPR/Cas9 platforms for in vitro studies of human development using hPSCs. *Development* 143: 4405-4418, 2016.
20. Bianconi E, Piovesan A, Facchin F, Beraudi A, Casadei R, Frabetti F, Vitale L, Pelleri MC, Tassani S, Piva F, Perez-Amodio S, Strippoli P, Canaider S. An estimation of the number of cells in the human body. *Ann Hum Biol* 40: 463-471, 2013.
21. Bikard D, Jiang W, Samai P, Hochschild A, Zhang F, Marraffini LA. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res* 41: 7429-7437, 2013.
22. Black JB, Adler AF, Wang HG, D'Ippolito AM, Hutchinson HA, Reddy TE, Pitt GS, Leong KW, Gersbach CA. Targeted Epigenetic Remodeling of Endogenous Loci by CRISPR/Cas9-Based Transcriptional Activators Directly Converts Fibroblasts to Neuronal Cells. *Cell Stem Cell* 19: 406-414, 2016.
23. Boettcher M, Tian R, Blau J, Markegard E, Wu D, Biton A, Zaitlen N, McCormick F, Kampmann M, McManus MT. Decoding directional genetic dependencies through orthogonal CRISPR/Cas screens. *bioRxiv* 2017.
24. Braff JL, Yaung SJ, Esvelt KM, Church GM. Characterization of Cas9-Guide RNA Orthologs. *Cold Spring Harb Protoc* 2016: 2016.
25. Braun CJ, Bruno PM, Horlbeck MA, Gilbert LA, Weissman JS, Hemann MT. Versatile in vivo regulation of tumor phenotypes by dCas9-mediated transcriptional perturbation. *Proc Natl Acad Sci U S A* 113: E3892-3900, 2016.
26. Braun SMG, Kirkland JG, Chory EJ, Husmann D, Calarco JP, Crabtree GR. Rapid and reversible epigenome editing by endogenous chromatin regulators. *Nature communications* 8: 560, 2017.
27. Breunig CT, Durovic T, Neuner AM, Baumann V, Wiesbeck MF, Koferle A, Gotz M, Ninkovic J, Stricker SH. One step generation of customizable gRNA vectors for multiplex CRISPR approaches through string assembly gRNA cloning (STAgR). *PLoS One* 13: e0196015, 2018.
28. Briner AE, Donohoue PD, Gomaa AA, Selle K, Slorach EM, Nye CH, Haurwitz RE, Beisel CL, May AP, Barrangou R. Guide RNA functional modules direct Cas9 activity and orthogonality. *Mol Cell* 56: 333-339, 2014.
29. Bubeck F, Hoffmann MD, Harteveld Z, Aschenbrenner S, Bietz A, Waldhauer MC, Borner K, Fakhiri J, Schmelas C, Dietz L, Grimm D, Correia BE, Eils R, Niopek D. Engineered anti-CRISPR proteins for optogenetic control of CRISPR-Cas9. *Nat Methods* 15: 924-927, 2018.
30. Buckley RH. Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. *Annu Rev Immunol* 22: 625-655, 2004.
31. Bultmann S and Stricker SH. Entering the post-epigenomic age: back to epigenetics. *Open Biol* 8: 2018.
32. Campa CC, Weisbach NR, Santinha AJ, Incarnato D, Platt RJ. Multiplexed genome engineering by Cas12a and CRISPR arrays encoded on single transcripts. *Nat Methods* 16: 887-893, 2019.
33. Cano-Rodriguez D, Gjaltema RA, Jilderda LJ, Jellema P, Dokter-Fokkens J, Ruiters MH, Rots MG. Writing of H3K4Me3 overcomes epigenetic silencing in a sustained but context-dependent manner. *Nature communications* 7: 12284, 2016.
34. Cantara WA, Crain PF, Rozenski J, McCloskey JA, Harris KA, Zhang X, Vendeix FA, Fabris D, Agris PF. The RNA Modification Database, RNAMDB: 2011 update. *Nucleic Acids Res* 39: D195-201, 2011.
35. Carmo-Fonseca M. The contribution of nuclear compartmentalization to gene regulation. *Cell* 108: 513-521, 2002.
36. Cermak T, Curtin SJ, Gil-Humanes J, Cegan R, Kono TJY, Konecna E, Belanto JJ, Starker CG, Mathre JW, Greenstein RL, Voytas DF. A Multipurpose Toolkit to Enable Advanced Genome Engineering in Plants. *Plant Cell* 29: 1196-1217, 2017.

37. Chakraborty S, Ji H, Kabadi AM, Gersbach CA, Christoforou N, Leong KW. A CRISPR/Cas9-based system for reprogramming cell lineage specification. *Stem Cell Reports* 3: 940-947, 2014.
38. Chang YK, Hwang JS, Chung TY, Shin YJ. SOX2 Activation Using CRISPR/dCas9 Promotes Wound Healing in Corneal Endothelial Cells. *Stem Cells* 36: 1851-1862, 2018.
39. Chao JA, Patskovsky Y, Almo SC, Singer RH. Structural basis for the coevolution of a viral RNA-protein complex. *Nat Struct Mol Biol* 15: 103-105, 2008.
40. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, E PRI, Lin S, Kiani S, Guzman CD, Wiegand DJ, Ter-Ovanesyan D, Braff JL, Davidsohn N, Housden BE, Perrimon N, Weiss R, Aach J, Collins JJ, Church GM. Highly efficient Cas9-mediated transcriptional programming. *Nat Methods* 12: 326-328, 2015.
41. Chavez A, Tuttle M, Pruitt BW, Ewen-Campen B, Chari R, Ter-Ovanesyan D, Haque SJ, Cecchi RJ, Kowal EJ, Buchthal J, Housden BE, Perrimon N, Collins JJ, Church G. Comparison of Cas9 activators in multiple species. *Nat Methods* 2016.
42. Chen LF, Lin YT, Gallegos DA, Hazlett MF, Gomez-Schiavon M, Yang MG, Kalmeta B, Zhou AS, Holtzman L, Gersbach CA, Grandl J, Buchler NE, West AE. Enhancer Histone Acetylation Modulates Transcriptional Bursting Dynamics of Neuronal Activity-Inducible Genes. *Cell Rep* 26: 1174-1188 e1175, 2019.
43. Chen T, Gao D, Zhang R, Zeng G, Yan H, Lim E, Liang FS. Chemically Controlled Epigenome Editing through an Inducible dCas9 System. *J Am Chem Soc* 139: 11337-11340, 2017.
44. Chen X, Wei M, Liu X, Song S, Wang L, Yang X, Song Y. Construction and validation of the CRISPR/dCas9-EZH2 system for targeted H3K27Me3 modification. *Biochem Biophys Res Commun* 511: 246-252, 2019.
45. Cheng AW, Jillette N, Lee P, Plaskon D, Fujiwara Y, Wang W, Taghbalout A, Wang H. Casilio: a versatile CRISPR-Cas9-Pumilio hybrid for gene regulation and genomic labeling. *Cell Res* 26: 254-257, 2016.
46. Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW, Rangarajan S, Shivalila CS, Dadon DB, Jaenisch R. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res* 23: 1163-1171, 2013.
47. Cheng J, Roden CA, Pan W, Zhu S, Baccei A, Pan X, Jiang T, Kluger Y, Weissman SM, Guo S, Flavell RA, Ding Y, Lu J. A Molecular Chipper technology for CRISPR sgRNA library generation and functional mapping of noncoding regions. *Nature communications* 7: 11178, 2016.
48. Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* 24: 132-141, 2014.
49. Choudhury SR, Cui Y, Lubecka K, Stefanska B, Irudayaraj J. CRISPR-dCas9 mediated TET1 targeting for selective DNA demethylation at BRCA1 promoter. *Oncotarget* 2016.
50. Chow RD, Guzman CD, Wang G, Schmidt F, Youngblood MW, Ye L, Errami Y, Dong MB, Martinez MA, Zhang S, Renauer P, Bilguvar K, Gunel M, Sharp PA, Zhang F, Platt RJ, Chen S. AAV-mediated direct in vivo CRISPR screen identifies functional suppressors in glioblastoma. *Nat Neurosci* 20: 1329-1341, 2017.
51. Colasante G, Lignani G, Brusco S, Di Berardino C, Carpenter J, Giannelli S, Valassina N, Bido S, Ricci R, Castoldi V, Marenna S, Church T, Massimino L, Morabito G, Benfenati F, Schorge S, Leocani L, Kullmann DM, Broccoli V. dCas9-Based Scn1a Gene Activation Restores Inhibitory Interneuron Excitability and Attenuates Seizures in Dravet Syndrome Mice. *Molecular therapy : the journal of the American Society of Gene Therapy* 2019.
52. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819-823, 2013.
53. Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, Zhang F. RNA editing with CRISPR-Cas13. *Science* 358: 1019-1027, 2017.

54. Cress BF, Toparlak OD, Guleria S, Lebovich M, Stieglitz JT, Englaender JA, Jones JA, Linhardt RJ, Koffas MA. CRISPathBrick: Modular Combinatorial Assembly of Type II-A CRISPR Arrays for dCas9-Mediated Multiplex Transcriptional Repression in E. coli. *ACS Synth Biol* 4: 987-1000, 2015.
55. Cui Y, Xu J, Cheng M, Liao X, Peng S. Review of CRISPR/Cas9 sgRNA Design Tools. *Interdiscip Sci* 10: 455-465, 2018.
56. Dahlman JE, Abudayyeh OO, Joung J, Gootenberg JS, Zhang F, Konermann S. Orthogonal gene knockout and activation with a catalytically active Cas9 nuclease. *Nat Biotechnol* 33: 1159-1161, 2015.
57. Dai W, Xu X, Wang D, Wu J, Wang J. Cancer therapy with a CRISPR-assisted telomerase-activating gene expression system. *Oncogene* 38: 4110-4124, 2019.
58. Davis KM, Pattanayak V, Thompson DB, Zuris JA, Liu DR. Small molecule-triggered Cas9 protein with improved genome-editing specificity. *Nat Chem Biol* 11: 316-318, 2015.
59. de Solis CA, Ho A, Holehonnur R, Ploski JE. The Development of a Viral Mediated CRISPR/Cas9 System with Doxycycline Dependent gRNA Expression for Inducible In vitro and In vivo Genome Editing. *Front Mol Neurosci* 9: 70, 2016.
60. de Wit E and de Laat W. A decade of 3C technologies: insights into nuclear organization. *Genes Dev* 26: 11-24, 2012.
61. Degirmenci U, Li J, Lim YC, Siang DTC, Lin S, Liang H, Sun L. Silencing an insulin-induced lncRNA, LncASIR, impairs the transcriptional response to insulin signalling in adipocytes. *Scientific reports* 9: 5608, 2019.
62. Dong C, Hao GF, Hua HL, Liu S, Labena AA, Chai G, Huang J, Rao N, Guo FB. Anti-CRISPRdb: a comprehensive online resource for anti-CRISPR proteins. *Nucleic Acids Res* 46: D393-D398, 2018.
63. Dow LE, Fisher J, O'Rourke KP, Muley A, Kastenhuber ER, Livshits G, Tschaharganeh DF, Socci ND, Lowe SW. Inducible in vivo genome editing with CRISPR-Cas9. *Nat Biotechnol* 33: 390-394, 2015.
64. Droge W and Schipper HM. Oxidative stress and aberrant signaling in aging and cognitive decline. *Aging Cell* 6: 361-370, 2007.
65. Duellman T, Doll A, Chen X, Wakamiya R, Yang J. dCas9-mediated transcriptional activation of tissue inhibitor of metalloproteinases. *Metalloproteinases Med* 4: 63-73, 2017.
66. Dukhovny A, Lamkiewicz K, Chen Q, Fricke M, Jabrane-Ferrat N, Marz M, Jung JU, Sklan EH. A CRISPR activation screen identifies genes protecting from Zika virus infection. *J Virol* 2019.
67. Ehrke-Schulz E, Schiwon M, Leitner T, David S, Bergmann T, Liu J, Ehrhardt A. CRISPR/Cas9 delivery with one single adenoviral vector devoid of all viral genes. *Scientific reports* 7: 17113, 2017.
68. Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat Methods* 10: 1116-1121, 2013.
69. Friedmann T. A brief history of gene therapy. *Nat Genet* 2: 93-98, 1992.
70. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 31: 822-826, 2013.
71. Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol* 32: 279-284, 2014.
72. Fukushima HS, Takeda H and Nakamura R. Targeted in vivo epigenome editing of H3K27me3. *Epigenetics Chromatin* 12: 17, 2019.
73. Furuhata Y, Nihongaki Y, Sato M, Yoshimoto K. Control of Adipogenic Differentiation in Mesenchymal Stem Cells via Endogenous Gene Activation Using CRISPR-Cas9. *ACS Synth Biol* 6: 2191-2197, 2017.
74. Gallego-Bartolome J, Gardiner J, Liu W, Papikian A, Ghoshal B, Kuo HY, Zhao JM, Segal DJ, Jacobsen SE. Targeted DNA demethylation of the Arabidopsis genome using the human TET1 catalytic domain. *Proc Natl Acad Sci U S A* 115: E2125-E2134, 2018.

75. Galonska C, Charlton J, Mattei AL, Donaghey J, Clement K, Gu H, Mohammad AW, Stamenova EK, Cacchiarelli D, Klages S, Timmermann B, Cantz T, Scholer HR, Gnirke A, Ziller MJ, Meissner A. Genome-wide tracking of dCas9-methyltransferase footprints. *Nature communications* 9: 597, 2018.
76. Gao D and Liang FS. Chemical Inducible dCas9-Guided Editing of H3K27 Acetylation in Mammalian Cells. *Methods Mol Biol* 1767: 429-445, 2018.
77. Gao X, Tsang JC, Gaba F, Wu D, Lu L, Liu P. Comparison of TALE designer transcription factors and the CRISPR/dCas9 in regulation of gene expression by targeting enhancers. *Nucleic Acids Res* 42: e155, 2014.
78. Gao Y, Xiong X, Wong S, Charles EJ, Lim WA, Qi LS. Complex transcriptional modulation with orthogonal and inducible dCas9 regulators. *Nat Methods* 13: 1043-1049, 2016.
79. Garcia-Bloj B, Moses C, Sgro A, Plani-Lam J, Arooj M, Duffy C, Thiruvengadam S, Sorolla A, Rashwan R, Mancera RL, Leisewitz A, Swift-Scanlan T, Corvalan AH, Blancafort P. Waking up dormant tumor suppressor genes with zinc fingers, TALEs and the CRISPR/dCas9 system. *Oncotarget* 7: 60535-60554, 2016.
80. Gasperini M, Hill AJ, McFaline-Figueroa JL, Martin B, Kim S, Zhang MD, Jackson D, Leith A, Schreiber J, Noble WS, Trapnell C, Ahituv N, Shendure J. A Genome-wide Framework for Mapping Gene Regulation via Cellular Genetic Screens. *Cell* 176: 377-390 e319, 2019.
81. Geyer PK, Vitalini MW and Wallrath LL. Nuclear organization: taking a position on gene expression. *Curr Opin Cell Biol* 23: 354-359, 2011.
82. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154: 442-451, 2013.
83. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. *Gene* 363: 15-23, 2005.
84. Goldman RD, Gruenbaum Y, Moir RD, Shumaker DK, Spann TP. Nuclear lamins: building blocks of nuclear architecture. *Genes Dev* 16: 533-547, 2002.
85. Gonzalez F, Zhu Z, Shi ZD, Lelli K, Verma N, Li QV, Huangfu D. An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. *Cell Stem Cell* 15: 215-226, 2014.
86. Goyal A, Fiskin E, Gutschner T, Polycarpou-Schwarz M, Gross M, Neugebauer J, Gandhi M, Caudron-Herger M, Benes V, Diederichs S. A cautionary tale of sense-antisense gene pairs: independent regulation despite inverse correlation of expression. *Nucleic Acids Res* 45: 12496-12508, 2017.
87. Grade S and Gotz M. Neuronal replacement therapy: previous achievements and challenges ahead. *NPJ Regen Med* 2: 29, 2017.
88. Graf R, Li X, Chu VT, Rajewsky K. sgRNA Sequence Motifs Blocking Efficient CRISPR/Cas9-Mediated Gene Editing. *Cell Rep* 26: 1098-1103 e1093, 2019.
89. Guenzl PM and Barlow DP. Macro lncRNAs: a new layer of cis-regulatory information in the mammalian genome. *RNA Biol* 9: 731-741, 2012.
90. Han K, Jeng EE, Hess GT, Morgens DW, Li A, Bassik MC. Synergistic drug combinations for cancer identified in a CRISPR screen for pairwise genetic interactions. *Nat Biotechnol* 35: 463-474, 2017.
91. Hao N, Shearwin KE and Dodd IB. Programmable DNA looping using engineered bivalent dCas9 complexes. *Nature communications* 8: 1628, 2017.
92. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 405: 486-489, 2000.
93. Harris H. *The Birth of the Cell.* New Haven, CT: Yale University Press, 1999.
94. Hartenian E and Doench JG. Genetic screens and functional genomics using CRISPR/Cas9 technology. *The FEBS journal* 282: 1383-1393, 2015.

95. Hassig CA, Fleischer TC, Billin AN, Schreiber SL, Ayer DE. Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell* 89: 341-347, 1997.
96. He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 333: 1303-1307, 2011.
97. Heaton BE, Kennedy EM, Dumm RE, Harding AT, Sacco MT, Sachs D, Heaton NS. A CRISPR Activation Screen Identifies a Pan-avian Influenza Virus Inhibitory Host Factor. *Cell Rep* 20: 1503-1512, 2017.
98. Helm M and Motorin Y. Detecting RNA modifications in the epitranscriptome: predict and validate. *Nat Rev Genet* 18: 275-291, 2017.
99. Hemphill J, Borchardt EK, Brown K, Asokan A, Deiters A. Optical Control of CRISPR/Cas9 Gene Editing. *J Am Chem Soc* 137: 5642-5645, 2015.
100. Herold M, Bartkuhn M and Renkawitz R. CTCF: insights into insulator function during development. *Development* 139: 1045-1057, 2012.
101. Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol* 2015.
102. Hinz JM, Laughery MF and Wyrick JJ. Nucleosomes Inhibit Cas9 Endonuclease Activity in Vitro. *Biochemistry* 54: 7063-7066, 2015.
103. Hobert O. Regulatory logic of neuronal diversity: terminal selector genes and selector motifs. *Proc Natl Acad Sci U S A* 105: 20067-20071, 2008.
104. Hoffmann MD, Aschenbrenner S, Grosse S, Rapti K, Domenger C, Fakhiri J, Mastel M, Borner K, Eils R, Grimm D, Niopek D. Cell-specific CRISPR-Cas9 activation by microRNA-dependent expression of anti-CRISPR proteins. *Nucleic Acids Res* 2019.
105. Horlbeck MA, Witkowsky LB, Guglielmi B, Replogle JM, Gilbert LA, Villalta JE, Torigoe SE, Tjian R, Weissman JS. Nucleosomes impede Cas9 access to DNA in vivo and in vitro. *eLife* 5: 2016.
106. Horvath P, Romero DA, Coute-Monvoisin AC, Richards M, Deveau H, Moineau S, Boyaval P, Fremaux C, Barrangou R. Diversity, activity, and evolution of CRISPR loci in Streptococcus thermophilus. *J Bacteriol* 190: 1401-1412, 2008.
107. Hotchkiss RD. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. *J Biol Chem* 175: 315-332, 1948.
108. Hou Z, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ, Thomson JA. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc Natl Acad Sci U S A* 110: 15644-15649, 2013.
109. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 31: 827-832, 2013.
110. Hu J, Lei Y, Wong WK, Liu S, Lee KC, He X, You W, Zhou R, Guo JT, Chen X, Peng X, Sun H, Huang H, Zhao H, Feng B. Direct activation of human and mouse Oct4 genes using engineered TALE and Cas9 transcription factors. *Nucleic Acids Res* 42: 4375-4390, 2014.
111. Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, Liu DR. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* 556: 57-63, 2018.
112. Huang YH, Su J, Lei Y, Brunetti L, Gundry MC, Zhang X, Jeong M, Li W, Goodell MA. DNA epigenome editing using CRISPR-Cas SunTag-directed DNMT3A. *Genome Biol* 18: 176, 2017.
113. Isaac RS, Jiang F, Doudna JA, Lim WA, Narlikar GJ, Almeida R. Nucleosome breathing and remodeling constrain CRISPR-Cas9 function. *eLife* 5: 2016.
114. Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333: 1300-1303, 2011.

115. Jackson DA. Structure-function relationships in eukaryotic nuclei. *Bioessays* 13: 1-10, 1991.
116. Jacobs JZ, Ciccaglione KM, Tournier V, Zaratiegui M. Implementation of the CRISPR-Cas9 system in fission yeast. *Nature communications* 5: 5344, 2014.
117. Jenuwein T and Allis CD. Translating the histone code. *Science* 293: 1074-1080, 2001.
118. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816-821, 2012.
119. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. *eLife* 2: e00471, 2013.
120. Jost M, Chen Y, Gilbert LA, Horlbeck MA, Krenning L, Menchon G, Rai A, Cho MY, Stern JJ, Prota AE, Kampmann M, Akhmanova A, Steinmetz MO, Tanenbaum ME, Weissman JS. Combined CRISPRi/a-Based Chemical Genetic Screens Reveal that Rigosertib Is a Microtubule-Destabilizing Agent. *Mol Cell* 68: 210-223 e216, 2017.
121. Jost M, Santos DA, Saunders RA, Horlbeck MA, Hawkins JS, Scaria SM, Norman TM, Hussmann JA, Liem CR, Gross CA, Weissman JS. Titrating gene expression using libraries of systematically attenuated CRISPR guide RNAs. *Nat Biotechnol* 2020.
122. Joung J, Engreitz JM, Konermann S, Abudayyeh OO, Verdine VK, Aguet F, Gootenberg JS, Sanjana NE, Wright JB, Fulco CP, Tseng YY, Yoon CH, Boehm JS, Lander ES, Zhang F. Genome-scale activation screen identifies a lncRNA locus regulating a gene neighbourhood. *Nature* 548: 343-346, 2017.
123. Kabadi AM, Ousterout DG, Hilton IB, Gersbach CA. Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. *Nucleic Acids Res* 42: e147, 2014.
124. Kantor B, Tagliafierro L, Gu J, Zamora ME, Ilich E, Grenier C, Huang ZY, Murphy S, Chiba-Falek O. Downregulation of SNCA Expression by Targeted Editing of DNA Methylation: A Potential Strategy for Precision Therapy in PD. *Molecular therapy : the journal of the American Society of Gene Therapy* 26: 2638-2649, 2018.
125. Kearns NA, Genga RM, Enuameh MS, Garber M, Wolfe SA, Maehr R. Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. *Development* 141: 219-223, 2014.
126. Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, Garber M, Maehr R. Functional annotation of native enhancers with a Cas9-histone demethylase fusion. *Nat Methods* 2015.
127. Kelley ML, Strezoska Z, He K, Vermeulen A, Smith A. Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing. *J Biotechnol* 233: 74-83, 2016.
128. Kemaladewi DU, Bassi PS, Erwood S, Al-Basha D, Gawlik KI, Lindsay K, Hyatt E, Kember R, Place KM, Marks RM, Durbeej M, Prescott SA, Ivakine EA, Cohn RD. A mutation-independent approach for muscular dystrophy via upregulation of a modifier gene. *Nature* 572: 125-130, 2019.
129. Khare SP, Habib F, Sharma R, Gadewal N, Gupta S, Galande S. HIstome--a relational knowledgebase of human histone proteins and histone modifying enzymes. *Nucleic Acids Res* 40: D337-342, 2012.
130. Kiani S, Beal J, Ebrahimkhani MR, Huh J, Hall RN, Xie Z, Li Y, Weiss R. CRISPR transcriptional repression devices and layered circuits in mammalian cells. *Nat Methods* 11: 723-726, 2014.
131. Kiani S, Chavez A, Tuttle M, Hall RN, Chari R, Ter-Ovanesyan D, Qian J, Pruitt BW, Beal J, Vora S, Buchthal J, Kowal EJ, Ebrahimkhani MR, Collins JJ, Weiss R, Church G. Cas9 gRNA engineering for genome editing, activation and repression. *Nat Methods* 12: 1051-1054, 2015.
132. Kim JM, Kim K, Schmidt T, Punj V, Tucker H, Rice JC, Ulmer TS, An W. Cooperation between SMYD3 and PC4 drives a distinct transcriptional program in cancer cells. *Nucleic Acids Res* 43: 8868-8883, 2015.
133. Kim JS, Lee HJ and Carroll D. Genome editing with modularly assembled zinc-finger nucleases. *Nat Methods* 7: 91; author reply 91-92, 2010.

134. Kim SK, Han GH, Seong W, Kim H, Kim SW, Lee DH, Lee SG. CRISPR interference-guided balancing of a biosynthetic mevalonate pathway increases terpenoid production. *Metab Eng* 38: 228-240, 2016.
135. Kipniss NH, Dingal P, Abbott TR, Gao Y, Wang H, Dominguez AA, Labanieh L, Qi LS. Engineering cell sensing and responses using a GPCR-coupled CRISPR-Cas system. *Nature communications* 8: 2212, 2017.
136. Klann TS, Black JB, Chellappan M, Safi A, Song L, Hilton IB, Crawford GE, Reddy TE, Gersbach CA. CRISPR-Cas9 epigenome editing enables high-throughput screening for functional regulatory elements in the human genome. *Nat Biotechnol* 35: 561-568, 2017.
137. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JR, Aryee MJ, Joung JK. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523: 481-485, 2015.
138. Koferle A and Stricker SH. A Universal Protocol for Large-scale gRNA Library Production from any DNA Source. *JoVE* e56264, 2017.
139. Koferle A, Worf K, Breunig C, Baumann V, Herrero J, Wiesbeck M, Hutter LH, Gotz M, Fuchs C, Beck S, Stricker SH. CORALINA: a universal method for the generation of gRNA libraries for CRISPR-based screening. *BMC Genomics* 17: 917, 2016.
140. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 517: 583-588, 2015.
141. Kuscu C, Arslan S, Singh R, Thorpe J, Adli M. Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. *Nat Biotechnol* 32: 677-683, 2014.
142. Kweon J, Jang AH, Kim DE, Yang JW, Yoon M, Rim Shin H, Kim JS, Kim Y. Fusion guide RNAs for orthogonal gene manipulation with Cas9 and Cpf1. *Nature communications* 8: 1723, 2017.
143. Kwon DY, Zhao YT, Lamonica JM, Zhou Z. Locus-specific histone deacetylation using a synthetic CRISPR-Cas9-based HDAC. *Nature communications* 8: 15315, 2017.
144. Lanctot C, Cheutin T, Cremer M, Cavalli G, Cremer T. Dynamic genome architecture in the nuclear space: regulation of gene expression in three dimensions. *Nat Rev Genet* 8: 104-115, 2007.
145. Lane AB, Strzelecka M, Ettinger A, Grenfell AW, Wittmann T, Heald R. Enzymatically Generated CRISPR Libraries for Genome Labeling and Screening. *Dev Cell* 34: 373-378, 2015.
146. Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat Protoc* 8: 2180-2196, 2013.
147. Lee TI and Young RA. Transcriptional regulation and its misregulation in disease. *Cell* 152: 1237-1251, 2013.
148. Lei Y, Zhang X, Su J, Jeong M, Gundry MC, Huang YH, Zhou Y, Li W, Goodell MA. Targeted DNA methylation in vivo using an engineered dCas9-MQ1 fusion protein. *Nature communications* 8: 16026, 2017.
149. Leung RK and Whittaker PA. RNA interference: from gene silencing to gene-specific therapeutics. *Pharmacol Ther* 107: 222-239, 2005.
150. Li E, Bestor TH and Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69: 915-926, 1992.
151. Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nat Biotechnol* 31: 688-691, 2013.
152. Li S and Mason CE. The pivotal regulatory landscape of RNA modifications. *Annu Rev Genomics Hum Genet* 15: 127-150, 2014.
153. Lian J, HamediRad M, Hu S, Zhao H. Combinatorial metabolic engineering using an orthogonal tri-functional CRISPR system. *Nature communications* 8: 1688, 2017.

154. Lin F, Dong L, Wang W, Liu Y, Huang W, Cai Z. An Efficient Light-Inducible P53 Expression System for Inhibiting Proliferation of Bladder Cancer Cell. *Int J Biol Sci* 12: 1273-1278, 2016.
155. Linder B, Grozhik AV, Olarerin-George AO, Meydan C, Mason CE, Jaffrey SR. Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome. *Nat Methods* 12: 767-772, 2015.
156. Linder CC. Genetic Variables That Influence Phenotype. *ILAR Journal* 47: 132-140, 2006.
157. Liu KI, Ramli MN, Woo CW, Wang Y, Zhao T, Zhang X, Yim GR, Chong BY, Gowher A, Chua MZ, Jung J, Lee JH, Tan MH. A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing. *Nat Chem Biol* 12: 980-987, 2016.
158. Liu P, Chen M, Liu Y, Qi LS, Ding S. CRISPR-Based Chromatin Remodeling of the Endogenous Oct4 or Sox2 Locus Enables Reprogramming to Pluripotency. *Cell Stem Cell* 22: 252-261 e254, 2018.
159. Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, Shu J, Dadon D, Young RA, Jaenisch R. Editing DNA Methylation in the Mammalian Genome. *Cell* 167: 233-247 e217, 2016.
160. Liu XS, Wu H, Krzisch M, Wu X, Graef J, Muffat J, Hnisz D, Li CH, Yuan B, Xu C, Li Y, Vershkov D, Cacace A, Young RA, Jaenisch R. Rescue of Fragile X Syndrome Neurons by DNA Methylation Editing of the FMR1 Gene. *Cell* 172: 979-992 e976, 2018.
161. Liu Y, Gong W, Huang CC, Herr W, Cheng X. Crystal structure of the conserved core of the herpes simplex virus transcriptional regulatory protein VP16. *Genes Dev* 13: 1692-1703, 1999.
162. Liu Y, Yu C, Daley TP, Wang F, Cao WS, Bhate S, Lin X, Still C, 2nd, Liu H, Zhao D, Wang H, Xie XS, Ding S, Wong WH, Wernig M, Qi LS. CRISPR Activation Screens Systematically Identify Factors that Drive Neuronal Fate and Reprogramming. *Cell Stem Cell* 23: 758-771 e758, 2018.
163. Liu Y, Zeng Y, Liu L, Zhuang C, Fu X, Huang W, Cai Z. Synthesizing AND gate genetic circuits based on CRISPR-Cas9 for identification of bladder cancer cells. *Nature communications* 5: 5393, 2014.
164. Lupianez DG, Kraft K, Heinrich V, Krawitz P, Brancati F, Klopocki E, Horn D, Kayserili H, Opitz JM, Laxova R, Santos-Simarro F, Gilbert-Dussardier B, Wittler L, Borschiwer M, Haas SA, Osterwalder M, Franke M, Timmermann B, Hecht J, Spielmann M, Visel A, Mundlos S. Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. *Cell* 161: 1012-1025, 2015.
165. Ma D, Peng S, Huang W, Cai Z, Xie Z. Rational Design of Mini-Cas9 for Transcriptional Activation. *ACS Synth Biol* 7: 978-985, 2018.
166. Machnicka MA, Milanowska K, Osman Oglou O, Purta E, Kurkowska M, Olchowik A, Januszewski W, Kalinowski S, Dunin-Horkawicz S, Rother KM, Helm M, Bujnicki JM, Grosjean H. MODOMICS: a database of RNA modification pathways--2013 update. *Nucleic Acids Res* 41: D262-267, 2013.
167. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. *Nat Methods* 10: 977-979, 2013.
168. Maji B, Moore CL, Zetsche B, Volz SE, Zhang F, Shoulders MD, Choudhary A. Multidimensional chemical control of CRISPR-Cas9. *Nat Chem Biol* 13: 9-11, 2017.
169. Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol* 31: 833-838, 2013.
170. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. *Science* 339: 823-826, 2013.
171. Mandegar MA, Huebsch N, Frolov EB, Shin E, Truong A, Olvera MP, Chan AH, Miyaoka Y, Holmes K, Spencer CI, Judge LM, Gordon DE, Eskildsen TV, Villalta JE, Horlbeck MA, Gilbert LA, Krogan NJ, Sheikh SP, Weissman JS, Qi LS, So PL, Conklin BR. CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. *Cell Stem Cell* 18: 541-553, 2016.
172. Maniatis T, Efstratiadis A, Sim GK, Kafatos F. Amplification and characterization of eukaryotic structural genes. *Natl Cancer Inst Monogr* 9-10, 1978.

173. Maniatis T, Kee SG, Efstratiadis A, Kafatos FC. Amplification and characterization of a beta-globin gene synthesized in vitro. *Cell* 8: 163-182, 1976.
174. Maresch R, Mueller S, Veltkamp C, Ollinger R, Friedrich M, Heid I, Steiger K, Weber J, Engleitner T, Barenboim M, Klein S, Louzada S, Banerjee R, Strong A, Stauber T, Gross N, Geumann U, Lange S, Ringelhan M, Varela I, Unger K, Yang F, Schmid RM, Vassiliou GS, Braren R, Schneider G, Heikenwalder M, Bradley A, Saur D, Rad R. Multiplexed pancreatic genome engineering and cancer induction by transfection-based CRISPR/Cas9 delivery in mice. *Nature communications* 7: 10770, 2016.
175. Margolin JF, Friedman JR, Meyer WK, Vissing H, Thiesen HJ, Rauscher FJ, 3rd. Kruppel-associated boxes are potent transcriptional repression domains. *Proc Natl Acad Sci U S A* 91: 4509-4513, 1994.
176. Marx N, Grunwald-Gruber C, Bydlinski N, Dhiman H, Ngoc Nguyen L, Klanert G, Borth N. CRISPR-Based Targeted Epigenetic Editing Enables Gene Expression Modulation of the Silenced Beta-Galactoside Alpha-2,6-Sialyltransferase 1 in CHO Cells. *Biotechnol J* e1700217, 2018.
177. Matharu N, Rattanasopha S, Tamura S, Maliskova L, Wang Y, Bernard A, Hardin A, Eckalbar WL, Vaisse C, Ahituv N. CRISPR-mediated activation of a promoter or enhancer rescues obesity caused by haploinsufficiency. *Science* 363: 2019.
178. Matjusaitis M, Wagstaff LJ, Martella A, Baranowski B, Blin C, Gogolok S, Williams A, Pollard SM. Reprogramming of Fibroblasts to Oligodendrocyte Progenitor-like Cells Using CRISPR/Cas9-Based Synthetic Transcription Factors. *Stem Cell Reports* 2019.
179. Mauer J, Luo X, Blanjoie A, Jiao X, Grozhik AV, Patil DP, Linder B, Pickering BF, Vasseur JJ, Chen Q, Gross SS, Elemento O, Debart F, Kiledjian M, Jaffrey SR. Reversible methylation of m(6)Am in the 5' cap controls mRNA stability. *Nature* 541: 371-375, 2017.
180. Merkenschlager M and Nora EP. CTCF and Cohesin in Genome Folding and Transcriptional Gene Regulation. *Annu Rev Genomics Hum Genet* 17: 17-43, 2016.
181. Mochizuki Y, Chiba T, Kataoka K, Yamashita S, Sato T, Kato T, Takahashi K, Miyamoto T, Kitazawa M, Hatta T, Natsume T, Takai S, Asahara H. Combinatorial CRISPR/Cas9 Approach to Elucidate a Far-Upstream Enhancer Complex for Tissue-Specific Sox9 Expression. *Dev Cell* 46: 794-806 e796, 2018.
182. Montagutelli X. Effect of the genetic background on the phenotype of mouse mutations. *J Am Soc Nephrol* 11 Suppl 16: S101-105, 2000.
183. Morgan SL, Mariano NC, Bermudez A, Arruda NL, Wu F, Luo Y, Shankar G, Jia L, Chen H, Hu JF, Hoffman AR, Huang CC, Pitteri SJ, Wang KC. Manipulation of nuclear architecture through CRISPR-mediated chromosomal looping. *Nature communications* 8: 15993, 2017.
184. Morita S, Noguchi H, Horii T, Nakabayashi K, Kimura M, Okamura K, Sakai A, Nakashima H, Hata K, Nakashima K, Hatada I. Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions. *Nat Biotechnol* 34: 1060-1065, 2016.
185. Morris SA and Daley GQ. A blueprint for engineering cell fate: current technologies to reprogram cell identity. *Cell Res* 23: 33-48, 2013.
186. Moscou MJ and Bogdanove AJ. A simple cipher governs DNA recognition by TAL effectors. *Science* 326: 1501, 2009.
187. Moses C, Nugent F, Waryah CB, Garcia-Bloj B, Harvey AR, Blancafort P. Activating PTEN Tumor Suppressor Expression with the CRISPR/dCas9 System. *Mol Ther Nucleic Acids* 14: 287-300, 2019.
188. Nguyen DP, Miyaoka Y, Gilbert LA, Mayerl SJ, Lee BH, Weissman JS, Conklin BR, Wells JA. Ligand-binding domains of nuclear receptors facilitate tight control of split CRISPR activity. *Nature communications* 7: 12009, 2016.
189. Nguyen NT, He L, Martinez-Moczygemba M, Huang Y, Zhou Y. Rewiring Calcium Signaling for Precise Transcriptional Reprogramming. *ACS Synth Biol* 7: 814-821, 2018.
190. Nielsen AA and Voigt CA. Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks. *Mol Syst Biol* 10: 763, 2014.

191. Nihongaki Y, Kawano F, Nakajima T, Sato M. Photoactivatable CRISPR-Cas9 for optogenetic genome editing. *Nat Biotechnol* 33: 755-760, 2015.
192. Nishimasu H, Cong L, Yan WX, Ran FA, Zetsche B, Li Y, Kurabayashi A, Ishitani R, Zhang F, Nureki O. Crystal Structure of Staphylococcus aureus Cas9. *Cell* 162: 1113-1126, 2015.
193. Nissim L, Perli SD, Fridkin A, Perez-Pinera P, Lu TK. Multiplexed and programmable regulation of gene networks with an integrated RNA and CRISPR/Cas toolkit in human cells. *Mol Cell* 54: 698-710, 2014.
194. Noack F and Calegari F. Epitranscriptomics: A New Regulatory Mechanism of Brain Development and Function. *Front Neurosci* 12: 85, 2018.
195. Noack F, Pataskar A, Schneider M, Buchholz F, Tiwari VK, Calegari F. Assessment and site-specific manipulation of DNA (hydroxy-)methylation during mouse corticogenesis. *Life Sci Alliance* 2: 2019.
196. Nomura W, Matsumoto D, Sugii T, Kobayakawa T, Tamamura H. Efficient and Orthogonal Transcription Regulation by Chemically Inducible Artificial Transcription Factors. *Biochemistry* 57: 6452-6459, 2018.
197. O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, Doudna JA. Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* 516: 263-266, 2014.
198. O'Geen H, Ren C, Nicolet CM, Perez AA, Halmai J, Le VM, Mackay JP, Farnham PJ, Segal DJ. dCas9-based epigenome editing suggests acquisition of histone methylation is not sufficient for target gene repression. *Nucleic Acids Res* 45: 9901-9916, 2017.
199. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 87: 953-959, 1996.
200. Okada M, Kanamori M, Someya K, Nakatsukasa H, Yoshimura A. Stabilization of Foxp3 expression by CRISPR-dCas9-based epigenome editing in mouse primary T cells. *Epigenetics Chromatin* 10: 24, 2017.
201. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99: 247-257, 1999.
202. Panne D. The enhanceosome. *Curr Opin Struct Biol* 18: 236-242, 2008.
203. Pawluk A, Amrani N, Zhang Y, Garcia B, Hidalgo-Reyes Y, Lee J, Edraki A, Shah M, Sontheimer EJ, Maxwell KL, Davidson AR. Naturally Occurring Off-Switches for CRISPR-Cas9. *Cell* 167: 1829-1838 e1829, 2016.
204. Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, Thakore PI, Glass KA, Ousterout DG, Leong KW, Guilak F, Crawford GE, Reddy TE, Gersbach CA. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat Methods* 10: 973-976, 2013.
205. Peter CJ, Saito A, Hasegawa Y, Tanaka Y, Nagpal M, Perez G, Alway E, Espeso-Gil S, Fayyad T, Ratner C, Dincer A, Gupta A, Devi L, Pappas JG, Lalonde FM, Butman JA, Han JC, Akbarian S, Kamiya A. In vivo epigenetic editing of Sema6a promoter reverses transcallosal dysconnectivity caused by C11orf46/Arl14ep risk gene. *Nature communications* 10: 4112, 2019.
206. Peterson BA, Haak DC, Nishimura MT, Teixeira PJ, James SR, Dangl JL, Nimchuk ZL. Genome-Wide Assessment of Efficiency and Specificity in CRISPR/Cas9 Mediated Multiple Site Targeting in Arabidopsis. *PLoS One* 11: e0162169, 2016.
207. Pflueger C, Tan D, Swain T, Nguyen T, Pflueger J, Nefzger C, Polo JM, Ford E, Lister R. A modular dCas9-SunTag DNMT3A epigenome editing system overcomes pervasive off-target activity of direct fusion dCas9-DNMT3A constructs. *Genome Res* 28: 1193-1206, 2018.
208. Pignani S, Zappaterra F, Barbon E, Follenzi A, Bovolenta M, Bernardi F, Branchini A, Pinotti M. Tailoring the CRISPR system to transactivate coagulation gene promoters in normal and mutated contexts. *Biochim Biophys Acta Gene Regul Mech* 1862: 619-624, 2019.
209. Piguet F, Alves S and Cartier N. Clinical Gene Therapy for Neurodegenerative Diseases: Past, Present, and Future. *Hum Gene Ther* 28: 988-1003, 2017.

210. Pinto BS, Saxena T, Oliveira R, Mendez-Gomez HR, Cleary JD, Denes LT, McConnell O, Arboleda J, Xia G, Swanson MS, Wang ET. Impeding Transcription of Expanded Microsatellite Repeats by Deactivated Cas9. *Mol Cell* 68: 479-490 e475, 2017.
211. Pollock C and Huang S. The perinucleolar compartment. *Cold Spring Harb Perspect Biol* 2: a000679, 2010.
212. Polstein LR and Gersbach CA. A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. *Nat Chem Biol* 11: 198-200, 2015.
213. Polstein LR, Perez-Pinera P, Kocak DD, Vockley CM, Bledsoe P, Song L, Safi A, Crawford GE, Reddy TE, Gersbach CA. Genome-wide specificity of DNA binding, gene regulation, and chromatin remodeling by TALE- and CRISPR/Cas9-based transcriptional activators. *Genome Res* 25: 1158-1169, 2015.
214. Poondla N, Chandrasekaran AP, Heese K, Kim KS, Ramakrishna S. CRISPR-mediated upregulation of DR5 and downregulation of cFLIP synergistically sensitize HeLa cells to TRAIL-mediated apoptosis. *Biochem Biophys Res Commun* 512: 60-65, 2019.
215. Prelich G. Gene overexpression: uses, mechanisms, and interpretation. *Genetics* 190: 841-854, 2012.
216. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152: 1173-1183, 2013.
217. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using Staphylococcus aureus Cas9. *Nature* 520: 186-191, 2015.
218. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154: 1380-1389, 2013.
219. Rauch BJ, Silvis MR, Hultquist JF, Waters CS, McGregor MJ, Krogan NJ, Bondy-Denomy J. Inhibition of CRISPR-Cas9 with Bacteriophage Proteins. *Cell* 168: 150-158 e110, 2017.
220. Regev A, Teichmann SA, Lander ES, Amit I, Benoist C, Birney E, Bodenmiller B, Campbell P, Carninci P, Clatworthy M, Clevers H, Deplancke B, Dunham I, Eberwine J, Eils R, Enard W, Farmer A, Fugger L, Gottgens B, Hacohen N, Haniffa M, Hemberg M, Kim S, Klenerman P, Kriegstein A, Lein E, Linnarsson S, Lundberg E, Lundeberg J, Majumder P, Marioni JC, Merad M, Mhlanga M, Nawijn M, Netea M, Nolan G, Pe'er D, Phillipakis A, Ponting CP, Quake S, Reik W, Rozenblatt-Rosen O, Sanes J, Satija R, Schumacher TN, Shalek A, Shapiro E, Sharma P, Shin JW, Stegle O, Stratton M, Stubbington MJT, Theis FJ, Uhlen M, van Oudenaarden A, Wagner A, Watt F, Weissman J, Wold B, Xavier R, Yosef N, Human Cell Atlas Meeting P. The Human Cell Atlas. *eLife* 6: 2017.
221. Richter F, Fonfara I, Bouazza B, Schumacher CH, Bratovic M, Charpentier E, Moglich A. Engineering of temperature- and light-switchable Cas9 variants. *Nucleic Acids Res* 44: 10003-10014, 2016.
222. Roundtree IA, Evans ME, Pan T, He C. Dynamic RNA Modifications in Gene Expression Regulation. *Cell* 169: 1187-1200, 2017.
223. Rousseau BA, Hou Z, Gramelspacher MJ, Zhang Y. Programmable RNA Cleavage and Recognition by a Natural CRISPR-Cas9 System from Neisseria meningitidis. *Mol Cell* 69: 906-914 e904, 2018.
224. Ryan OW, Skerker JM, Maurer MJ, Li X, Tsai JC, Poddar S, Lee ME, DeLoache W, Dueber JE, Arkin AP, Cate JH. Selection of chromosomal DNA libraries using a multiplex CRISPR system. *eLife* 3: 2014.
225. Sadowski I, Ma J, Triezenberg S, Ptashne M. GAL4-VP16 is an unusually potent transcriptional activator. *Nature* 335: 563-564, 1988.
226. Sakuma T, Nishikawa A, Kume S, Chayama K, Yamamoto T. Multiplex genome engineering in human cells using all-in-one CRISPR/Cas9 vector system. *Scientific reports* 4: 5400, 2014.

227. Sander JD and Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol* 32: 347-355, 2014.
228. Saunderson EA, Stepper P, Gomm JJ, Hoa L, Morgan A, Allen MD, Jones JL, Gribben JG, Jurkowski TP, Ficz G. Hit-and-run epigenetic editing prevents senescence entry in primary breast cells from healthy donors. *Nature communications* 8: 1450, 2017.
229. Schaefer M, Pollex T, Hanna K, Lyko F. RNA cytosine methylation analysis by bisulfite sequencing. *Nucleic Acids Res* 37: e12, 2009.
230. Schwartz S, Bernstein DA, Mumbach MR, Jovanovic M, Herbst RH, Leon-Ricardo BX, Engreitz JM, Guttman M, Satija R, Lander ES, Fink G, Regev A. Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. *Cell* 159: 148-162, 2014.
231. Seipel K, Georgiev O and Schaffner W. Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. *EMBO J* 11: 4961-4968, 1992.
232. Sekiya T, Kato K, Kawaguchi A, Nagata K. Involvement of CTCF in transcription regulation of EGR1 at early G1 phase as an architecture factor. *Scientific reports* 9: 329, 2019.
233. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343: 84-87, 2014.
234. Shang W, Wang F, Fan G, Wang H. Key elements for designing and performing a CRISPR/Cas9-based genetic screen. *J Genet Genomics* 44: 439-449, 2017.
235. Shao J, Wang M, Yu G, Zhu S, Yu Y, Heng BC, Wu J, Ye H. Synthetic far-red light-mediated CRISPR-dCas9 device for inducing functional neuronal differentiation. *Proc Natl Acad Sci U S A* 115: E6722-E6730, 2018.
236. Shariati SA, Dominguez A, Xie S, Wernig M, Qi LS, Skotheim JM. Reversible Disruption of Specific Transcription Factor-DNA Interactions Using CRISPR/Cas9. *Mol Cell* 74: 622-633 e624, 2019.
237. Shechner DM, Hacisuleyman E, Younger ST, Rinn JL. Multiplexable, locus-specific targeting of long RNAs with CRISPR-Display. *Nat Methods* 12: 664-670, 2015.
238. Shen Z, Zhang X, Chai Y, Zhu Z, Yi P, Feng G, Li W, Ou G. Conditional knockouts generated by engineered CRISPR-Cas9 endonuclease reveal the roles of coronin in C. elegans neural development. *Dev Cell* 30: 625-636, 2014.
239. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119: 941-953, 2004.
240. Shin J, Jiang F, Liu JJ, Bray NL, Rauch BJ, Baik SH, Nogales E, Bondy-Denomy J, Corn JE, Doudna JA. Disabling Cas9 by an anti-CRISPR DNA mimic. *Sci Adv* 3: e1701620, 2017.
241. Shrimp JH, Grose C, Widmeyer SRT, Thorpe AL, Jadhav A, Meier JL. Chemical Control of a CRISPR-Cas9 Acetyltransferase. *ACS Chem Biol* 13: 455-460, 2018.
242. Shyamsunder P, Shanmugasundaram M, Mayakonda A, Dakle P, Teoh WW, Han L, Kanojia D, Lim MC, Fullwood M, An O, Yang H, Jizhong S, Hossain MZ, Madan V, Koeffler HP. Identification of a novel enhancer of CEBPE essential for granulocytic differentiation. *Blood* 2019.
243. Spitz F and Furlong EE. Transcription factors: from enhancer binding to developmental control. *Nat Rev Genet* 13: 613-626, 2012.
244. Srivastava A. In vivo tissue-tropism of adeno-associated viral vectors. *Curr Opin Virol* 21: 75-80, 2016.
245. Stadhouders R, Filion GJ and Graf T. Transcription factors and 3D genome conformation in cell-fate decisions. *Nature* 569: 345-354, 2019.
246. Stepper P, Kungulovski G, Jurkowska RZ, Chandra T, Krueger F, Reinhardt R, Reik W, Jeltsch A, Jurkowski TP. Efficient targeted DNA methylation with chimeric dCas9-Dnmt3a-Dnmt3L methyltransferase. *Nucleic Acids Res* 45: 1703-1713, 2017.

247. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 507: 62-67, 2014.
248. Stricker SH and Gotz M. DNA-Methylation: Master or Slave of Neural Fate Decisions? *Front Neurosci* 12: 5, 2018.
249. Stricker SH, Koferle A and Beck S. From profiles to function in epigenomics. *Nat Rev Genet* 18: 51-66, 2017.
250. Su J, Huang YH, Cui X, Wang X, Zhang X, Lei Y, Xu J, Lin X, Chen K, Lv J, Goodell MA, Li W. Homeobox oncogene activation by pan-cancer DNA hypermethylation. *Genome Biol* 19: 108, 2018.
251. Sun S, Xiao J, Huo J, Geng Z, Ma K, Sun X, Fu X. Targeting ectodysplasin promotor by CRISPR/dCas9-effector effectively induces the reprogramming of human bone marrow-derived mesenchymal stem cells into sweat gland-like cells. *Stem Cell Res Ther* 9: 8, 2018.
252. Suzuki T, Ueda H, Okada S, Sakurai M. Transcriptome-wide identification of adenosine-to-inosine editing using the ICE-seq method. *Nat Protoc* 10: 715-732, 2015.
253. Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, Sur M, Zhang F. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotechnol* 33: 102-106, 2015.
254. Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, Fukuda M, Takeda N, Niida H, Kato H, Shinkai Y. G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* 16: 1779-1791, 2002.
255. Tak YE, Kleinstiver BP, Nunez JK, Hsu JY, Horng JE, Gong J, Weissman JS, Joung JK. Inducible and multiplex gene regulation using CRISPR-Cpf1-based transcription factors. *Nat Methods* 14: 1163-1166, 2017.
256. Tan M, Luo H, Lee S, Jin F, Yang JS, Montellier E, Buchou T, Cheng Z, Rousseaux S, Rajagopal N, Lu Z, Ye Z, Zhu Q, Wysocka J, Ye Y, Khochbin S, Ren B, Zhao Y. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell* 146: 1016-1028, 2011.
257. Tanay A and Regev A. Scaling single-cell genomics from phenomenology to mechanism. *Nature* 541: 331-338, 2017.
258. Tang X, Zheng X, Qi Y, Zhang D, Cheng Y, Tang A, Voytas DF, Zhang Y. A Single Transcript CRISPR-Cas9 System for Efficient Genome Editing in Plants. *Mol Plant* 9: 1088-1091, 2016.
259. Tessarz P and Kouzarides T. Histone core modifications regulating nucleosome structure and dynamics. *Nat Rev Mol Cell Biol* 15: 703-708, 2014.
260. Thakore PI, D'Ippolito AM, Song L, Safi A, Shivakumar NK, Kabadi AM, Reddy TE, Crawford GE, Gersbach CA. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nat Methods* 2015.
261. Thompson DB, Aboulhouda S, Hysolli E, Smith CJ, Wang S, Castanon O, Church GM. The Future of Multiplexed Eukaryotic Genome Engineering. *ACS Chem Biol* 13: 313-325, 2018.
262. Tong Y, Sun J, Wong CF, Kang Q, Ru B, Wong CN, Chan AS, Leung SY, Zhang J. MICMIC: identification of DNA methylation of distal regulatory regions with causal effects on tumorigenesis. *Genome Biol* 19: 73, 2018.
263. Trapnell C. Defining cell types and states with single-cell genomics. *Genome Res* 25: 1491-1498, 2015.
264. Truong VA, Hsu MN, Kieu Nguyen NT, Lin MW, Shen CC, Lin CY, Hu YC. CRISPRai for simultaneous gene activation and inhibition to promote stem cell chondrogenesis and calvarial bone regeneration. *Nucleic Acids Res* 2019.
265. Vad-Nielsen J, Gammelgaard KR, Daugaard TF, Nielsen AL. Cause-and-Effect relationship between FGFR1 expression and epithelial-mesenchymal transition in EGFR-mutated non-small cell lung cancer cells. *Lung Cancer* 132: 132-140, 2019.

266. van Steensel B and Furlong EEM. The role of transcription in shaping the spatial organization of the genome. *Nat Rev Mol Cell Biol* 20: 327-337, 2019.
267. Vaquerizas JM, Kummerfeld SK, Teichmann SA, Luscombe NM. A census of human transcription factors: function, expression and evolution. *Nat Rev Genet* 10: 252-263, 2009.
268. Vazquez-Vilar M, Bernabe-Orts JM, Fernandez-Del-Carmen A, Ziarsolo P, Blanca J, Granell A, Orzaez D. A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard. *Plant Methods* 12: 10, 2016.
269. Verma N, Pan H, Dore LC, Shukla A, Li QV, Pelham-Webb B, Teijeiro V, Gonzalez F, Krivtsov A, Chang CJ, Papapetrou EP, He C, Elemento O, Huangfu D. TET proteins safeguard bivalent promoters from de novo methylation in human embryonic stem cells. *Nat Genet* 50: 83-95, 2018.
270. Vickaryous MK and Hall BK. Human cell type diversity, evolution, development, and classification with special reference to cells derived from the neural crest. *Biol Rev Camb Philos Soc* 81: 425-455, 2006.
271. Villamizar O, Waters SA, Scott T, Saayman S, Grepo N, Urak R, Davis A, Jaffe A, Morris KV. Targeted Activation of Cystic Fibrosis Transmembrane Conductance Regulator. *Molecular therapy : the journal of the American Society of Gene Therapy* 27: 1737-1748, 2019.
272. Vo BT, Kwon JA, Li C, Finkelstein D, Xu B, Orr BA, Sherr CJ, Roussel MF. Mouse medulloblastoma driven by CRISPR activation of cellular Myc. *Scientific reports* 8: 8733, 2018.
273. Vojta A, Dobrinic P, Tadic V, Bockor L, Korac P, Julg B, Klasic M, Zoldos V. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Res* 2016.
274. Waaijers S, Portegijs V, Kerver J, Lemmens BB, Tijsterman M, van den Heuvel S, Boxem M. CRISPR/Cas9-targeted mutagenesis in Caenorhabditis elegans. *Genetics* 195: 1187-1191, 2013.
275. Waddington CH. The epigenotype. 1942. *Int J Epidemiol* 41: 10-13, 2012.
276. Waddington CH. *The strategy of the genes; a discussion of some aspects of theoretical biology*. London,: Allen & Unwin, 1957, p. ix, 262 p.
277. Waddington CH. Towards a theoretical biology. *Nature* 218: 525-527, 1968.
278. Wang C, Shen L, Fu Y, Yan C, Wang K. A Simple CRISPR/Cas9 System for Multiplex Genome Editing in Rice. *J Genet Genomics* 42: 703-706, 2015.
279. Wang G, Zhao N, Berkhout B, Das AT. CRISPR-Cas based antiviral strategies against HIV-1. *Virus Res* 244: 321-332, 2018.
280. Wang H, Guo R, Du Z, Bai L, Li L, Cui J, Li W, Hoffman AR, Hu JF. Epigenetic Targeting of Granulin in Hepatoma Cells by Synthetic CRISPR dCas9 Epi-suppressors. *Mol Ther Nucleic Acids* 11: 23-33, 2018.
281. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 343: 80-84, 2014.
282. Weber J, Ollinger R, Friedrich M, Ehmer U, Barenboim M, Steiger K, Heid I, Mueller S, Maresch R, Engleitner T, Gross N, Geumann U, Fu B, Segler A, Yuan D, Lange S, Strong A, de la Rosa J, Esposito I, Liu P, Cadinanos J, Vassiliou GS, Schmid RM, Schneider G, Unger K, Yang F, Braren R, Heikenwalder M, Varela I, Saur D, Bradley A, Rad R. CRISPR/Cas9 somatic multiplex-mutagenesis for high-throughput functional cancer genomics in mice. *Proc Natl Acad Sci U S A* 112: 13982-13987, 2015.
283. Wei S, Zou Q, Lai S, Zhang Q, Li L, Yan Q, Zhou X, Zhong H, Lai L. Conversion of embryonic stem cells into extraembryonic lineages by CRISPR-mediated activators. *Scientific reports* 6: 19648, 2016.
284. Wei Y, Lang J, Zhang Q, Yang CR, Zhao ZA, Zhang Y, Du Y, Sun Y. DNA methylation analysis and editing in single mammalian oocytes. *Proc Natl Acad Sci U S A* 116: 9883-9892, 2019.
285. Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, Kagey MH, Rahl PB, Lee TI, Young RA. Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* 153: 307-319, 2013.
286. Wilson LOW, O'Brien AR and Bauer DC. The Current State and Future of CRISPR-Cas9 gRNA Design Tools. *Front Pharmacol* 9: 749, 2018.

287. Wolfe SA, Nekludova L and Pabo CO. DNA recognition by Cys2His2 zinc finger proteins. *Annu Rev Biophys Biomol Struct* 29: 183-212, 2000.
288. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 458: 766-770, 2009.
289. Wong AS, Choi GC, Cui CH, Pregernig G, Milani P, Adam M, Perli SD, Kazer SW, Gaillard A, Hermann M, Shalek AK, Fraenkel E, Lu TK. Multiplexed barcoded CRISPR-Cas9 screening enabled by CombiGEM. *Proc Natl Acad Sci U S A* 113: 2544-2549, 2016.
290. Wong TWY and Cohn RD. Therapeutic Applications of CRISPR/Cas for Duchenne Muscular Dystrophy. *Curr Gene Ther* 17: 301-308, 2017.
291. Wu X, Scott DA, Kriz AJ, Chiu AC, Hsu PD, Dadon DB, Cheng AW, Trevino AE, Konermann S, Chen S, Jaenisch R, Zhang F, Sharp PA. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nat Biotechnol* 32: 670-676, 2014.
292. Xie C, Chen YL, Wang DF, Wang YL, Zhang TP, Li H, Liang F, Zhao Y, Zhang GY. SgRNA Expression of CRIPSR-Cas9 System Based on MiRNA Polycistrons as a Versatile Tool to Manipulate Multiple and Tissue-Specific Genome Editing. *Scientific reports* 7: 5795, 2017.
293. Xie K, Minkenberg B and Yang Y. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc Natl Acad Sci U S A* 112: 3570-3575, 2015.
294. Xie S, Duan J, Li B, Zhou P, Hon GC. Multiplexed Engineering and Analysis of Combinatorial Enhancer Activity in Single Cells. *Mol Cell* 66: 285-299 e285, 2017.
295. Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B, Wang XC, Chen QJ. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biol* 14: 327, 2014.
296. Xiong T, Meister GE, Workman RE, Kato NC, Spellberg MJ, Turker F, Timp W, Ostermeier M, Novina CD. Targeted DNA methylation in human cells using engineered dCas9-methyltransferases. *Scientific reports* 7: 6732, 2017.
297. Xu C, Qi X, Du X, Zou H, Gao F, Feng T, Lu H, Li S, An X, Zhang L, Wu Y, Liu Y, Li N, Capecchi MR, Wu S. piggyBac mediates efficient in vivo CRISPR library screening for tumorigenesis in mice. *Proc Natl Acad Sci U S A* 114: 722-727, 2017.
298. Xu L, Zhao L, Gao Y, Xu J, Han R. Empower multiplex cell and tissue-specific CRISPR-mediated gene manipulation with self-cleaving ribozymes and tRNA. *Nucleic Acids Res* 45: e28, 2017.
299. Xu X, Li J, Zhu Y, Xie B, Wang X, Wang S, Xie H, Yan H, Ying Y, Lin Y, Liu B, Wang W, Zheng X. CRISPR-ON-Mediated KLF4 overexpression inhibits the proliferation, migration and invasion of urothelial bladder cancer in vitro and in vivo. *Oncotarget* 8: 102078-102087, 2017.
300. Xu X, Tao Y, Gao X, Zhang L, Li X, Zou W, Ruan K, Wang F, Xu GL, Hu R. A CRISPR-based approach for targeted DNA demethylation. *Cell Discov* 2: 16009, 2016.
301. Xue Z, Cui C, Liao Z, Xia S, Zhang P, Qin J, Guo Q, Chen S, Fu Q, Yin Z, Ye Z, Tang Y, Shen N. Identification of LncRNA Linc00513 Containing Lupus-Associated Genetic Variants as a Novel Regulator of Interferon Signaling Pathway. *Front Immunol* 9: 2967, 2018.
302. Yamaguchi H, Hopf FW, Li SB, de Lecea L. In vivo cell type-specific CRISPR knockdown of dopamine beta hydroxylase reduces locus coeruleus evoked wakefulness. *Nature communications* 9: 5211, 2018.
303. Yamazaki T, Fujikawa C, Kubota A, Takahashi A, Hirose T. CRISPRa-mediated NEAT1 lncRNA upregulation induces formation of intact paraspeckles. *Biochem Biophys Res Commun* 504: 218-224, 2018.
304. Yamazaki T, Hatano Y, Handa T, Kato S, Hoida K, Yamamura R, Fukuyama T, Uematsu T, Kobayashi N, Kimura H, Yamagata K. Targeted DNA methylation in pericentromeres with genome editing-based artificial DNA methyltransferase. *PLoS One* 12: e0177764, 2017.

305. Yan Q, Xu K, Xing J, Zhang T, Wang X, Wei Z, Ren C, Liu Z, Shao S, Zhang Z. Multiplex CRISPR/Cas9-based genome engineering enhanced by Drosha-mediated sgRNA-shRNA structure. *Scientific reports* 6: 38970, 2016.
306. Yang J, Rajan SS, Friedrich MJ, Lan G, Zou X, Ponstingl H, Garyfallos DA, Liu P, Bradley A, Metzakopian E. Genome-Scale CRISPRa Screen Identifies Novel Factors for Cellular Reprogramming. *Stem Cell Reports* 12: 757-771, 2019.
307. Yeo NC, Chavez A, Lance-Byrne A, Chan Y, Menn D, Milanova D, Kuo CC, Guo X, Sharma S, Tung A, Cecchi RJ, Tuttle M, Pradhan S, Lim ET, Davidsohn N, Ebrahimkhani MR, Collins JJ, Lewis NE, Kiani S, Church GM. An enhanced CRISPR repressor for targeted mammalian gene regulation. *Nat Methods* 15: 611-616, 2018.
308. Yin H, Song CQ, Suresh S, Kwan SY, Wu Q, Walsh S, Ding J, Bogorad RL, Zhu LJ, Wolfe SA, Koteliansky V, Xue W, Langer R, Anderson DG. Partial DNA-guided Cas9 enables genome editing with reduced off-target activity. *Nat Chem Biol* 14: 311-316, 2018.
309. Yin L, Maddison LA, Li M, Kara N, LaFave MC, Varshney GK, Burgess SM, Patton JG, Chen W. Multiplex Conditional Mutagenesis Using Transgenic Expression of Cas9 and sgRNAs. *Genetics* 200: 431-441, 2015.
310. Yoshida M, Yokota E, Sakuma T, Yamatsuji T, Takigawa N, Ushijima T, Yamamoto T, Fukazawa T, Naomoto Y. Development of an integrated CRISPRi targeting DeltaNp63 for treatment of squamous cell carcinoma. *Oncotarget* 9: 29220-29232, 2018.
311. Yoshioka S, Fujii W, Ogawa T, Sugiura K, Naito K. Development of a mono-promoter-driven CRISPR/Cas9 system in mammalian cells. *Scientific reports* 5: 18341, 2015.
312. Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, Lim WA. Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell* 160: 339-350, 2015.
313. Zaret KS and Carroll JS. Pioneer transcription factors: establishing competence for gene expression. *Genes Dev* 25: 2227-2241, 2011.
314. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell* 2015.
315. Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, Wu WY, Scott DA, Severinov K, van der Oost J, Zhang F. Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nat Biotechnol* 35: 31-34, 2017.
316. Zetsche B, Volz SE and Zhang F. A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nat Biotechnol* 33: 139-142, 2015.
317. Zhang F, Wang L, Qi B, Zhao B, Ko EE, Riggan ND, Chin K, Qiao H. EIN2 mediates direct regulation of histone acetylation in the ethylene response. *Proc Natl Acad Sci U S A* 114: 10274-10279, 2017.
318. Zhang X, Wen H and Shi X. Lysine methylation: beyond histones. *Acta Biochim Biophys Sin (Shanghai)* 44: 14-27, 2012.
319. Zhang Y, Tian S, Xiong J, Zhou Y, Song H, Liu C. JQ-1 Inhibits Colon Cancer Proliferation via Suppressing Wnt/beta-Catenin Signaling and miR-21. *Chem Res Toxicol* 31: 302-307, 2018.
320. Zheng Y, Shen W, Zhang J, Yang B, Liu YN, Qi H, Yu X, Lu SY, Chen Y, Xu YZ, Li Y, Gage FH, Mi S, Yao J. CRISPR interference-based specific and efficient gene inactivation in the brain. *Nat Neurosci* 21: 447-454, 2018.
321. Zhou H, Liu J, Zhou C, Gao N, Rao Z, Li H, Hu X, Li C, Yao X, Shen X, Sun Y, Wei Y, Liu F, Ying W, Zhang J, Tang C, Zhang X, Xu H, Shi L, Cheng L, Huang P, Yang H. In vivo simultaneous transcriptional activation of multiple genes in the brain using CRISPR-dCas9-activator transgenic mice. *Nat Neurosci* 21: 440-446, 2018.

322. Zhou VW, Goren A and Bernstein BE. Charting histone modifications and the functional organization of mammalian genomes. *Nat Rev Genet* 12: 7-18, 2011.
323. Zhu H, Zhang L, Tong S, Lee CM, Deshmukh H, Bao G. Spatial control of in vivo CRISPR–Cas9 genome editing via nanomagnets. *Nature Biomedical Engineering* 2018.
324. Ziller MJ, Ortega JA, Quinlan KA, Santos DP, Gu H, Martin EJ, Galonska C, Pop R, Maidl S, Di Pardo A, Huang M, Meltzer HY, Gnirke A, Heckman CJ, Meissner A, Kiskinis E. Dissecting the Functional Consequences of De Novo DNA Methylation Dynamics in Human Motor Neuron Differentiation and Physiology. *Cell Stem Cell* 22: 559-574 e559, 2018.

Table 1: Examples of transcriptional engineering employing targeted gene activation.

Load	Cell(s)	Target gene(s)	Transciptional changes (up to)	Publication
dCas9-VP48, dCas9-VP160	HeLa, early mouse embryos	IL1RN, Sox2, Oct4	10x	(46)
dCas9-VP64	HEK293T	NTF3	>500x	(167)
dCas9-VP64	HEK293T	IL1RN, ASCL1, NANOG, HBG1/2, MYOD1, VEGFA, TERT, IL1B, IL1R2	IL1RN (>2000x), ASCL1 (250x), NANOG (13x), HBG1/2 (>100x), MYOD1 (50x), VEGFA (2x), TERT (2x), IL1B (10x), IL1R2 (20x)	(204)
dCas9-VP64	B-cell lymphoblastic leukemia cells	Mgmt	30x	(25)
dCas9-VP64	In vivo (mouse kidney)	Sim1a	3x	(177)
dCas9-VP64, dCas9-VP160, dCas9-VPR	NSC34, HEK293T	TIMP 1/2/3	TIMP 1/2/3 (30x)	(65)
dCas9-VP64, SAM	HEK293T	Rex1, Oct4	Rex1 (30x)	(169)
dCas9-VP64 MPH	HeLa	DR5	5x	(214)
dCas9-VP160	NCI-H460	NOP14-AS1	10x	(86)
dCas9-VPR, SAM	in vivo (Drosophila m.)	Luciferase	1000x	(319)
SAM	NSCLC	FGFR1	50x	(265)
SAM	HAP1, U2OS	NEAT1	3-4x	(303)
SAM, MPH	HEK293T	ASCL1 and various other factors	40x-600x	(140)
SAM	HeLa	Linc00513	15x	(301)
SAM	Fibroblasts	Oct4, Sox2	15x-100x	(158)
SPH	in vivo (mouse brain)	Ascl1, Neurog2, NeurdoD1, Acta1, Dkk1, Slc6a4, Rnf43, Bcl2, Znf3, Prdm16, Miat, Halgr, Fendrr, Lncpint	1-100x	(321)
MPH	HEK293T	IL1B, HBG1, ZFP42	20- 6000x	(56)
Butterfly, dCas9-VP64, dCas9-VPR, SAM, SunTag, dCas9-P300, dCas9-VP160,	HEK293T	Ascl1, NeuroD1, TTN, HBG1, RHOXF2, ACTC1	10x to 10000x	(41)
Casilio	HEK293T	Oct4	6000x	(45)
Cpf1-VPR, Cpf1-P65	HEK293T	HBB, AR, NPY1R	80x	(255)
dCas9(SP) dCas9(NM) with chemically dimerizing VPR	HEK293T	ASCL1, TTN, RHOXF,IL1RN	ASCL1 (90x), TTN (25x), RHOXF (180x), IL1RN (750x)	(11)
mini-dCas9-VTR	HEK293T	eBFP2 Reporter	1200x	(165)
MCP-VP64	HEK293T	CXCR4	5x	(312)

Table 2: Examples of targeted gene activation inducing cell state changes.

Load	Cell(s)	Target gene(s)	Transciptional changes (up to)	Publication
dCas9-VP64	mESCs	Cdx2, Gata6	Cdx2 (>100x)	(283)
dCas9-VP48 **dCas9-VP96** **dCas9-VP192**	Fibroblasts, hESC	Oct4, Sox2, Nanog, LIN28, KLF4, CDH1	Oct4 (100x), Sox2(30x), Nanog(25x), LIN28(5x), KLF4(3x), CDH1 (70x)	(9)
dCas9-VPR	iPSCs	MIAT, NeuroD1, Ascl1, RhoxF2, TTN, ACTC1	MIAT (280x), NeuroD1 (87x), Ascl1 (4600x), RhoxF2 (18000x), TTN (20000x), ACTC1 (330x)	(40)
SAM	Mesenchymal stem cells	PPARG, CEBPA, KLF5	PPARG (15x), CEBPA (10000x), KLF5 (90x)	(73)
Butterfly	Fibroblasts	MyoD1	80x	(37)
Butterfly	HEK293T, Fibroblasts	Ascl1, Myt1l, Brn2	Ascl1 (6000x)	(22)

Table 3: Examples of transcriptional engineering employing targeted gene repression.

Load	Cell(s)	Target gene(s)	Transciptional changes (up to)	Publication
dCAS9	HEK293T	mRFP Reporter	300x repression	(216)
dCas9-KRAB	K562	HBE1	10x repression	(260)
dCas9-KRAB–MeCP2	HEK293T, SH-SY5Y	YFP Reporter, CANX, CXCR4, CHK1, SEL1L, ARPC2, MAPK3, BRCA1, BLM, GZMM, MAPK3, RHOA	10x repression	(307)
PP7-SID	HEK293T	LUC reporter	5x repression	(237)
dCas9-KRAB, dCas9-Mxi1	S. cerevisae, HEK293T, HeLa	Tef1, GFP, CD71,CXCR4	3x - 53x repression	(82)

Table 4: Examples of epigenome editing targeting DNA modifications.

dCas9-	Cell(s)	Target genes	5mC (abs.incr.)	mRNA / Protein	Phenotype	Publication
DNMT3A (cat.dom.)	hESC (DNMT3A K.O.)	PAX6, ARX (RE)	Partial rescue (↑20-40%)	Rescue (↑)	Rescue of motor neurogenesis and repression of floor plate induction	(324)
DNMT3A (cat.dom.)	mESCs (Dnmt3a/b D.K.O. + transient repression of DNMT1)	Dazl, Foxa2, Id3, Foxb1, H19, Fboxo40 (P)	(↑10-40%)	No sign. change	n.s.	(75)
DNMT3A (cat.dom.) **Tet1 (cat.dom.)**	Gastric cell cancer line (AGS)	WNT5B, SOX9, FGFR1, KLF9, HDAC11, APOC3, APOA1… (RE)	(↑) (↓)	20-70% (↓) 1.5-5-fold (↑)	Cell-migration	(262)
DNMT3A (602-912)	HEK293T	IL6ST, BACH2 (P)	(↑30-60%)	40-50% (↓)	n.s.	(273)
DNMT3A (IF A1 IF A2 cat.dom. SunTag)	HEK293T	BACH2, HOXA5, KLF4 (P)	(↑2-80%)	~80% (↓) DNMT3A1-SunTag (HOXA5)	n.s.	(112)
DNMT3A (cat.dom. E756A SunTag)	HEK293T	DLX1	(↑~60%)	2-fold (↑)	n.s.	(250)
DNMT3A (cat.dom.)	Hep3B Hek 293T	GRN (P)	(↑~50%)	~30% (↓)	Proliferation (↓)	(280)
Dnmt3A (cat.dom.) Dnmt3L	HEK293T	EpCAM, CXCR4, TFRC (↓)	(↑20-40%)	~40-50% (↓)	n.s.	(246)
Dnmt3A (cat.dom.) Dnmt3L	Myo-epithelial donor cells	p16, RASSF, HIC1, PTEN …	(↑20-60%)	10-60% (↓)	Senescence (↓)	(228)
DNMT3A (cat.dom.) SunTag	MCF-7 HeLa	UNC5C, BCL3, DACH1 (P)	(↑20-50%)	n.s.	n.s.	(207)
DNMT3A (cat.dom.)	hIPSC	SNCA	(↑10-60%)	~20% (↓)	Cell viability (↑)	(124)
DNMT3A (f.length)	mESC	SNRNP-GFP reporter	(↑35-70%)	GFP-loss in up to 70% of cells	n.s.	(159)
DNMT3A, DNMT3L KRAB	K562 HEK293T	IFNAR1, VEGFA, B2M	(↑ up to 100 %)	Up to 500-fold (↓)	n.s.	(4)

MQ1 (q147l)	HEK293T, K562 in vivo	HOXA5, HOXA6, Igf2/H19 DMR …	(↑35-70%) HOXA5	40% (↓) HOXA5	n.s.	(148)
SSS1 (cat.dom.)	Embryos (2-cell stage)	Major satellites	(↑20%)	n.s.	Heterochromatin index (↑)	(304)
SSS1 (cat.dom.) Split	HEK293T E.Coli (reporter plasmids)	SALL2 P2 E1a (P)	(↑5-20%) SALL2 P2	n.s.	n.s.	(296)
Tet1	mEmbryo	Dchs1	(↓40-70%)	2.5-fold (↑)	Proliferation (↓)	(195)
Tet1	Tet TKO hESCs	Pax6	n.s.	400-fold (↑)	Neural differentiation (↑)	(269)
Tet1 (cat.dom.)	iPSCs neurons (FX52)	FMR1	(↓60-100%)	481-fold (↑)	Rescue of FXS Phenotype	(160)
Tet1 (cat.dom.)	NPC	Sox1	(10-50% ↓)	n.s.	Neural stem cell reprogramming (↑) (+dCas9-VP64)	(16)
Tet1 (cat.dom.)	HeLa MCF7	BRCA1	(↓10-50%)	2.5-fold (↑)	Proliferation (↓)	(49)
Tet1 (cat.dom.)	mESCs, neurons C3H10T1/2 MEFs, …	Bdnf, MyoD	(↓35-60%)	3-fold (↑)	fibroblast to myoblast conversion (↑)	(159)
Tet1 (cat.dom.) SunTag	ESCs E14 VZ	GFAP, H19	(↓60-90%)	1.7-fold (↑)	n.s.	(184)
Tet1 (cat.dom.) SunTag	Arabidop.	FWA	(↓50%)	8-fold (↑)	Changes in flowering time and leaf number	(74)
Tet1, Dnmt	mouse oocytes	Agouti Snrp	(↑80%; ↓10%)	n.s.	n.s.	(284)
TET1, (cat.dom.) DNMT3A (cat.dom.) SunTag	CHO	ST6GAL1	n.s.	>30-fold (↑) ~80% (↓)	Changes in glycan structure	(176)

Table 5: Examples of epigenome editing targeting histone marks.

dCas9-	Cell(s)	Target genes	Histone modifications	mRNA / Protein	Phenotype	Publication
P300 (cat.dom.)	HEK293T	IL1RN, MYOD, Oct4, β-globin	H3K27Ac (↑10-fold)	10-10000-fold (↑)	n.s.	(101)
P300 (cat.dom.)	T-cells (68-41, Primary)	Foxp3	H3K27Ac ↑	3-fold (↑)	Treg signature (↑)	(200)
EIN2-C (C-ter. dom.)	Arabidop.	EBF2	H3K14Ac (↑10-fold)	2-3-fold (↑)	Plant size (↓)	(317)
P300 (cat.dom.)	HEK293T	Oct4	H3K27Ac ↑	5-20-fold (↑)	n.s.	(110)
P300 (cat.dom.)	HEK293T	IL1RN	H3K27Ac (↑~25%)	30-fold (↑)	n.s.	(43)
P300 (cat.dom.)	K562 HEK293T	β-globin Her2	H3K27ac (↑4-fold)	n.s.	n.s.	(136)
P300 (cat.dom.)	HEK293T	IL1RN, RHOXF2, TTN	n.s.	100-10000-fold (↑)	n.s.	(241)
P300, HDAC8	neurons	Fos, Npas4	H3Ac (↑50% P300 ↓40% HDAC	1.5-fold (↑)	neuronal spiking frequency	(42)
HDAC3	N2a	Smn1, Mecp2, Isl1	n.s.	80% (↓)	n.s.	(143)
P300	MEFs	Oct4	H3Ac (↑4-fold)	10^5-fold (↑)	Pluripotency (↑)	(158)
LSD1	mESCs	Oct4, Tbx3	H3K4me2 (↓ > 90%)	90% (↓)	Cell morphology	(126)
PRDM9 (cat.dom.)	HEK293T A549	ICAM1, RASSIF1A, EPCAM, PLOD2	H3K4me3 (↑60%)	8-fold (↑)	n.s.	(33)
Suv39h1/ HP1c	HEK293T mESCs	CXCR4, Nkx2.9	H3K9me3 (↑150-fold)	50-80% (↓)	n.s.	(26)
SUV39H1, G9A, Ezh2, Fog1	HCT116	Her2, MYC, EpCAM	H3K9me3 (↑30-fold), H3K27me3 (↓20%)	80% (↓)	n.s.	(198)
EZH2 (cat.dom.)	Hep3B HEK293T	GRN	H3K27me3 (↑1.5-fold)	↓	Proliferation (↓)	(280)
Ezh2	In vivo (Medaka)	Arhgap35, Pfkfb4a, …	H3K27me3 (↑10-fold)	25% (↓)	n.s.	(72)
SMYD3	HEK293T	FNP1	H3K4me3 (↑2-fold)	4-fold (↑)	n.s.	(132)

Fig. 1

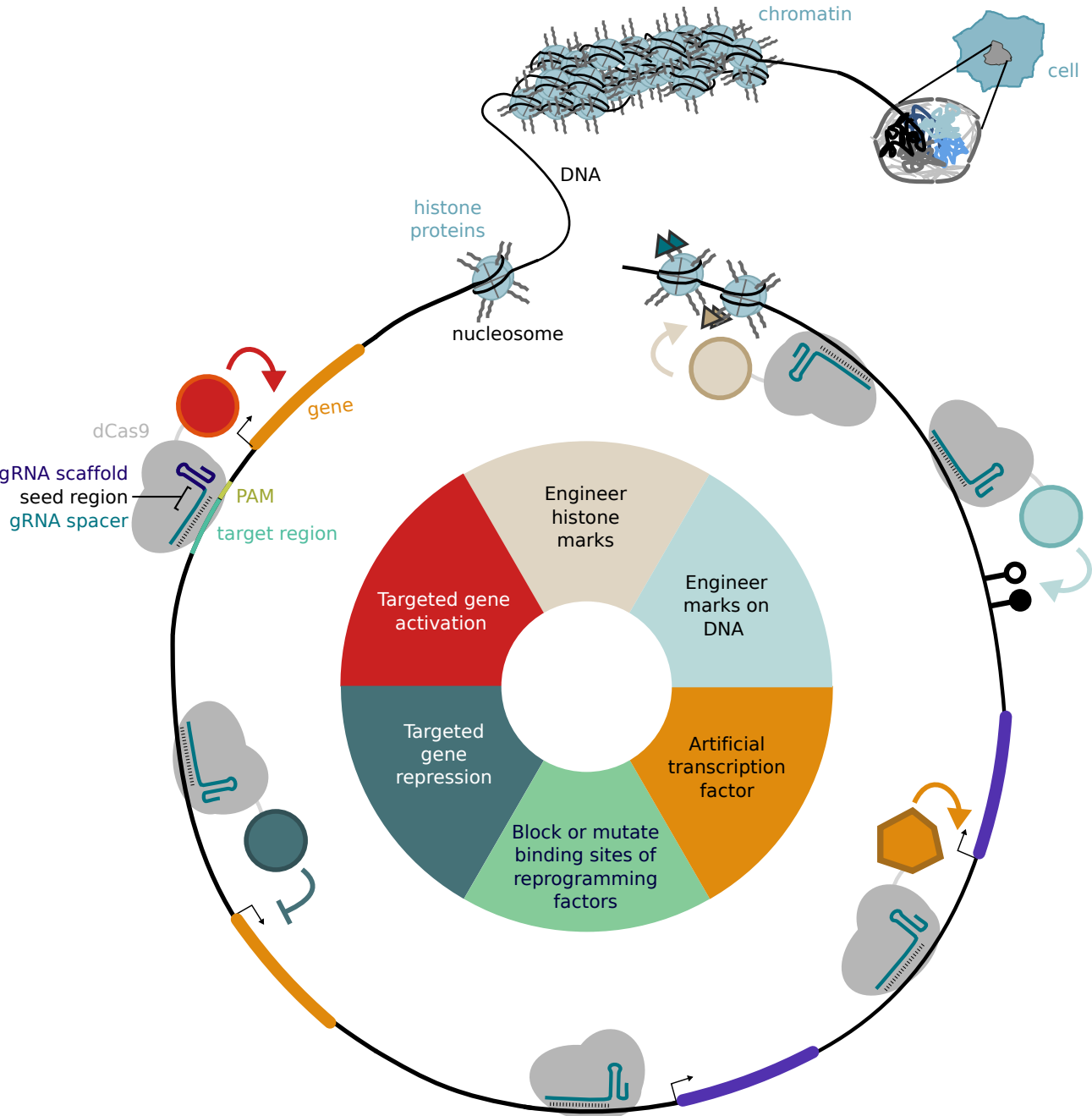
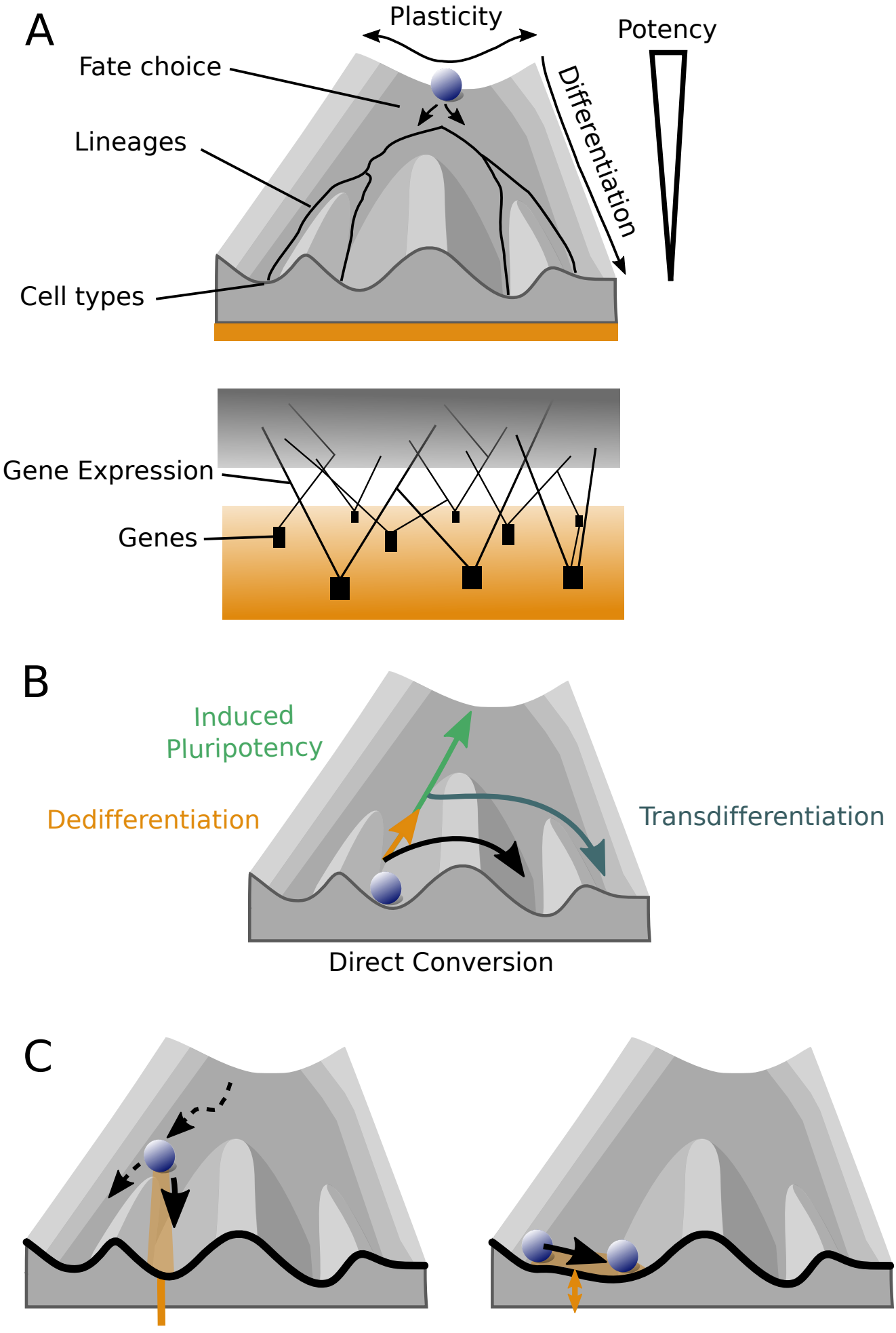
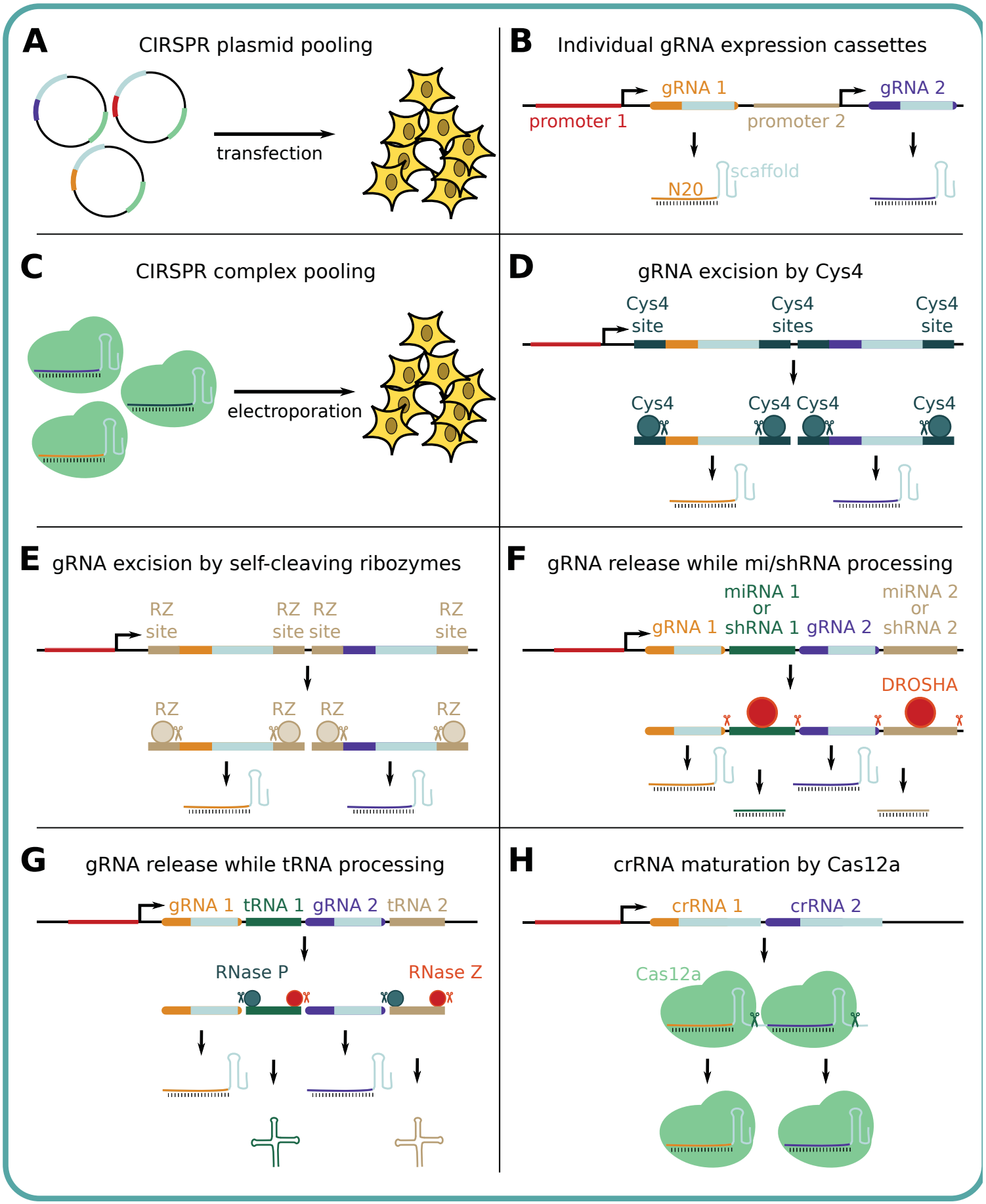

chromatin
cell
DNA
histone proteins
nucleosome
dCas9
gRNA scaffold
seed region
gRNA spacer
PAM
target region
gene
Engineer histone marks
Engineer marks on DNA
Artificial transcription factor
Block or mutate binding sites of reprogramming factors
Targeted gene repression
Targeted gene activation

Fig. 2


A
Plasticity
Potency
Fate choice
Lineages
Differentiation
Cell types
Gene Expression
Genes
B
Induced
Pluripotency
Dedifferentiation
Transdifferentiation
Direct Conversion
C
Master Transcription Factor
Reprogramming Factor


A
CIRSPR plasmid pooling
transfection
B
Individual gRNA expression cassettes
gRNA 1
gRNA 2
promoter 1
promoter 2
N20
scaffold
C
CIRSPR complex pooling
electroporation
D
gRNA excision by Cys4
Cys4 site
Cys4 sites
Cys4 site
Cys4
Cys4
Cys4
Cys4
E
gRNA excision by self-cleaving ribozymes
RZ site
RZ site site
RZ site
RZ
RZ
RZ
RZ
F
gRNA release while mi/shRNA processing
miRNA 1 or shRNA 1
miRNA 2 or shRNA 2
gRNA 1
gRNA 2
DROSHA
G
gRNA release while tRNA processing
gRNA 1
tRNA 1
gRNA 2
tRNA 2
RNase P
RNase Z
H
crRNA maturation by Cas12a
crRNA 1
crRNA 2
Cas12a

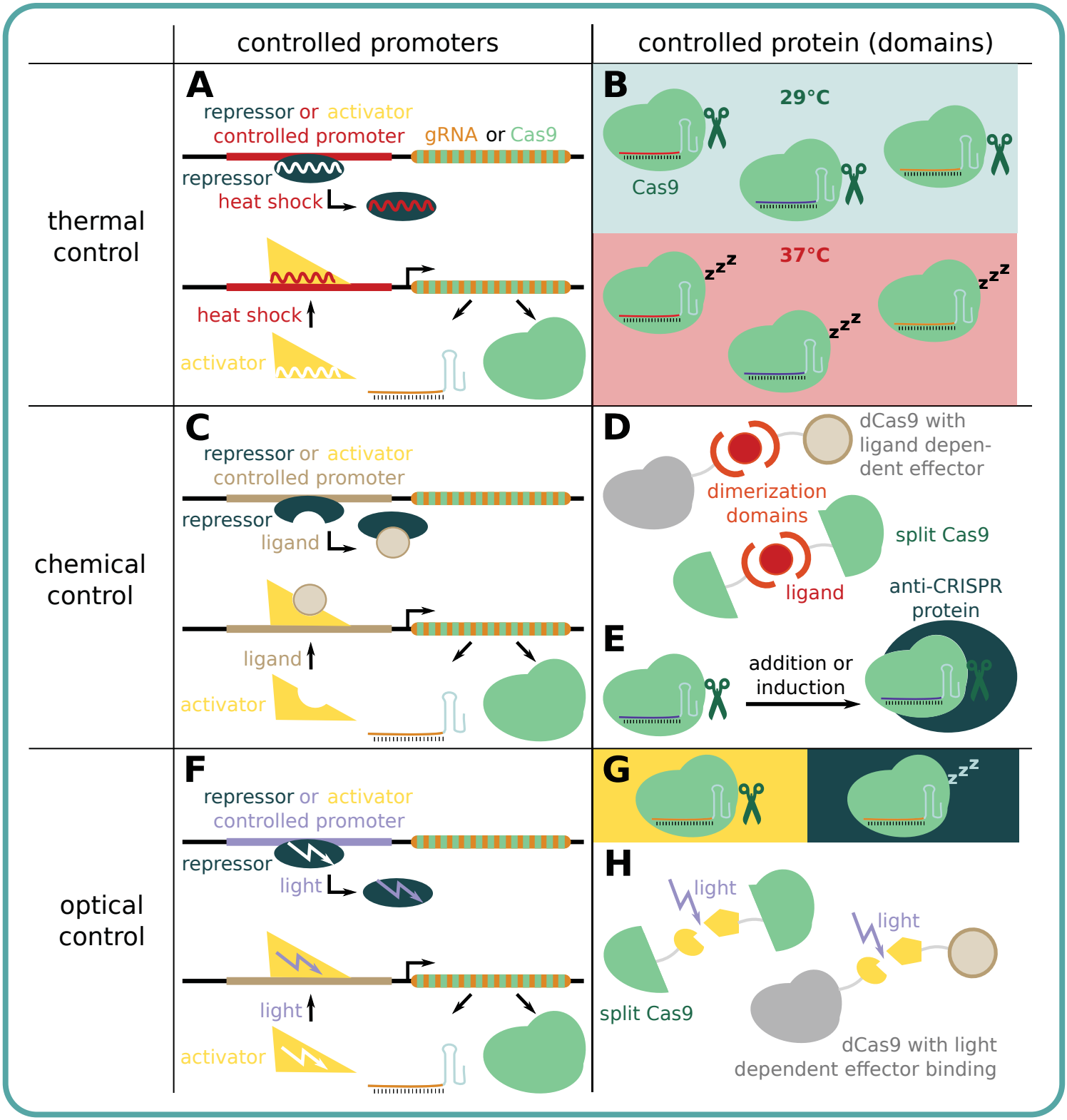

controlled promoters
controlled protein (domains)
thermal control
chemical control
optical control
A
repressor or activator
controlled promoter
gRNA or Cas9
repressor
heat shock
heat shock
activator
B
29°C
Cas9
37°C
C
repressor or activator
controlled promoter
repressor
ligand
ligand
activator
D
dCas9 with ligand depen-dent effector
dimerization domains
split Cas9
ligand
anti-CRISPR protein
E
addition or induction
F
repressor or activator
controlled promoter
repressor
light
light
activator
G
H
light
light
split Cas9
dCas9 with light dependent effector binding

Fig. 5

Targeted gene activation

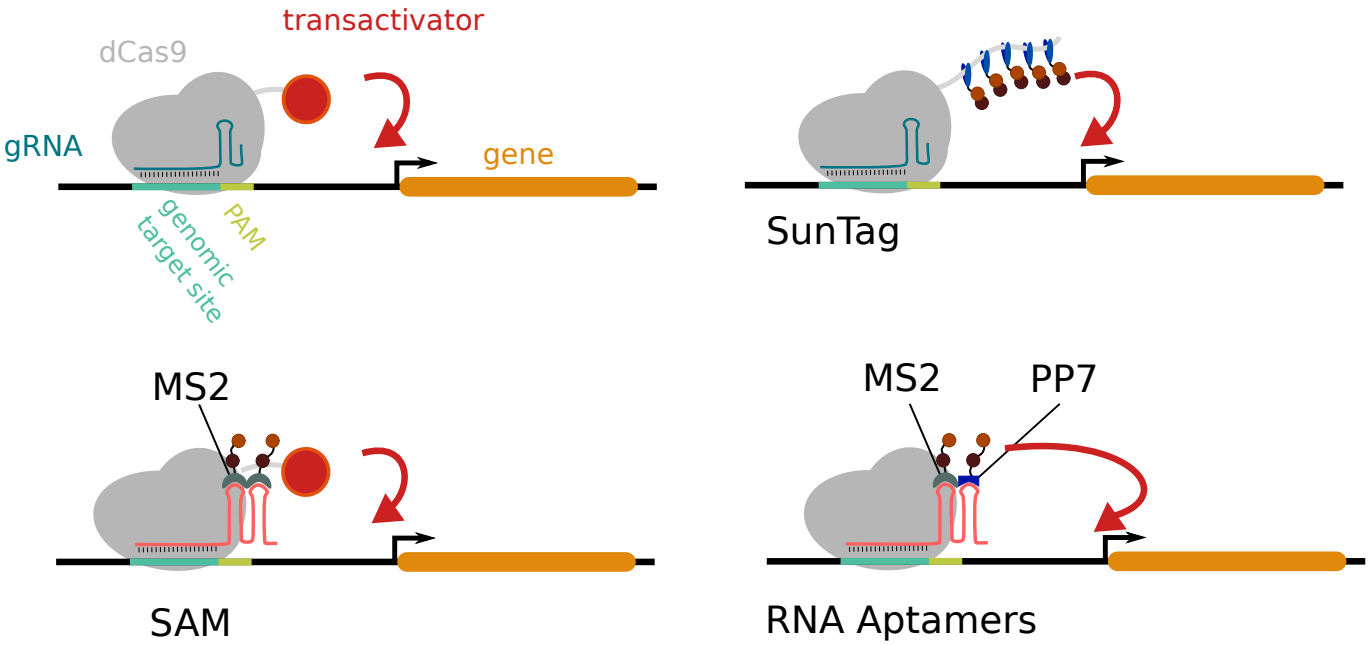


Fig. 6

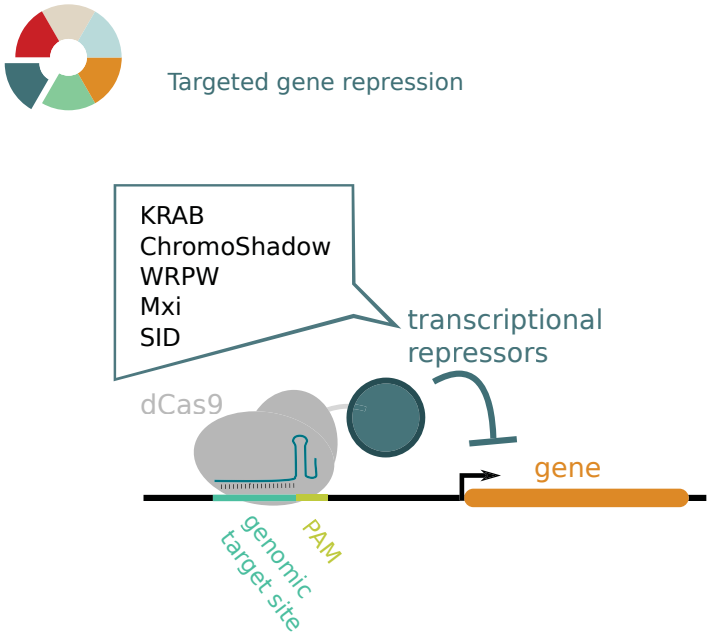

Targeted gene repression
KRAB
ChromoShadow
WRPW
Mxi
SID
transcriptional
repressors
dCas9
gene
genomic
target site
PAM

Fig. 7

Block or mutate binding sites of reprogramming factors

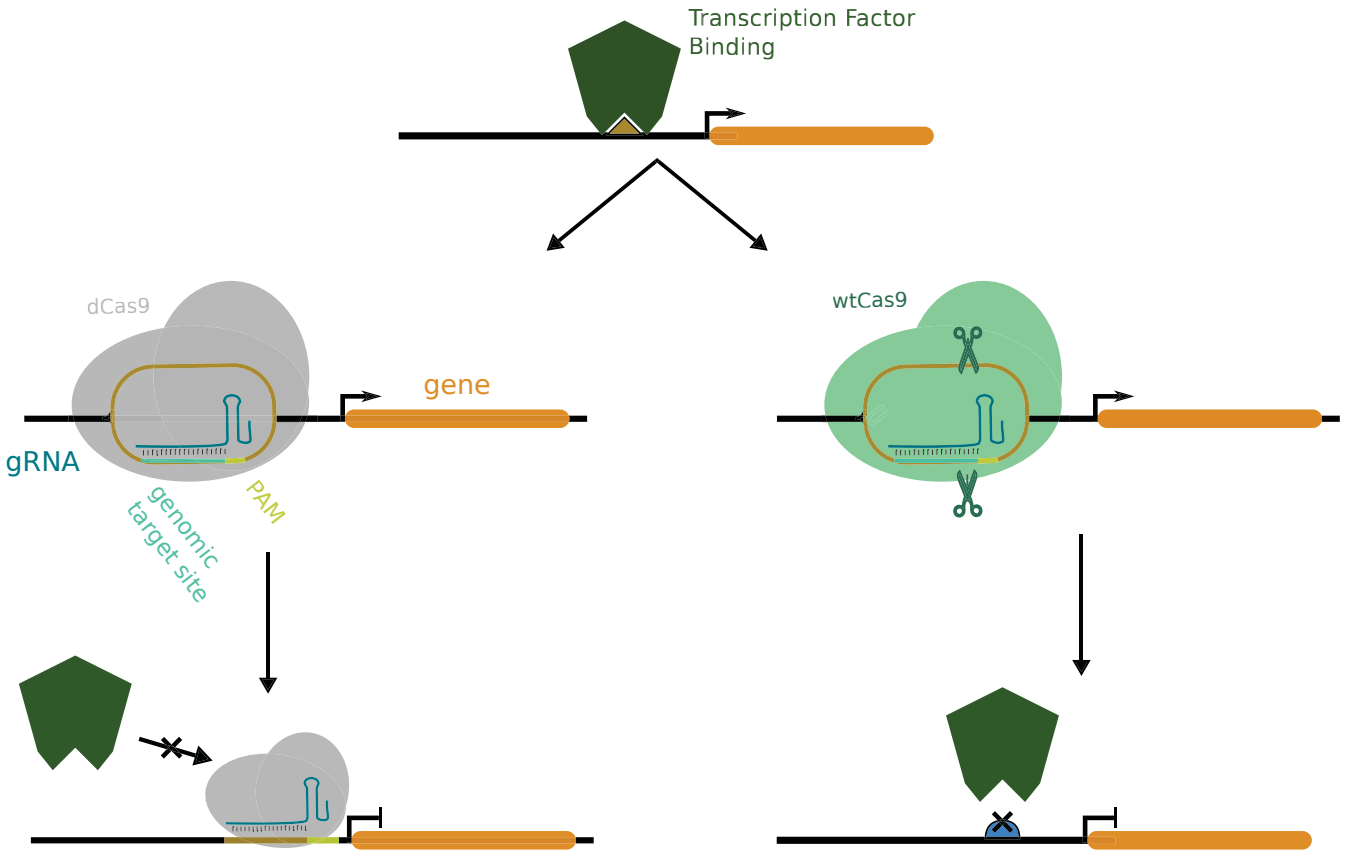


Fig. 8

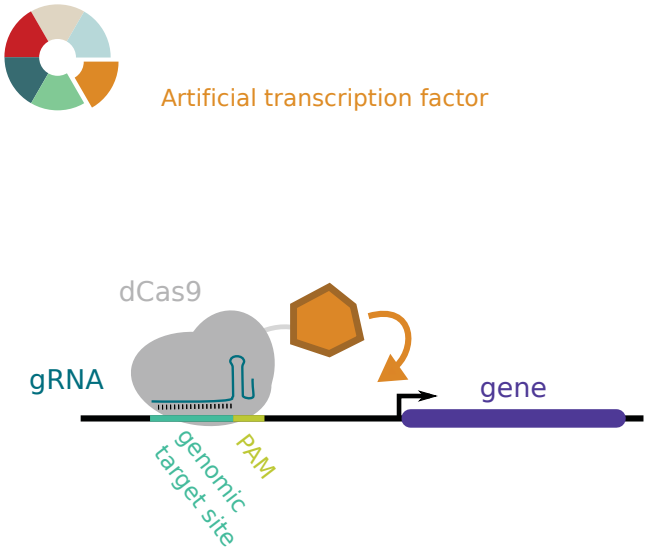

Artificial transcription factor
dCas9
gRNA
gene
genomic target site
PAM

Fig. 9

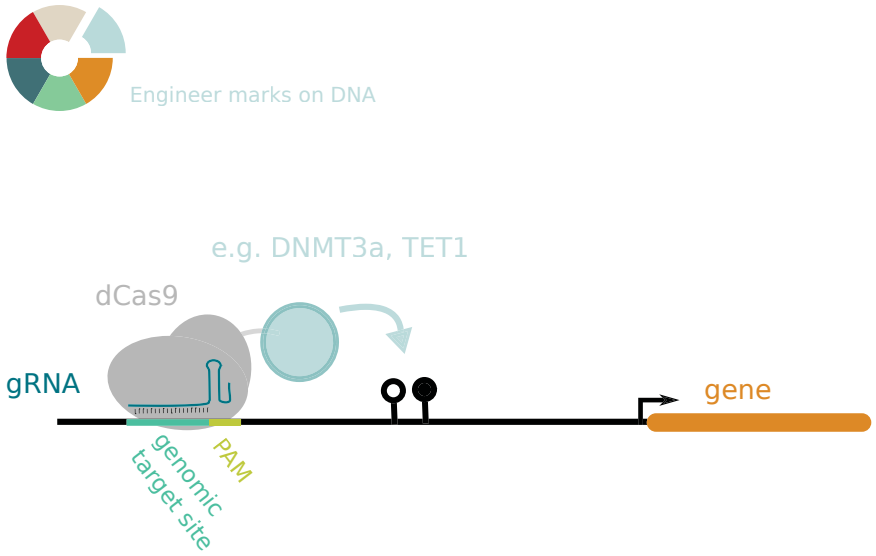
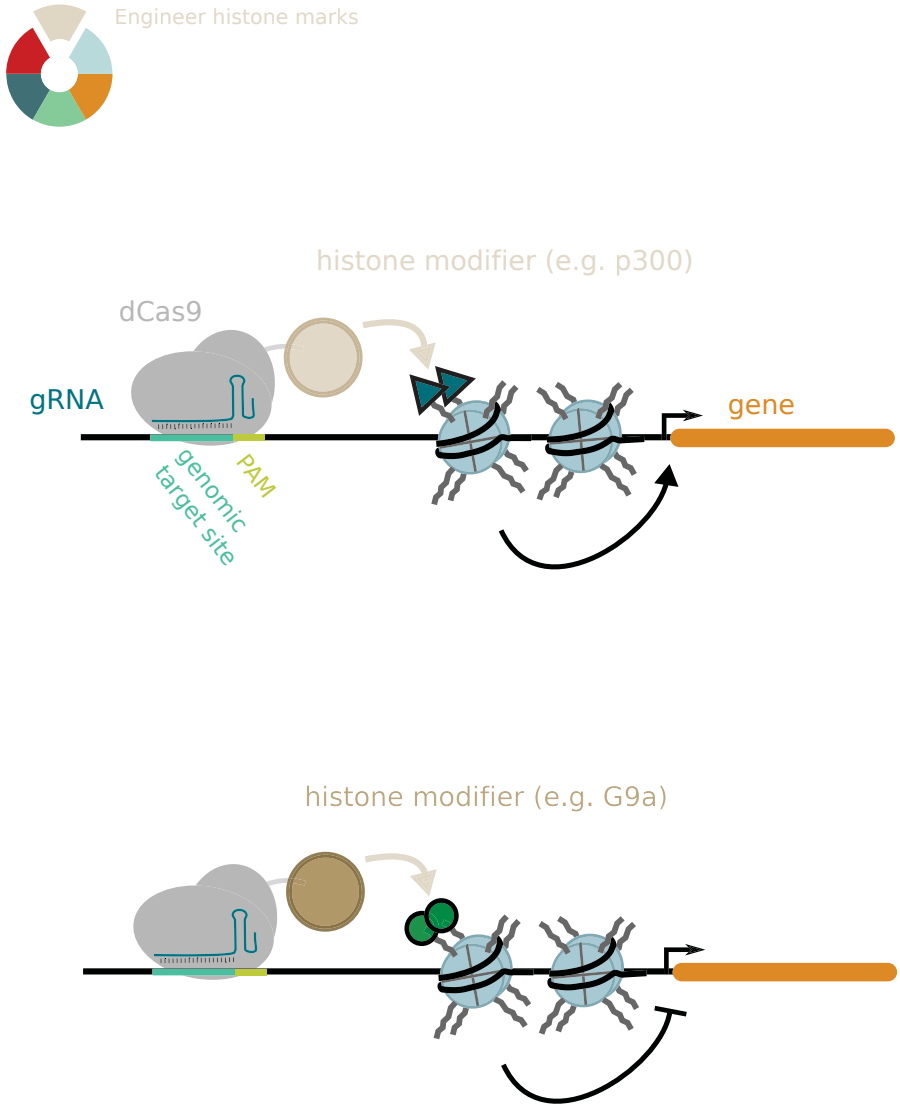
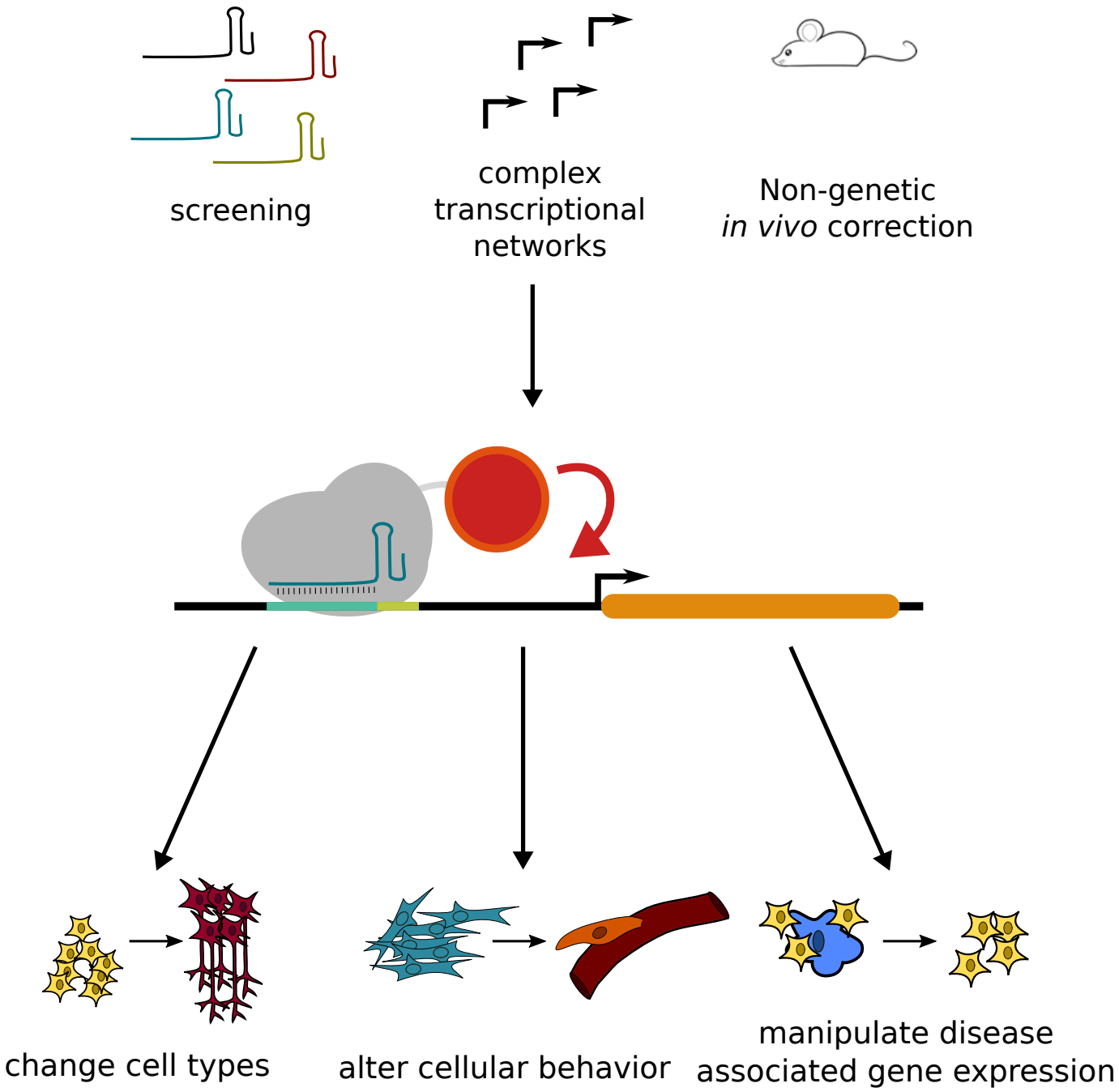

Engineer marks on DNA
e.g. DNMT3a, TET1
dCas9
gRNA
gene
PAM
genomic target site

Fig. 10


Engineer histone marks
histone modifier (e.g. p300)
dCas9
gRNA
gene
genomic target site
PAM
histone modifier (e.g. G9a)


screening
complex transcriptional networks
Non-genetic *in vivo* correction
change cell types
alter cellular behavior
manipulate disease associated gene expression