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Epigenetic editing: How cutting-edge targeted epigenetic modification might provide novel avenues for autoimmune disease therapy☆

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Abstract

Autoimmune diseases are enigmatic and complex, and most been associated with epigenetic changes. Epigenetics describes changes in gene expression related to environmental influences mediated by a variety of effectors that alter the three-dimensional structure of chromatin and facilitate transcription factor or repressor binding. Recent years have witnessed a dramatic change and acceleration in epigenetic editing approaches, spurred on by the discovery and later development of the CRISPR/Cas9 system as a highly modular and efficient site-specific DNA binding domain. The purpose of this article is to offer a review of epigenetic editing approaches to date, with a focus on alterations of DNA methylation, and to describe a few prominent published examples of epigenetic editing. We will also offer as an example work done by our laboratory demonstrating epigenetic editing of the *FOXP3* gene in human T cells. Finally, we discuss briefly the future of epigenetic editing in autoimmune disease.

1. Introduction

Autoimmune diseases are complex and heterogeneous, and have presented researchers with significant challenges, both in understanding pathogenesis and also developing successful therapeutics. The past decade has witnessed numerous publications demonstrating epigenetic associations with autoimmune diseases, but these are hampered by their inability to test causality; that is, do epigenetic changes *associated* with autoimmune disease *lead* to that disease. Until recently, editing patterns of epigenetic marks controlling gene expression was difficult at best. However, over the past several years, novel modular technologies have begun to emerge allowing precise targeting of proteins within the nucleus to specific DNA locations. This, coupled with ever-increasing advances in the understanding of epigenetic regulation, has blossomed into the field of epigenetic engineering. Although still in its

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infancy, a modular and highly specific approach to epigenetic editing has the potential to be a game changing, both in the way we interrogate the biological consequences of the myriad epigenetic modifications that have been associated with autoimmune disease, and in the way we approach the treatment of epigenetically-linked diseases by direct modification.

In this review, we will first introduce a brief history of epigenetics and epigenetic editing approaches, starting with zinc finger protein- (ZFP-) and transcription-activator-like-effector- (TALE-) based systems, then focusing on examples of modern dCas9-based approaches. We will offer examples of their use, both in vitro and, in one case, in vivo (Fig. 1). We will then describe the potential application of epigenetic approaches to autoimmune disease, offering a brief overview of epigenetic changes in autoimmune disease with a particular focus on regulatory T cells (T-regs). As an example, we will share our laboratory's preliminary work on epigenetic editing of T cells to produce overexpression of *FOXP3*, a key step in the development and function of T-regs. Finally, we will offer our thoughts on the future of epigenetic editing as both a research technique and potential clinical therapeutic in autoimmune disease and specifically highlight a few of the challenges faced by researchers in the coming years.

2. A brief introduction to epigenetics

Epigenetics is the scientific field which focuses on mechanisms used by eukaryotic cells to integrate environmental inputs and the underlying genetic code to produce a pattern of gene transcription [1]. Originally defined as heritable changes in gene expression or function not caused by alterations in the underlying genetic code, a more modern definition, "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states," better encapsulates the broad implications of epigenetics on cellular function [2]. There are at least three fundamental mechanisms by which epigenetic control is implemented, including DNA methylation, post-translational modification of histone tails, and the functions of noncoding RNAs (ncRNAs). Working in concert with various structural proteins, epigenetic effectors alter the three-dimensional structure of DNA and allow for compression/condensation and relaxation of the DNA double helix. Additionally, they provide support for gene suppression by the recruitment of additional pro- and anti-transcriptional machinery. Cellular epigenetic patterns are indispensable for embryonic development and are a major mechanism whereby gene transcription patterns are set, changed, and propagated through differentiation. Although once thought to be semi-permanent, changing only with cell division, it is now recognized that epigenetic modifications undergo frequent, active modifications. This plasticity is a key feature of epigenetic regulation that makes epigenetic editing feasible; e.g. cellular enzymes exist which catalyze modifications at each level of epigenetic control which may be harnessed and redirected to alter epigenetic states at particular genomic locations. In order to understand the utility of targeted epigenetic effector fusion proteins, it is useful to first have a firm grasp on how endogenous epigenetic patterns are created and maintained; we will focus on DNA methylation and histone sidechain alterations, as these are the epigenetic modifications that have been leveraged to date in epigenetic editing experiments.

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Methylation of genomic DNA occurs on the 5′ carbon position of the pyrimidine ring of cytosine residues, found in most eukaryotic genomes. Although traditionally described in the context of palindromic CpG dinucleotides, it is now understood that other motifs (e.g. C-H-G or C-H-H, where H is a non-G base) are carry methylation modifications in embryonic tissue and induced pluripotent stem cells. Most of cytosine residues in the human genome are methylated [3]. CpG pairs are widely distributed, including gene promoters, gene bodies, and intragenic regions; however, their transcriptionally relevant activity is generally ascribed to two regions. The first are within 5′ promoters upstream of a particular gene; about 70% of mammalian promoters contain short CpG-dense regions in these locations known as CpG islands. As our group and others have shown, the most important regions tend to be located between 1000 bp and 800 bp upstream of the transcription start site and in distal upstream enhancer elements. Enhancers are regulatory regions of DNA distant from (but usually within about 10 kb of) target genes, which contain transcription factor binding sites and can activate gene transcription from afar. They are characteristically associated with an open chromatin configuration and specific histone sidechain modifications of the nucleosomes flanking them, including H3K4me3 (histone 3 lysine 4 trimethylation) and H3K4me1 (histone 3 lysine 4 methylation), discussed later. In some disease states, the methylation state at these enhancers is actually better correlated with gene transcription than is methylation of traditional promoter elements.

The addition of methyl groups to cytosine residues is provided by a set of evolutionarily conserved set of DNA methyltransferases (DNMTs). These include the maintenance DNA methyltransferase DNMT1, which recognizes hemimethylated cytosines and copies DNA methylation information to the newly synthesized DNA strands, active during cellular division to copy epigenetic information to nascent DNA strands. DNMT3a and DNMT3b are de novo DNA methyltransferases and act to set global DNA methylation patterns during development and periods of epigenetic silencing. DNMT3 ligand (DNMT3L) is a recently discovered, catalytically inactive member of the DNMT family that works in concert with DNMT3a and DNMT3b to establish genomic methylation patterns. Each DNMT interacts with a wide variety of other enzyme complexes during chromatin remodeling, including various noncoding RNA molecules, helicases (which “unwind” the DNA double helix), and others.

Demethylation of DNA occurs via both active and passive mechanisms [4]. Passive demethylation, also known as replication-dependent dilution, occurs when normal copying of the DNA methylation pattern onto newly synthesized DNA strands during replication fails. With subsequent rounds of cell division, the original methylated cytosine pattern is diluted out of the subsequent cell pool. This is of particular importance in some autoimmune disease states; as will be discussed below, lupus T cells have reduced ERK pathway signaling resulting in decreased expression of DNMT1 which is associated with global hypomethylation. Interestingly, genome-wide loss of DNA methylation is seen in specific developmental stages (i.e. pre-implantation embryonic development and germ cell development), probably allowing cells to set up a “clean slate” for subsequent differentiation. Active DNA demethylation, a recently described phenomenon, involves the methylcytosine-to-hydroxymethylcytosine conversion and subsequent base excision repair to unmethylated cytosine, mediated by ten eleven ten gene family members (TET). We now

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recognize this first step, the conversion of methylcytosine to hydroxymethylcytosine, as the most critical and rate-limiting one in active DNA demethylation.

Histones are a highly conserved set of proteins which serve principally to stabilize and organize DNA within the nucleus. The functional unit of histones, the nucleosome, is composed of repeating octamers each containing dimers of the four core histone proteins (H2A, H2B, H3, and H4), along with a double-wrap (180–200 bp) of DNA around its outer surface and a single histone H1 intra-nucleosomal “linker”. Post-translational modifications (PTM) of certain histone amino acids make these residues relatively more positively or negatively charged and can thus attract (condense) or repel (open) the phosphate backbone of DNA [19]. Since the original description of this gene regulation function of histones, a plethora of modifications to core histone proteins have been described. Histone protein PTMs localize to the N-terminal tail of the histone amino acid sequence, which is the “exposed” surface end, making interaction with other proteins possible. Modifications generally occur on lysine residues, although others have been described, including arginine, serine, and threonine. The various modifications, which include acetylation, methylation, ubiquitination, sumoylation, deimination, and poly(ADP)-ribosylation, among others [20].

Acetylation of terminal lysine residues of the four core histone proteins (H2A, H2B, H3, and H4) removes the natural positively charged state of the histone protein and reduces its interaction with negatively charged DNA phosphate background, leading to a less condensed (euchromatic) chromatin structure which is more accessible to transcription factors and generally is associated with an increase in gene transcription. Histone acetyltransferases (HAT) lead to increases in acetylation. Deacetylation leading to condensation of chromatin around nucleosomes and gene silencing (heterochromatin) is mediated by histone deacetylases, many of which have defined pharmacological inhibitors. The other widely studied histone modification is methylation, again generally occurring on lysine or arginine residues of histone tails. Unlike histone acetylation, the effects on gene regulation of histone methylation depend on the location of the modified position and the number or density of methyl groups added; for example, methylation of lysine 4 of the histone 3 protein (H3K4me) is frequently found in enhancer regions, whereas trimethylation of the same site (H3K4me3) is frequently found in active promoter regions. Conversely, trimethylation of H3K9 and H3K27 are commonly associated with inactive gene transcription [22].

Epigenetic modifications coordinate with each other to facilitate gene expression control. For example, DNA methylation facilitates the rearrangement of the histone sidechain pattern to establish a transcriptionally repressive chromatin structure. A variety of methyl-DNA-binding enzymes have been described, generally falling into two families: the methyl-CpG binding domain-containing proteins (MBDs) and the Kaiso [9] (human zinc finger proteins that bind methylated DNA) family. These proteins generally associated with other enzymes which have histone deacetylase activity in order to establish silent chromatin and are indispensable during embryonic development. One example is methyl cytosine binding protein 2 (MeCP2), which binds to methylated DNA and associates with a whole variety of repressor enzymes including nuclear receptor co-repressor 1 (NcoR1), a protein which recruits histone deacetylases to repress gene activation among others [23, 24].

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3. History of epigenetic editing approaches to date

Any sequence-specific epigenetic editing platform must, by necessity, contain at least two parts: a DNA binding module and a catalytic, active epigenetic modification module (Fig. 1). The enzymes which catalyze the addition of methyl groups to DNA (the DNA methyltransferases in eukaryotes and M.SssI in prokaryotes), as described above, have been known for years [5], whereas those enzymes responsible for active DNA demethylation (DNA glycosylases within the ten-eleven-ten or TET family) remained elusive until only a few years ago [6]. Unlike the relatively restricted set of DNA methylation effectors, there are dozens of histone PTM effectors, many of which have been leveraged in epigenetic editing approaches.

3.1. The problem of DNA localization

A key and persistent problem in epigenetic editing has been the delivery of these epigenetic modification motifs in a highly sequence-specific manner. To date, three DNA localization (DNA binding) systems have been used in fusion proteins to target epigenetic modifications to particular locations in the genome. The first to be implemented were zinc finger proteins (ZFPs). ZFPs are common, endogenous DNA binding proteins found in most eukaryotes, and consist of about 30 amino acids split among an alpha helix and two beta sheets linked by a zinc ion. Three amino acids within the alpha-helix interact with three base pairs in the major groove of DNA to afford specificity [7]. Although the three-base pair specificity produced by a single ZFP is not specific enough to be useful when targeting the large human genome. For this reason, combinations of six zinc fingers into a single DNA binding module (recognizing 18 base pairs of DNA, mathematically likely to be unique within the genome) has become commonplace [8], and there are a number of articles demonstrating methods for ZFP design and optimization [9–12] In fact, there now exist several free software tools to easily design ZFPs to target any input DNA sequence (see Zinc Finger Tools from the Scripps institute, or the Zinc Finger Consortium sponsored by the Joung Lab at Harvard). Sequence-specific ZFPs linked with DNA nuclease domains are even available commercially (i.e. Sigma-Aldrich, St. Louis, MO). Although originally thought of as only mildly immunogenic owing to similarity with endogenous ZFPs, more recent studies have indicated that this may not be the case [13]. Furthermore, ZFPs are able to specifically bind to modified DNA bases (i.e. methylated cytosine residues), allowing fine targeting of epigenetic modifications. Despite their usefulness, ZFPs have several drawbacks. Perhaps the most difficult to overcome is the high cost and lack of modularity of this system. For a new genomic location to be targeted, an entirely new ZFP must be designed and synthesized.

TALEs are proteins secreted from *Xanthomonas* bacteria upon infection of certain plants via a Type III secretion system [14]. Their natural function is to recognize and bind to specific plant DNA sequences in order to activate expression of genes which aid their infection. TALEs' DNA binding activity is mediated by a central repeat domain consisting of a variable number of 33–34 amino acid repeats; substitutions of amino acids 12 and 13, which are responsible for binding corresponding DNA nucleotides, afford them sequence specificity [15]. Similar to ZFPs, synthetic combination of these TALE repeats has allowed researchers to target nearly any DNA sequence with a high degree of specificity;

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furthermore, they are able to be engineered to bind to modified DNA bases. Also, like ZFPs, cloning of repeat TALE arrays is tedious, technically challenging, and expensive. Their large size and lack of an endogenous analogue has led many to worry about potential immunogenicity if used in vivo in human patients. A final challenge, at least from an epigenetic editing perspective, is that dense local DNA methylation inhibits the binding of most TALEs, limiting their usefulness in editing techniques aimed at reducing DNA methylation [16]. Nonetheless, TALEs have been successfully used for epigenetic editing in a number of studies.

The recent discovery of an endogenous bacterial adaptive immune system, mediated by genetically-coded "memory" and effector proteins known as Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) and Cas9 respectively, has greatly simplified the design and implementation of DNA targeting domains for a variety of purposes. The CRISPR/Cas9 system consists of a single endonuclease protein (Cas9) which in turn binds to RNAs encoded by CRISPR loci. These RNAs contain within them a ~20 bp localization sequence just upstream of a protospacer adjacent motif (5′NGG-3′, PAM) which directly binds to host DNA and lends sequence specificity. Within its natural host, the CRISPR/Cas9 system affords bacteria the ability to recognize and cut invading viral DNA [17]. The two nuclease domains of Cas9 have been experimentally inactivated by introducing two amino acid mutations, D10A (Asp10 → Ala10) and H840A (His840 → Ala840), leading to a highly specific and modular DNA binding system known as defective Cas9 (dCas9). Unlike TALEs and ZFPs, retargeting of a dCas9-based system requires only synthesizing and replacing the 20 bp localization sequence within the targeting RNA gene (usually identified as a guide RNA or gRNA). Although there were initial concerns regarding the sequence specificity of CRISPR and the potential for off-target effects [18], refinement in the design of guide RNAs and Cas9 proteins have largely resolved this concern (review, [19]).

3.2. Advantages of epigenetic editing over other forms of gene therapy

Epigenetic editing using the techniques outlined in this review offer several advantages over more traditional genetic editing and gene expression control techniques. Firstly, we expect that epigenetic modification would allow for "hit and run" editing, expanded in more detail later. Briefly, an acute change in epigenetic pattern should allow for a durable change in gene expression, at least until the epigenetic pattern is modified again by either a natural or synthetic process. Secondly, epigenetic editing allows researchers to explore the consequences of repressing or inducing a gene via its "natural" control mechanisms. This will allow more conclusive evidence to be drawn regarding the functional significance of alterations in gene expression; traditional techniques rely on introducing the open reading frame (ORF) of a gene to be overexpressed via an extrachromosomal plasmid, and do not recapitulate a gene's local chromatin environment, epigenetic modification allows gene expression to be manipulated in situ. The long-lasting effects of epigenetic modifications will also presumably allow for long-term gene repression. This is in contrast to more traditional interfering-RNA (RNAi)-based approaches, which are more transient in nature. Direct genetic editing via CRISPR/Cas9 (nuclease-competent) have also been recently described as a mechanism to interrupt a coding sequence and permanently turn genes off,

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although this permanence is a two-edged sword, as genes cannot be easily repaired to a competent state without editing them a second time. In this regard, epigenetic editing can be seen as a comfortable middle ground, offering medium- to long-term gene expression modification while leaving the underlying genetic sequence intact. It should be noted here, as discussed above, that epigenetic marks are heritable through cellular division. This means that changes induced by epigenetic editing should be passed through subsequent generations of cellular division, again offering a semi-permanent solution to gene expression modulation without having to modify the underlying sequence. Finally, epigenetic editing offers the only pathway by which to elucidate the consequences of specific epigenetic modifications; that is, to "prove" whether previously-associated epigenetic changes actually cause a disease state or are simply associated with it.

3.3. Examples of epigenetic editing approaches to date

The first examples of epigenetic editing in the literature were by Xu and colleagues in 1997 [20] and Snowden et al. in 2002 [21]. Snowden linked an engineered ZFP targeting a location 434 bp downstream of the transcription start site of human *VEGF-A* transcription start site, separately fused to the catalytic domain of histone methyltransferases G9a and SUV39H1 and deacetylase v-ErbA. Targeting a repressive histone tail pattern to this locus was successful in substantially reducing *VEGF* gene expression. The first modern example of DNA methylation editing was published in 2007 by Li et al. [22]. They fused the catalytic domains of murine Dnmt3a to one of two engineered ZFPs directed to promoter regions of the human herpes simplex virus (HSV). They then generated a reporter system consisting of HSV promoter elements and luciferase and transfected the ZFP-3a fusion protein and demonstrated significant reduction in luciferase activity (~50%) with concomitant increases in DNA methylation within the targeted region. Foreshadowing potential future clinical applications, they also transfected their ZFP-3a fusion into human cell lines during HSV-1 lytic infection and demonstrated a substantial reduction in HSV-1 titer, presumably owing to viral gene silencing during infection. Similar repression of VEGF-A expression was noted in 2012 by Siddique et al. by targeting DNA methyltransferases [23].

Maeder et al. in 2013 were one of the first groups to create a targeted demethylation system. They constructed a TALE-TET1 fusion containing multiple fused TALE domains targeting *KLF4*, a gene encoding a transcription factor important in differentiation, proliferation, and pluripotency, among others [24]. The group produced TALE-TET1 fusions using both full-length *TET1* and the catalytic domain of *TET1* alone. Importantly, they demonstrate that fusions containing the catalytic domain were actually better at demethylating targeted CpG sites than the full-length fusions, and that significant demethylation in the promoter regions of the targeted genes was accompanied by increases in gene expression. They also tested using a variety of lengths of linker (the short amino acid sequence that links DNA binding domains, here TALE repeats, to the catalytic domain of interest), but did not find any appreciable differences. It should be noted that this group included data in the supplementary material regarding ZFP-TET1 fusions, which demonstrate similar demethylation efficiency. Chen et al. went on to demonstrate similar findings for ZFP-TET1 and ZFP-TET2 plasmids in 2014 [25]. They did not see demethylation with ZFP-TET3 fusions, however.

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Moving towards clinical applicability, Falahi and colleagues in 2013 published a ZFP-G9a fusion protein targeted to the *HER2/Neu* oncogene, important in many forms of cancer, and inhibited by the FDA-approved drug Imatinib [26]. They reasoned that epigenetic silencing of this oncogene may offer several advantages over traditional drug therapy, not least of which would be overcoming Imatinib resistance, which is a substantial problem in the oncology field. They did indeed demonstrate targeted histone H3K9 methylation, along with repression of oncogene expression by about 40% in *HER2/Neu +* SKOV3 breast cancer cell lines. Importantly, epigenetic editing of this oncogene was also associated with a reduction in cell metabolic activity and inhibition in cell growth. Many other groups have since validated the approach of fusing ZFPs [22, 23, 25–34], TALEs [24, 35–37], or dCas9 [33, 35, 38–43] DNA-binding domains with an epigenetic effector to modulate local epigenetic marks and gene expression.

The field of epigenetic editing expanded substantially following the creation of dCas9, as it represented a highly modular and easily-implemented DNA binding system. The first strategies to modulate gene expression with dCas9 actually included no effectors at all, and used the bulky dCas9 protein targeted to specific gene locations to sterically hinder transcription [44, 45]. This strategy was not found to be robust in mammalian cells, however. Subsequent studies leveraged single fusion proteins of dCas9 fused with short-term transcriptional activator and repressor domains (i.e. VP64, KRAB), which resulted in significant gene expression modifications while the dCas9-fusion was present, but were not epigenetically modifying per se [46–48]. In 2015 Konermann et al. demonstrated that massive multiplexing of simultaneous gene activation (of every RefSeq coding gene) using a modified dCas9-VP64 system with 70,290 guide RNAs was achievable [49]. True “epigenetic targeting”, including dCas9 fusions combined with histone acetylases like p300 [35] and CBP [50], histone demethylase LSD1 [38], and DNA methylation/demethylation DNMT3a [42, 51], MQ1 [40], and TET1 [52] have been more recently described.

3.4. Refining epigenetic editing techniques

Until just the past year, epigenetic editing approaches have consisted of single fusion proteins; e.g. a DNA binding domain (dCas9, for example) fused to a flexible linker fused to an epigenetically active catalytic domain (TET1, for example). A refinement to this approach, which has yielded significant improvements in both modularity and activity, was presented by Morita et al. in 2016 [53]. They leveraged the SUNTAG [54] system, which consists of two parts. The first is a DNA localization domain linked with a tandem repeat of multiple copies (in this case 10) of the 19-amino acid GCN4 peptide separated by small linkers. The second part of the SUNTAG system consists of an effector domain of interest (originally the transcription activator domain VP64) fused with the single-chain variable fragment of an anti-GCN4 antibody. This system offers a couple of core advantages over previous fusion protein epigenetic effectors. First, it makes the system highly modular, as switching to another epigenetic effector domain is as simple as substituting a new anti-GCN4-effector plasmid in transfection experiments. The second is that it allows multiple effector domains to be linked to a single dCas9 targeting molecule, which increases the likelihood of achieving any epigenetic editing effect. For example, Morita et al. produced a dCas9-SUNTAG-TET1 system to target the H19 gene in embryonic stem cells. Unlike

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previous dCas9-TET1 direct fusion proteins, which produced about 30–50% demethylation of target sequences, Morita and colleagues were able to demonstrate near-100% demethylation efficiency in transfected- and flow-sorted cells, a remarkable achievement. This SUNTAG epigenetic editing approach has subsequently been used to increase local methylation levels by Huang and colleagues [55, 56]. They *HOXA5, BACH2*, and *KLF4*, and demonstrated increased DNA methylation of between 30% and 80%, with concomitant gene expression suppression of ~80%.

One important aspect of epigenetic editing that has not received much attention in the literature is the notion of "stickiness"; e.g. the durability of epigenetic changes induced by editing techniques in the absence of the editing construct itself. Theoretically, this concept of stickiness is one of the major draws of an epigenetic editing technique (as opposed to, say, targeting a gene activator like VP64 or gene suppressor like RNAi, both of which have mechanisms of action limited to their presence within a cell). The ability of epigenetic editing to induce long-term transcriptional and function consequences, including transgenerational epigenetic modification, has been recently demonstrated [57, 58] and coined "hit-and-run" epigenetic editing. As an example, Saunderson et al. in 2017 [58] targeted dCas9-Dnmt3a-Dnmt3l to in vitro cell cultures of both an immortalized cell line (1089 cells) and primary myoepithelial cells. They demonstrated persistence of hypermethylation of the targeted genes (*HIC1, RASSF1A, RASSF1B, RASSF1C, p16*, and *p14*) to 10 days post-transfection, after the dCas9 construct was no longer detectable. Remarkably, increased methylation induced by epigenetic editing was persistent in *RASSF1* and *CDKN2A* out to 40 days in primary myoepithelial cells, with consistent repression of gene expression. Hypermethylation targeted only to p16 prevented primary myoepithelial cells from becoming senescent and induced a cancer-like cell proliferation.

Finally, several examples of epigenetic editing in vivo have recently been published [21, 40, 59, 60]. One recent example by Lei and colleagues [40] leveraged a dCas9-MQ1 fusion. MQ1 is a highly active yet small (396 amino acid) DNA methyltransferase created by mapping the amino acid sequences of the bacterial *M. SssI* methyltransferase onto the human codon map and performing codon optimization. They introduced this dCas9-MQ1 construct along with gRNA sequences targeting the maternally-imprinted *Igf2/H19* locus into mouse zygotes by microinjection. They subsequently analyzed DNA methylation levels of this locus in 3-week-old animals' tail clipping DNA. DNA methylation increases of ~20% were found in this region, reinforcing both the activity of this epigenetic approach in vivo and the persistence of epigenetic modification through cellular differentiation and multiple cycles of cellular division.

3.5. The problem of mistargeting

All genetic and epigenetic modification platforms have suffered from some degree of mistargeting; that is, of "tolerating" mismatches in targeting regions and producing either genetic or epigenetic changes in inappropriate locations. Substantial efforts in the past several years have been undertaken to improving the targeting specificity, particularly of CRISPR/Cas9/dCas9-based systems. Much of this work has been done in the more-well-established field of genetic editing, and these fall into three main categories. The first is

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improvement in the structure of the Cas9 protein itself to facilitate tighter binding of guide RNA sequences to the appropriate DNA location. Various mutations have been introduced into the *Streptococcus pyogenes* Cas9 protein which have facilitated improved specificity. One such variant, a quadruple-mutation (N479a/R661A/Q695A/Q926A) high-fidelity Cas9 dubbed Sp-Cas9-HF1, was demonstrated by Kleinstiver et al. in 2016 to have nearly-undetectable levels of off-target gene editing [61]. Another approach to improve specificity involves modification of gRNAs themselves. Interestingly, in a pivotal study published by Fu and colleagues in 2014, it was noted that a simple truncation of the gRNA length to 17-to-18 nucleotides instead of the standard 20 demonstrated low to undetectable levels of off-target editing [19]. A final, and more radical, approach to limiting off target binding is the use of paired or split enzymatic activity. First demonstrated by Tsai et al. in 2014, this approach utilizes two gRNAs binding in close proximity to each other, each linked to Cas9 proteins fused with a sub-domain of the enzyme effector of choice (in this case, a dimeric version of the Fok1 nuclease). If either fusion protein is guided to an off-target location without the second fusion protein being present, DNA modification does not proceed, as the required enzymatic activity is not present. This was shown to increase specificity up to 1500-fold in traditional dCas9 genetic editing [62]. This approach has demonstrated efficacy in epigenetic editing as well [22, 63], as presented most recently in an article by Stepper et al. from 2017, in which a dimeric dCas9-Dnmt3a-Dnmt3l DNA methyltransferase was generated and targeted to three gene promoters. The group noted substantial increases in DNA methylation at targeted regions with only mild off-target methylation in two different genomic regions [63]. Moving forward, specificity concerns must be taken into account when designing and implementing epigenetic editing strategies, particularly as we move towards clinical applications of these techniques. Off-targeted effects must be closely evaluated and defined, and a combination of the above approaches implemented to minimize the potential for unintentional epigenetic disruption.

4. Applications of epigenetic editing in autoimmune disease research and therapy

4.1. A brief review of epigenetics and autoimmune disease

The human immune system is a highly organized, complex, and redundant system composed of at least several hundreds of different cell types, all with a slightly different transcriptional programming to accomplish particular tasks. The healthy human immune system consists of an intricate balance of proinflammatory cells, including the T helper 1 (Th1), Th2, Th17 subsets of CD4+ T cells, and anti-inflammatory regulatory cells, including CD25+ FOXP3hi + regulatory T (Treg) cells. Each of these distinct cellular subtypes expresses its own set of cell surface markers and produces a unique set of cytokines [64]. This phenotypic differentiation is in large part driven by a cell subset-specific set of master transcription factors that, when expressed, result in a particular transcriptional program unique to each cell subset [65–67]. It comes as no surprise, given the requirement for long-lasting gene transcription control during differentiation, that epigenetic mechanisms are at the heart of regulating these transcription factors, as well as the subsequent transcriptional programs that define immune cell subsets. Indeed, even nuanced cellular subsets can be robustly identified by differences in epigenetic patterns [68] which may be used to define the appropriate subset

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to which an unknown cell type belongs based on epigenetic data alone [69]. In fact, the ability to estimate the composition of a mixed whole blood sample based on genome-wide DNA methylation data is a feature built in to many modern epigenetics statistical analysis software pipelines [70].

Given the importance of epigenetics in development and maintenance of a healthy immune system, it follows that derangements in epigenetic control mechanisms would be a feature of autoimmune disease. Indeed, altered accessibility to chromatin cis-regulatory elements, including transcription factor-binding sites and enhancer elements (pro-transcriptional genomic regions distantly upstream or within introns of a protein-coding gene's transcription start site), is a consistent finding in various autoimmune diseases (see review [71]). As an example, we will next focus on the development of regulatory T-cells (T-regs) and the importance of the epigenetic status of the transcription factor *FOXP3*. We will then describe preliminary studies our laboratory has undertaken to study the consequences of modification of the DNA methylome of *FOXP3* in T-cell lines in vitro.

4.2. Introduction to regulatory T cells and FOXP3

Regulatory T cells (T-regs) are characterized by the expression of the transcription factor FOXP3 and the surface expression of CD25 and co-inhibitory receptors such as cytotoxic T lymphocyte antigen 4 (CTLA4). They secrete the anti-inflammatory cytokine IL-10 and are expanded by the presence of TGFβ. These cells play a key role in suppressing the expansion of autoreactive pathogenic T cells, thereby maintaining immune homeostasis. Deficiencies in the number or function of T-regs has been implicated in numerous autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, among others [72–74]. The expression of *FOXP3* is both necessary and sufficient to induce a T-reg-like phenotype in vitro as evidenced by in vitro studies involving transfection and overexpression of *FOXP3* [75]. The importance of this gene in T-reg development has been further demonstrated in studies of *FOXP3* deficiency in humans and mice. For example, mice carrying a nonsense mutation of *Foxp3*, also known as Scurfy mice, lack suppressive T cell function and suffer from constitutive immunological overactivity with substantial early mortality and an average lifespan of 3 weeks [76]. The human disease IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome, caused by *FOXP3* mutations, exhibit widely distributed autoimmune disease starting at only a few months of age [77, 78].

The initiation and maintenance of *FOXP3* expression is highly driven by particular sequences within its promoter and first exon, collectively known as conserved non-coding sequences (or CNSs), which include a number of transcription factor binding sites. An epigenetically open chromatin environment at each of these three CNSs is key to a stable *FOXP3* expression profile, although methylation at each CNS region has a particular function. Hypomethylation within the second CNS region (CNS2, within the first intron) occurs during T-reg development within the thymus and is important for maintenance of long-term *Foxp3* expression after nascent T-regs exit the thymus [79]. The demethylated state of this locus facilitates the binding of a variety of transcription factors also linked with T-reg development, including ETS1, STAT5, CREB, and REL [80]. A partially-methylated

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CNS2 region is characteristic of induced T-reg cells, which are less stable and may change phenotype to other, non-regulatory T-cell subtypes when exposed to a variety of inflammatory stimuli [79, 81, 82]. This demethylated state is maintained by the recruitment of aforementioned TET DNA glycosylases by methyl-binding domain protein 2 (MBD2) [83, 84].

CNS1 is located intronically, like CNS2. Its main function appears to be in peripheral T-regs' response to TGFβ-induced SMAD signaling via binding of activated SMAD3 [80, 85], and is particularly important in pregnancy [86]. CNS3 is located intronically between exon 1 and 2 and promotes the accumulation of histone marks associated with open chromatin and gene transcription. Demethylation of this region is found in T-reg cell precursors, even before *Foxp3* is expressed, highlighting its importance in epigenetic "poising". Interestingly, it does not appear to be involved in maintenance of *FOXP3* expression following induction [87, 88]. The promoter region of *FOXP3* also contains DNA methylation sites which are important in induction and long-term expression of the gene. Demethylation of the region is associated with both induced and "natural" T-regs, and partial demethylation is seen in naive CD4+ T-cells [89, 90]. This region includes binding sites for a variety of transcription factors, including NFAT, STAT5, AP1, and the Ets family.

Stabilization of *Foxp3* expression in mouse primary T cells via epigenetic editing was described by Okada et al. in 2017 [41]. They utilized dCas9-TET1 and dCas9-p300 (histone acetyltransferase) constructs targeted to the CNS2 (TSDR) region of murine *Foxp3*. After transducing the 68–41 murine T cell line, they noted demethylation of about 30%. In primary T cell experiments, utilizing induced T-regs as a transduction substrate, they found a partial stabilization effect, where dCas9-TET1 was able to reduce the loss of *Foxp3* expression after an inflammatory stimulus. dCas9-p300 targeting of the *Foxp3* promoter region was noted to strongly induce expression of the gene (mean 500-fold overexpression vs. control), and also somewhat protected transfected cells against losing *Foxp3* expression upon inflammatory stimulus.

4.3. Our laboratory's work on FOXP3-targeted epigenetic editing

Over the past several years, our laboratory has applied many of the aforementioned hit-and-run epigenetic editing techniques to *FOXP3* in human Jurkat T cells. We have leveraged dCas9-based fusion proteins, with both direct flexible linkages and, more recently, using the SUNTAG approach, and designed gRNAs targeting the *FOXP3* promoter, CNS2 (TSDR) and CNS3 (TGFβ sensor) regions (Fig. 2A).

After transfecting dCas9-SUNTAG-TET1 constructs into Jurkat cells via electroporation, either alone or in combination with each of the gRNAs and culturing for 48 h, we measured gene expression by RT-PCR. We found a nearly-significant increase in *FOXP3* expression with the dCas9-SUNTAG-TET1 alone, likely representing nonspecific binding (*vehicle*: 0.7 ± 0.2, *dC9-TET1-gRNA*: 3.1 ± 0.2 relative units vs. GAPDH, $p = 0.052$, Fig. 2B). Interestingly, targeting the CNS2 (TSDR) region increased gene expression the most, by >300-fold (*vehicle*: 0.7 ± 0.2, *dC9-TET1 + TSDR gRNA* 230 ± 10 relative units vs. GAPDH, $p < 0.0001$), whereas lesser but still quite significant increases were seen in both the promoter-targeting gRNA (*vehicle*: 0.7 ± 0.2, *dC9-TET1 + Prom. gRNA* 120 ± 20

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relative units vs. GAPDH, $p = 0.02$) and the CNS1-targeting gRNA (*vehicle*: 0.7 ± 0.2, *dC9-TET1 + CNS2 gRNA* 59 ± 10 relative units vs. GAPDH, $p = 0.001$). We then quantified DNA methylation of the TSDR region following transfection with the TSDR-targeting gRNA (Fig. 2C). We confirmed significant reductions in DNA methylation levels in 9 of the 11 CpG sites included in the region, with an overall average reduction of ~30%.

Furthermore, we found that epigenetically-edited Jurkat T-cells do indeed function as suppressive regulatory T cells (Fig. 3). Epigenetic editing of Jurkat cells was again induced using the dCas9-SUNTAG-TET1 system and gRNAs targeted to the same 3 regions mentioned previously. After a 3-day culture period, epigenetically-edited Jurkat cells were co-cultured in a 1:1 ratio with primary human effector T cells (CD4+, CD25−), which had previously been labeled with a cell tracing dye (Fig. 3A). Co-cultures were stimulated with anti-CD3/anti-CD28-coated beads and recombinant human IL2 at 30 U/mL culture media for 4 days, then cell division assessed with flow cytometry and an effector T-cell proliferation index calculated (Fig. 3B) [91]. Interestingly, epigenetic editing of the promoter, TSDR, and CNS2 were equally able to suppress effector T-cell division by 20–30% (dC9 + TET1 + promoter gRNA = 29% avg. suppression vs. dC9 + TET1 -gRNA, $p = 2E-5$, dC9 + TET1 + TSDR gRNA = 20% avg. suppression, $p = 0.008$, dC9 + TET1 + CNS1 gRNA 28% avg. suppression, $p = 3E-5$).

5. The future of epigenetic editing in autoimmune disease

The future of epigenetic editing in autoimmune diseases is bright indeed. As experience with the various methods of editing DNA methylation and histone PTMs increases, so too does the ease of use of the systems outlined above. The majority of epigenetic editing fusion protein expression vectors are available at low cost from plasmid repositories (i.e. Addgene), and, particularly with the dCas9-based systems, the cost of producing the short guide RNA coding sequences necessary to target nearly any gene of interest is remarkably low. Despite this, a few hurdles still stand in the way of future adoption of epigenetic editing techniques as clinical therapeutics. Perhaps the most daunting is the delivery of these large plasmids or proteins into cells of interest, either via protein transduction or nucleic acid transfer. The expression plasmid size for a dCas9-TET1 fusion (on the order of 10–14 kb), for example, exceeds the cargo capacity of most commonly-used viral vector platforms, including adeno-associated and adenoviruses, and is at the upper limit of lentivirus capacity. This may be addressed by delivering the individual parts of an epigenetic editing strategy separately; i.e. using a SUNTAG approach, delivering the dCas9-GCN and scFv-TET1 expression vectors individually. Such a strategy would nonetheless present stoichiometric complications, as dose would need to be adjusted to ensure the majority of target cells would indeed receive all parts of the editing system. Additional concerns relate to the potential immunogenicity of the DNA binding domains and effectors used, as mentioned previously.

Finally, it seems that multiple genes may need to be targeted to achieve a desired effect in a complex autoimmune disease state, and some with divergent desired outcome (i.e. in systemic lupus, one may desire to demethylate *FOXP3* while simultaneously methylating various Type-I interferon genes, known to be demethylated in lupus T cells [92]. This will likely require ex vivo loading of Cas9 fusion constructs with the appropriate targeting

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gRNA. Another fundamental problem that seems more solvable is the selection of appropriate genes and genomic locations for epigenetic editing. Although potentially daunting, the near-exponential growth of next-generation sequencing approaches and reductions in cost are rapidly increasing the epigenetic data available to researchers to help guide their interventions. We must keep in mind, though, that frequently used autoimmune disease model systems (i.e. the MRL*lpr* mouse in lupus) may have differing epigenetic control mechanisms than human patients; this problem will no doubt require multiple iterations of epigenetic testing to confirm activity in a particular disease.

6. Conclusion

Epigenetic editing, once thought a futuristic dream, has now become a reality. Leveraging cutting-edge and highly specific DNA binding domains, particularly nuclease-defective Cas9 (dCas9) proteins, along with older zinc finger proteins and TALENs, numerous groups have created epigenetic editing platforms to induce a wide variety of changes, including DNA methylation and demethylation and histone post-translational modifications. These approaches have not yet been implemented widely in immunological and autoimmune disease research; however, there is certainly great potential for this in the future, given the extensive existing data linking alterations in epigenetic states, particularly DNA methylation, with virtually every autoimmune disease. Although several roadblocks remain, including difficulty with introducing these large proteins or their encoding plasmids into appropriate target cells, the future for epigenetic editing in autoimmune disease is bright indeed.

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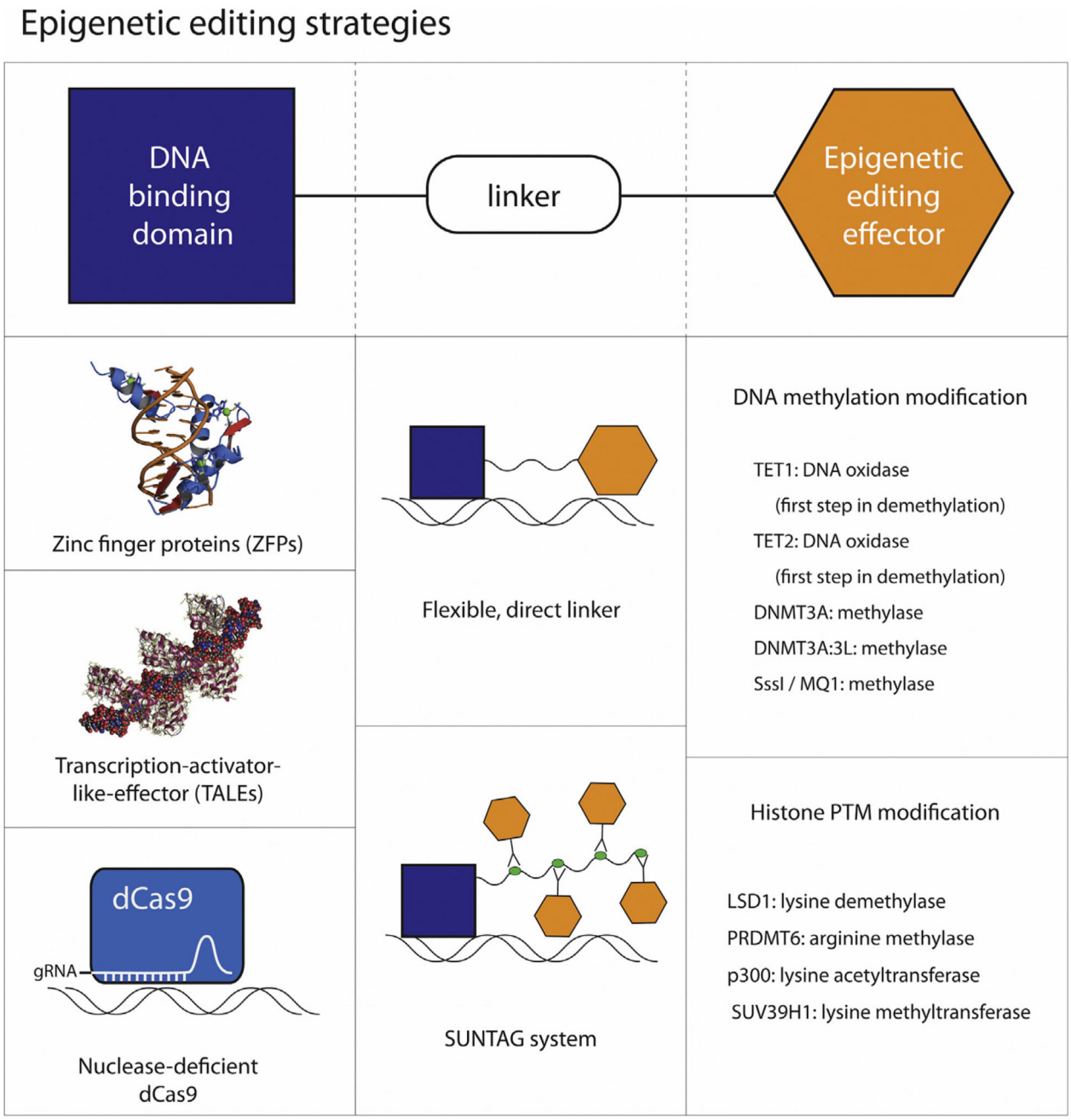

Fig. 1.
An overview of the structure of epigenetic editing strategies employed to date.

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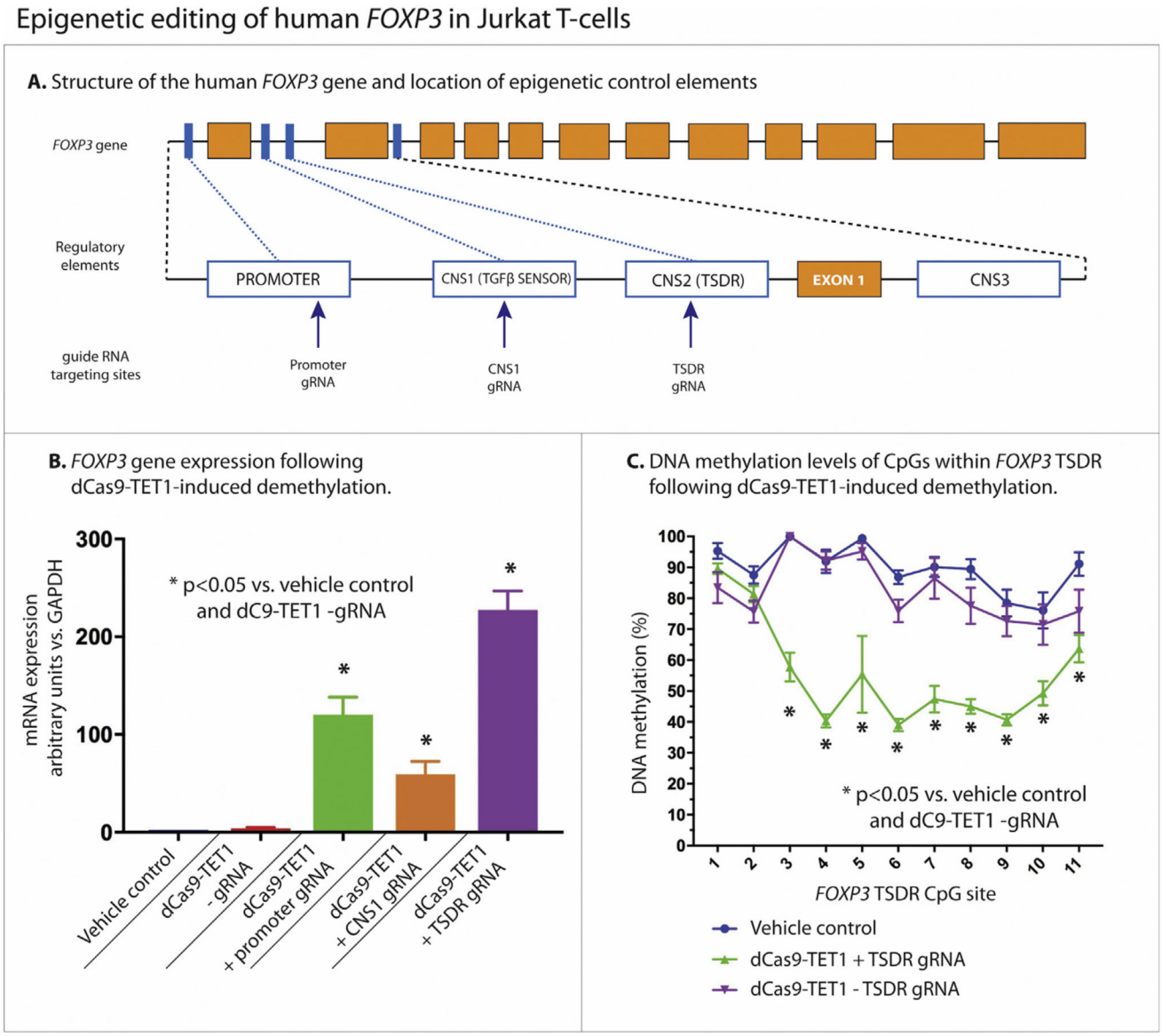

Fig. 2.

An example of epigenetic editing of the human *FOXP3* gene conducted by our laboratory. (A) Overview of the structure of the human *FOXP3* gene, important control regions represented as blue lines with exons represented as orange rectangles. (B) Induction of *FOXP3* gene expression following transfection of Jurkat cells with dCas9-Suntag-TET1 epigenetic editing machinery, combined with three guide RNAs (gRNAs) targeting differing key locations within the gene. (C) Decreases in DNA localized DNA methylation within the *FOXP3* TSDR region following dCas9-Suntag-TET1 transfection combined with a TSDR-targeting gRNA.

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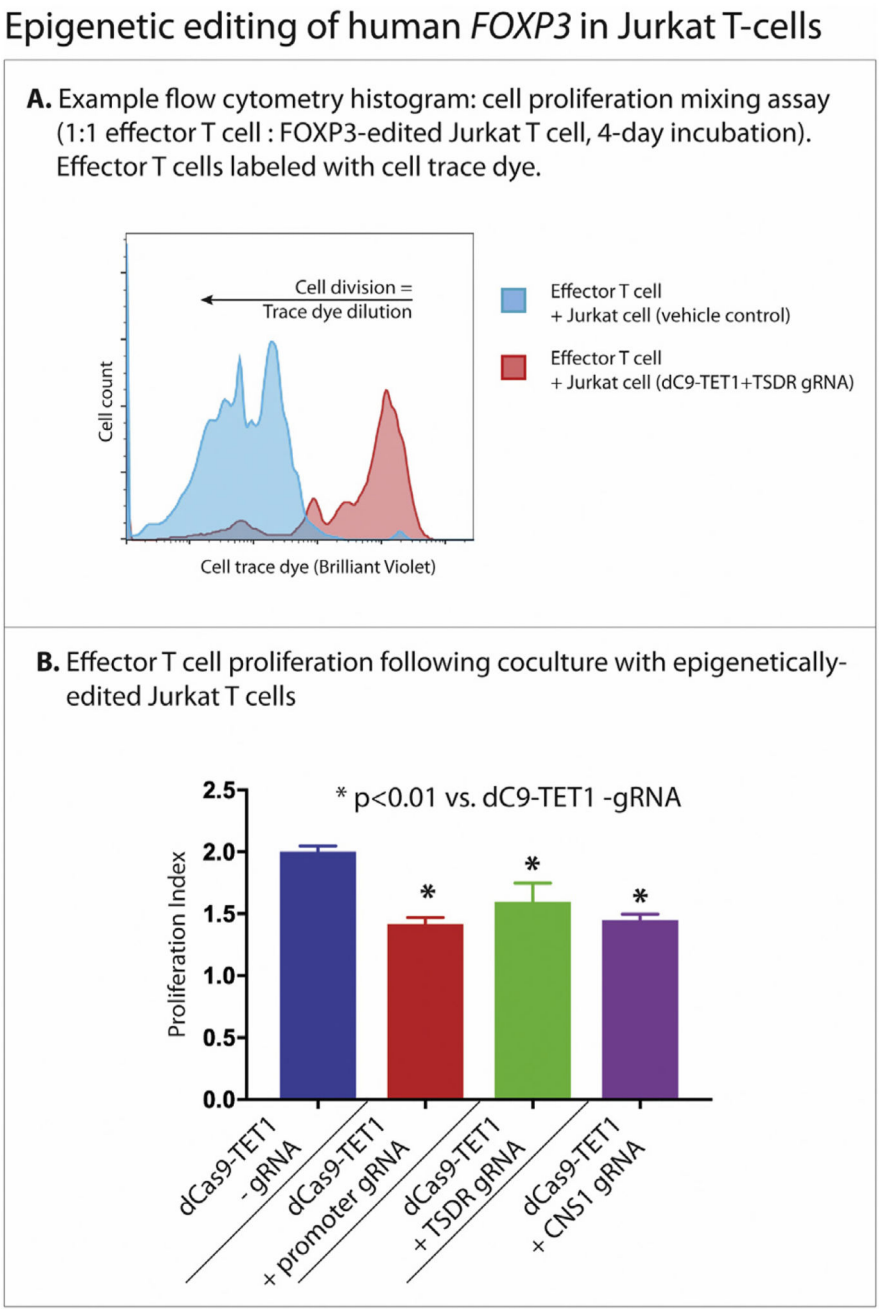

Fig. 3.

Co-culture cell proliferation studies demonstrating the induction of a regulatory phenotype following epigenetic editing and activation of *FOXP3* in human Jurkat T cell lines. Co-culture with primary CD4+, CD25− effector human T cells (T-eff). (A) Cell tracking dye dilution assay, flow cytometry histogram. T-eff cells mixed at a 1:1 ratio with dCas9-Suntag-TET1 + *FOXP3* TSDR gRNA-transfected Jurkat cells, stimulated with anti-CD4/anti-CD28 beads and rIL-2, then cultured for 4 days. (B) Proliferation index changes in T-eff following above 4-day co-culture experiments.