DNA Methylation Editing by CRISPR-guided Excision of 5-Methylcytosine

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Abstract

Tools for actively targeted DNA demethylation are required to increase our knowledge about regulation and specific functions of this important epigenetic modification. DNA demethylation in mammals involves TET-mediated oxidation of 5-methylcytosine (5-meC), which may promote its replication-dependent dilution and/or active removal through base excision repair (BER). However, it is still unclear whether oxidized derivatives of 5-meC are simply DNA demethylation intermediates or rather epigenetic marks on their own. Unlike animals, plants have evolved enzymes that directly excise 5-meC without previous modification. In this work, we have fused the catalytic domain of *Arabidopsis* ROS1 5-meC DNA glycosylase to a CRISPR-associated null-nuclease (dCas9) and analyzed its capacity for targeted reactivation of methylation-silenced genes, in comparison to other dCas9-effectors. We found that dCas9-ROS1, but not dCas9-TET1, is able to reactivate methylation-silenced genes and induce partial demethylation in a replication-independent manner. We also found that reactivation induced by dCas9-ROS1, as well as that achieved by two different CRISPR-based chromatin effectors (dCas9-VP160 and dCas9-p300), generally decreases with methylation density. Our results suggest that plant 5-meC DNA glycosylases are a valuable addition to the CRISPR-based toolbox for epigenetic editing.

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Introduction

DNA methylation (5-methylcytosine, 5-meC) is an epigenetic modification linked to gene repression that plays critical roles in cell differentiation, development, transposon silencing, genome imprinting, and X-chromosome inactivation [1—3]. Mammalian DNA methylation primarily affects symmetric CG dinucleotide sequences (also known as CpG sites), although significant non-CG methylation has been detected in pluripotent and brain cells [4]. Altered DNA methylation patterns are implicated in several pathological conditions, such as cancer and imprinting diseases [5,6].

Genomic DNA methylation patterns are dynamically controlled by antagonistic DNA methylation and demethylation processes [7]. In mammals, DNA methylation patterns are established by the DNA methyltransferase 3 (DNMT3) family of *de novo* methyltransferases and copied in post-replicative hemimethylated DNA by the maintenance methyltransferase DNMT1 [8]. Cytosine methylation may be removed through either passive or active processes. Passive demethylation involves dilution of 5-meC by DNA replication in the absence of methylation, whereas active demethylation requires enzymatic mechanisms for replication-independent removal of 5-meC [9].

Despite intense efforts, the identity of the enzymes involved in DNA demethylation in mammals has long remained elusive, and the very existence of active demethylation processes in mammalian cells has

been controversial [10,11]. However, accumulating evidence suggests that a family of alpha-ketoglutarate-dependent dioxygenases (Ten-Eleven Translocation, TET proteins) are implicated in mammalian DNA demethylation by catalyzing conversion of 5-meC to 5-hydroxymethylcytosine (5-hmeC), 5-formylcytosine (5-fC), and 5-carboxycytosine (5-caC) by consecutive oxidation reactions [12,13]. Both 5-fC and 5-caC can be excised by thymine DNA glycosylase (TDG), which may initiate their replacement with unmethylated cytosine in a base excision repair (BER) pathway [14]. TET proteins may also promote passive, replication-dependent DNA demethylation since 5-hmeC prevents maintenance DNA methylation [15]. Besides a role as intermediates in active and/or passive DNA demethylation, oxidized 5-meC derivatives may be independent epigenetic marks on their own and perform specific regulatory functions, since they have been found to be stable [16,17] and recognized by specific readers [18]. Unlike animals, plants have evolved a family of unique DNA glycosylases that directly remove 5-meC without prior modifications through an active BER demethylation pathway [19]. These enzymes, with no counterparts in animal cells, are typified by *Arabidopsis thaliana* REPRESSOR OF SILENCING 1 (ROS1) and its paralogs DEMETER (DME), and DEMETER-LIKE 2 and 3 (DML2 and DML3) [20−24].

Among all epigenetic modifications identified so far, DNA methylation is probably the best understood, but our knowledge of its specific functions is still incomplete [25]. Pioneering work showed that *in vitro* methylated DNA injected in cells is transcriptionally inactive [26,27], thus supporting the idea that DNA methylation is functionally associated with gene repression. Subsequent studies found that 5-meC may inhibit the binding of transcription factors and/or recruit Methyl-CpG-binding proteins (MBP) that, in turn, bind corepressors to inhibit transcription or modify chromatin (reviewed in Ref. [28]). However, it has been found that some transcription factors show affinity for methylated DNA [29]. Furthermore, the effect of DNA methylation on gene expression depends on sequence context, including the density of CpG sites and their location in cognate recognition sites of activating or repressing factors [30].

Advances in our understanding of the regulation and function of DNA methylation have been limited by the lack of appropriate tools to modify local methylation levels at specific sequences. Recently, the field of targeted methylation editing has received strong impulse by the development of the CRISPR/Cas technology. Mutationally deactivated Cas9 endonuclease (dCas9) may be used as an RNA-guided platform to target different types of effector proteins to specific sequences [31,32]. Several studies have reported targeted methylation [33−38], or hydromethylation [33,39−43] by expression of dCas9 fused to DNA methyltransferases or TET dioxygenases, respectively.

The use of TET-mediated oxidation to edit DNA methylation is problematic since it generates 5-meC derivatives that are stable and may have epigenetic roles on their own. A feasible alternative is to directly excise 5-meC through plant 5-meC DNA glycosylases. It has been recently reported that overexpression of *Arabidopsis* DME in human cells induces genome-wide DNA methylation changes and significant modifications in the cellular phenotype [44,45]. Furthermore, targeted demethylation and reactivation of a methylation-silenced reporter gene in human cells has been achieved by fusing the catalytic domain of *Arabidopsis* ROS1 and the DNA binding domain of yeast GAL4 [46]. In this work, we used a dCas9-ROS1 fusion protein to reactivate methylation-repressed genes, and compared its activity with that of different dCas9-effectors, including dCas9-TET1.

Results

Construction and expression of dCas9-effector fusion proteins

The different dCas9-effector proteins used in this study are shown in Fig. 1A. To generate a targeted 5-meC DNA glycosylase, we fused dCas9 to the catalytic domain of ROS1. As a control, we generated a catalytically inactive version containing a mutation in a conserved aspartate (D971 in full-length ROS1) that completely abolishes the enzymatic activity of the protein [22]. We selected TET1 as a second type of effector for targeted DNA demethylation and generated fusions of dCas9 to the wild-type protein [47] and to a mutant version with two substitutions (H1671Y and D1673A) that inactivate catalytic activity [12,47]. Additionally, we used previously described constructs with dCas9 fused to active and inactive versions of histone acetyltransferase p300 [48], as well as to the transcriptional activator VP160 [49]. The transient expression of every fusion protein in HEK293 cells was verified by Western blot analysis with anti-dCas9 (Fig. 1B).

Targeted reactivation by dCas9-ROS1 of a methylation-silenced reporter gene

We first compared the capacity of dCas9-ROS1 and dCas9-TET1 to reactivate a methylation-silenced luciferase reporter gene under the control of the minimal human herpesvirus 1 thymidylate kinase (TK) promoter [46]. We performed controlled *in vitro* methylation with M. *Sss*I DNA
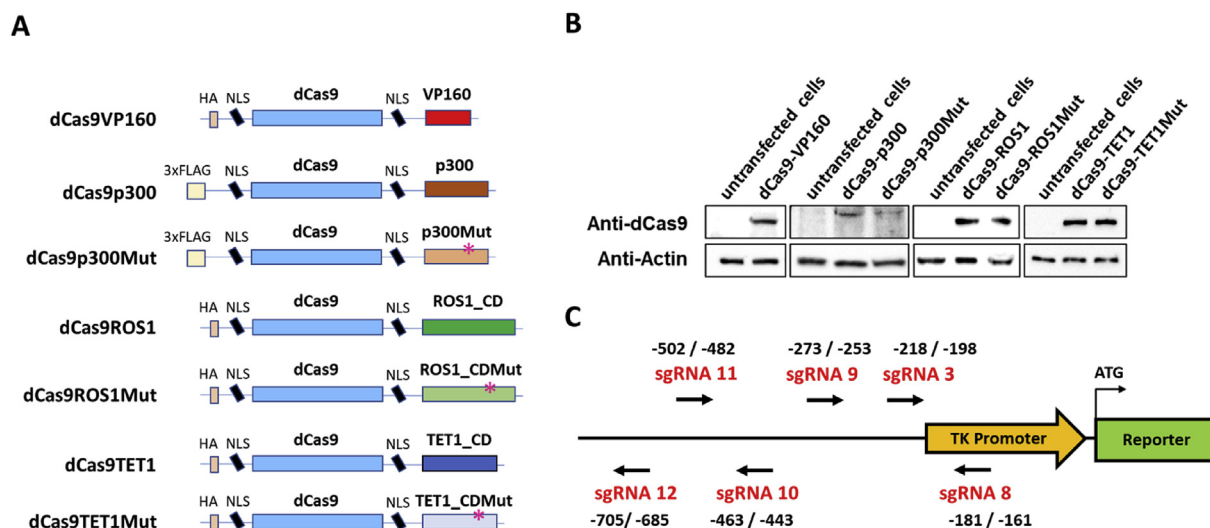
Fig. 1. Structure of dCas9-effectors and reporter constructs. (A) Schematic diagrams of dCas9-effectors used in this study. (B) Transient expression of dCas9-effector proteins in HEK293 cells. Western-blot analysis with an anti-Cas9 antibody was performed in cell extracts (80 μg) prepared 48 h after transfection. Actin was used as input control. (C) Reporter constructs contained the TK promoter fused to firefly luciferase or Green Fluorescent Protein (GFP) genes. Arrows indicate targeting sites of sgRNAs. Numbers indicate positions relative to ATG.

methyltransferase to achieve three different methylation levels (50, 75, and 100%), as assessed by *HpaII* sensitivity (Fig. S1). Six sgRNAs were designed to target a region spanning about 700 bp upstream of the initiation codon of the reporter gene, including the TK promoter (Fig. 1C). We cotransfected HEK293 cells with the methylated reporter plasmid and either dCas9-ROS1 or dCas9-TET1 effectors, together with single or multiple sgRNAs (Fig. 2). We found that dCas9-ROS1 was able to relieve repression induced by 50% and 75% methylation when targeted by most sgRNAs, although the effect was generally higher with combined sgRNAs. Importantly, luciferase activity levels in cells transfected with the mutant ROS1 version were similar to those detected in control cells transfected with either no sgRNA or with an empty vector containing no effector. Therefore, reactivation induced by ROS1 is dependent on its enzymatic activity. However, no reactivation was detected when the DNA methylation level was 100%. In comparison to dCas9-ROS1, no reactivation was detectable in cells expressing dCas9-TET1, irrespective of the sgRNA combination used and/or the level of DNA methylation. These results suggest that dCa9-ROS1 can be targeted to reactivate a methylation-silenced gene, but its effect is dependent on DNA methylation density.

Comparison of reactivation achieved by ROS1 and effectors not involved in DNA demethylation

We next asked whether reactivation levels achieved by ROS1 are similar when tested with a

different reporter gene and equivalent to those obtained by effectors not involved in a DNA demethylation pathway. We selected sgRNAs 3 and 9, either individually or in combination, for targeted reactivation of a TK-controlled GFP gene silenced by 50% methylation, and analyzed the effects of ROS1 and TET1 in comparison with p300 and VP160. We found that ROS1-induced GFP reactivation levels were similar to those observed with the luciferase reporter, and again a higher effect was achieved with combined sgRNAs (Fig. S2). In comparison, p300 and VP160 induced reactivation levels about 2.5-fold and 12-fold higher, respectively, than those induced by ROS1. Similarly to ROS1, VP160 was most effective when targeted by both sgRNAs, whereas p300 was somewhat more efficient when targeted by sgRNA 9 alone. As previously observed with the luciferase reporter, no reactivation of GFP was detectable with dCas9-TET1. Therefore, ROS1-mediated relieving of methylation-induced repression is achieved irrespectively of the targeted gene, being less effective than that achieved by either p300 or VP160.

Reactivation induced by different dCas9-effectors is methylation-density dependent

As shown above, derepression induced by ROS1 is abolished at high methylation levels (Fig. 2). We, therefore, asked whether reactivation exerted by VP160 and p300 is also methylation-density dependent. We cotransfected cells with TK-Luc plasmids displaying different DNA methylation levels (0, 10, 30, and 90%) and either targeted (sgRNAs 3 + 9) or
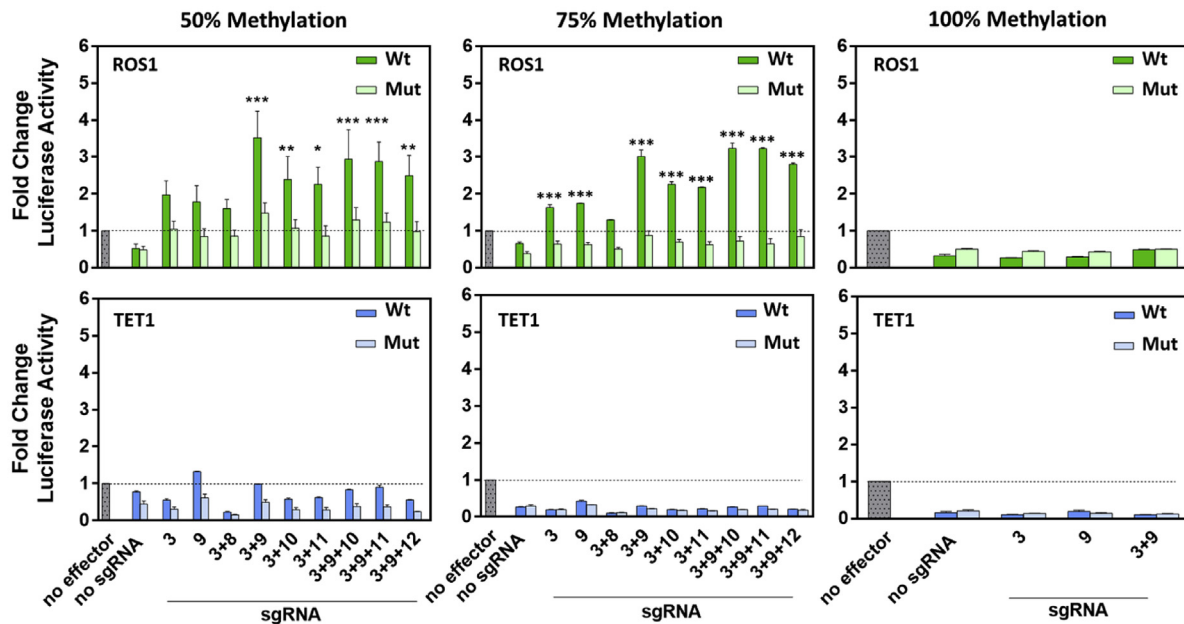
Fig. 2. Targeted reactivation by dCas9-ROS1 of a methylation-repressed luciferase reporter gene. A TK-luciferase reporter plasmid with different average methylation levels was cotransfected with dCas9-ROS1 or dCas9-TET1 effectors and single or multiple sgRNAs. Luciferase activity was determined 48 h after cotransfection and normalized to that detected with an empty vector (no effector). Values are means ± SE (error bars) from three independent transfection experiments. Asterisks indicate statistically significant differences (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; Student's unpaired t-test).

nontargeted dCas9-effector proteins (Fig. 3). Control transfections lacking either effector protein or sgRNA showed that, as expected, increased DNA methylation significantly decreased luciferase activity, which was reduced to 0.028−0.037% at 90% methylation. Expression of targeted dCas9-ROS1 decreased methylation-induced repression by about 2-fold in plasmids displaying either 10% or 30% methylation. Interestingly, expression was also induced in the unmethylated plasmid, which suggests that, as previously reported, transiently transfected DNA is subjected to *de novo* methylation [50]. Alternatively, ROS1 may have a DNA demethylation-independent role in transcriptional activation. In fact, we have previously shown that ROS1 actively interrogates unmethylated DNA in search of 5-meC [51], and therefore, may facilitate access to DNA independently of its demethylating activity. In any case, ROS1-induced expression was abolished at 90% methylation. As previously observed with GFP, dCas9-p300 achieved higher reactivation levels than dCas9-ROS1, but its effect was similarly reduced by increased DNA methylation levels and was also virtually abrogated at 90% methylation. In contrast, the reactivation pattern exerted by dCas9-VP160 was different, displaying increasing reactivation when methylation increased to 30%. Nevertheless, similarly to ROS1 and p300, VP160-induced reacti-

vation abruptly decreased when methylation reached 90%. As previously observed, no reactivation was detectable in cells expressing dCas9-TET1, regardless of the methylation level of the reporter plasmid. These results indicate that relief of methylation-induced repression by three different effectors (ROS1, p300, and V160) is methylation-density dependent, with ROS1 and p300 displaying similar patterns.

Transcriptional activation achieved by different effectors has a dissimilar impact on gene product activity

We next examined whether changes in luciferase activity induced by the different effectors correlate with mRNA levels of the luciferase reporter gene (Fig. 4). We co-transfected cells with a 50% methylated reporter plasmid and either targeted or nontargeted dCas9-effectors, and 48 h after co-transfection we measured luciferase activity and isolated total RNA. Levels of the firefly luciferase transcript were then analyzed by quantitative real-time PCR (qRT-PCR). We found that changes in luciferase activity and mRNA levels were generally highly correlated, although there were some differences among the different effectors. Thus, fold-change values for luciferase activity roughly
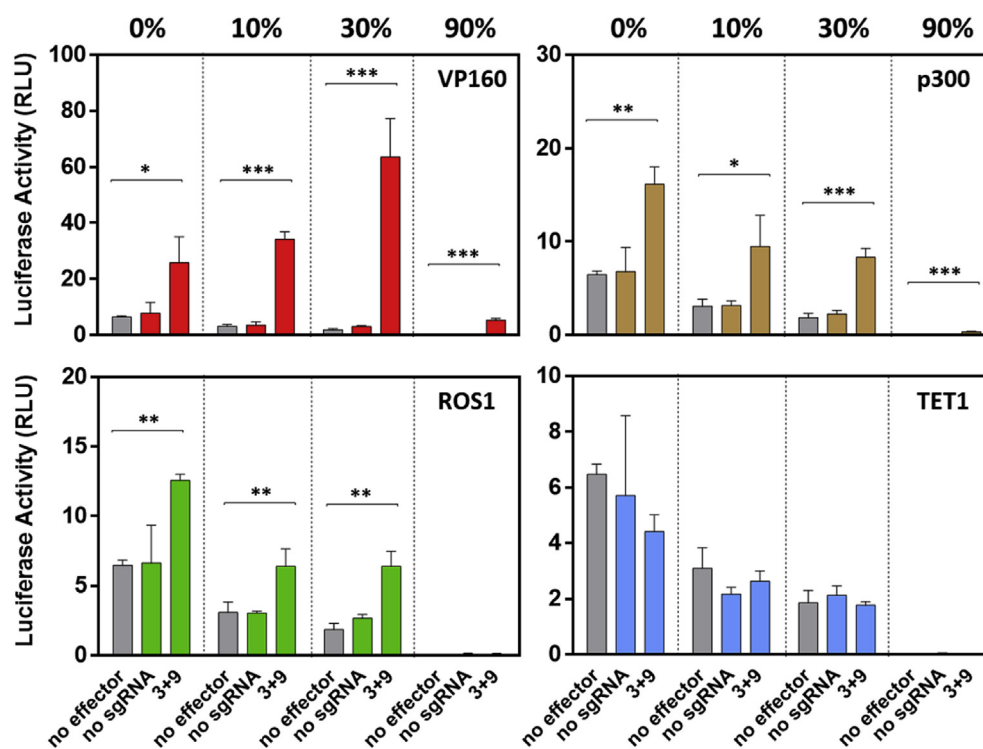
Fig. 3. Effect of methylation density on targeted reactivation induced by different dCas9-effector proteins. A TK-luciferase reporter plasmid with different average methylation levels (0, 10, 30, or 90%) was cotransfected with dCas9-effectors and two combined sgRNAs. Luciferase activity, determined 48 h after cotransfection, is shown in relative light units (RLU). Values are means $\pm$ SE (error bars) from two independent transfection experiments. Asterisks indicate statistically significant differences (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; Student's unpaired t-test).

paralleled those of mRNA levels when reactivation was targeted by dCas9-ROS1 or dCas9-p300. In contrast, increases in mRNA levels induced by dCas9-VP160 were about 3–5 times lower than the corresponding increments in luciferase activity, depending on the specific sgRNA(s) used. Finally, dCa9-TET1 induced small, but statistically significant increases in mRNA levels, although changes in luciferase activity were minimal. These results indicate that targeted transcriptional activation achieved by different effectors has a dissimilar impact on gene product activity, which likely reflects their different mechanisms of action.

No synergistic effects are detectable between dCas9-ROS1 and other dCas9-effectors

We next asked whether corecruitment of dCas9-ROS1 with different effector domains may synergistically enhance the reactivation of a methylation-silenced gene. We transfected different dCas9-effector expression plasmids into HEK293 cells, either individually or in various combinations (Fig. S3). In order to achieve comparable transfection efficiencies, the total amount of dCas9-effector expression plasmid was held constant (62.5 ng) in

all transfections. We did not observe synergistic increases in luciferase activity in any combination tested. In fact, combining different effectors led to decreased, rather than increased activity levels. Thus, wt ROS1 and wt p300 induced higher reactivation levels when tested individually than in combination. Reactivation was decreased further when cotransfections included either one or two mutant versions. Similarly, combining wt ROS1 with either wt or mutant TET1 also led to decreased reactivation. On the other hand, the high reactivation levels induced by VP160 were reduced when combined with either wt or mutant ROS1.

Corecruitment of downstream BER factors does not improve dCas9-ROS1-mediated reactivation

We next examined whether reactivation induced by dCas9-ROS1 might be increased by the corecruitment of additional BER factors acting downstream the 5-meC excision step. Plant 5-meC DNA glycosylases are bifunctional DNA glycosylases/lyases that remove 5-meC and cleave the phosphodiester backbone by β- or β, δ-elimination, generating single nucleotide gaps with either 3′-PUA (3-
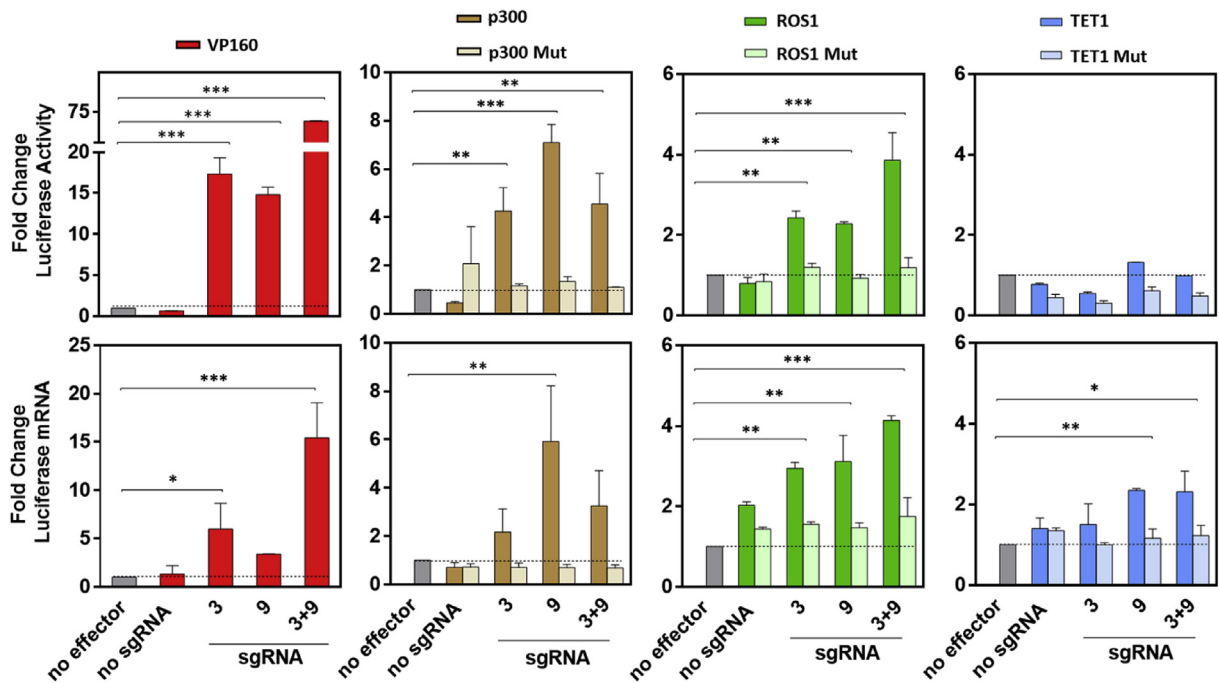
Fig. 4. Transcriptional reactivation induced by different dCas9-effector proteins. A TK-luciferase reporter plasmid with 50% average methylation was cotransfected with different dCas9-effectors and single or combined sgRNAs. Luciferase activity (top panels) and mRNA levels (bottom panels) were determined 48 h after cotransfection and normalized to those detected with an empty vector (no effector). Values are means $\pm$ SE (error bars) from two independent transfection experiments. Asterisks indicate statistically significant differences (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; Student's unpaired t-test).

phosphor-α, β-unsaturated aldehyde) or 3′-P (3′-phosphate) ends, respectively [22]. These noncanonical 3′ termini must be converted to 3′-OH ends before DNA polymerase and ligase activities complete the BER process. In *Arabidopsis*, 3′-P and 3′-PUA ends are processed by the DNA phosphatase ZDP and the 3′-phosphodiesterase activity of apurinic/apyrimidinic (AP) endonuclease APE1L, respectively [52,53]. Although human cells are endowed with the corresponding orthologs PNK and APE1, respectively, we reasoned that the corecruitment of ZDP and/or APE1L to DNA demethylation sites might improve the reactivation process.

We, therefore, constructed two additional effectors proteins by fusing dCas9 to ZDP and APE1L (Fig. S4A) and verified their transient expression in HEK293 cells by Western blot analysis (Fig. S4B). We then transfected expression plasmids for dCas9-ROS1, -ZDP, and -APE1L into cells either individually or in different combinations. As expected, in individual transfections, only cells expressing dCas9-ROS1 showed gene reactivation. However, the coexpression of dCas9-ROS1 with ZDP and/or APE1L led to decreased reactivation levels (Fig. S4C). Therefore, the corecruitment of downstream BER factors does not improve gene reactivation induced by ROS1.

Transcriptional reactivation induced by dCas9-ROS1 is concomitant with targeted, partial DNA demethylation

The results reported above indicate that dCas9-ROS1 can induce transcriptional reactivation of a methylation-silenced reporter gene in nonreplicating DNA. To examine whether such reactivation is accompanied of changes in DNA methylation, we cotransfected cells with 50% methylated reporter plasmid and either targeted or nontargeted dCas9-ROS1. For targeting, we used sgRNAs 3 and 9, either individually or in combination. As a control, we used the catalytically inactive mutant version of ROS1. After 48 h, we extracted plasmid DNA and performed methylation analysis by bisulfite DNA sequencing of a region upstream of the luciferase gene, including the TK promoter (Fig. 5). A total of 12 CpG sites distributed over a 100-bp region were analyzed. We found that cells expressing catalytically-active dCas9-ROS1 exhibited a modest, but statistically significant, decrease of DNA methylation levels at specific CpG sites when compared to those detected in cells expressing no sgRNA. Since dCas9-ROS1 is specifically targeted to the TK promoter of the reporter gene, partial local DNA demethylation may have a significant impact on
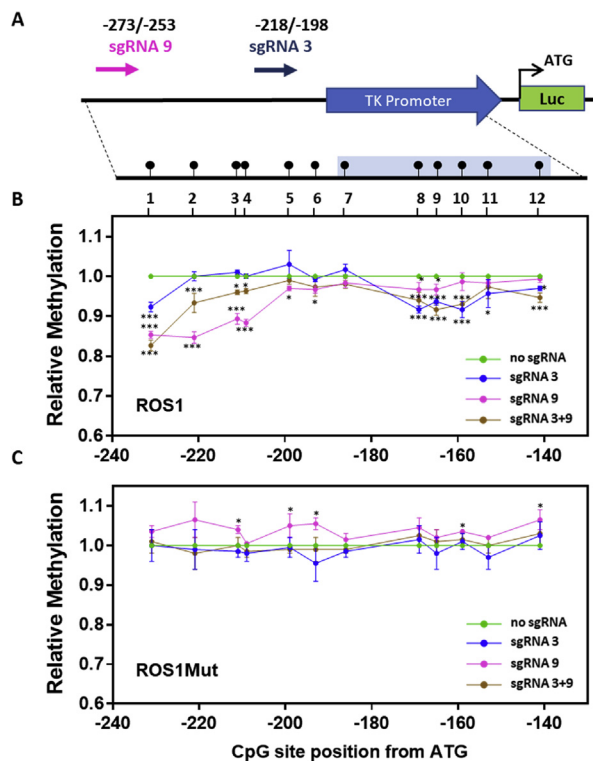
Fig. 5. DNA demethylation induced by dCas9-ROS1 on a methylated reporter gene. A TK-luciferase reporter plasmid with 50% average methylation was cotransfected with dCas9-ROS1 or its mutant version and single or combined sgRNAs. Plasmid DNA was re-isolated 48 h after cotransfection, bisulfite-treated, PCR-amplified, and pyrosequenced. Graphs show methylation at different positions normalized to that detected after cotransfection with no sgRNA. Values are means $\pm$ SE (error bars) from two independent transfection experiments. Asterisks indicate statistically significant differences (*: $P < 0.05$; ***: $P < 0.001$; Student's t-test).

transcriptional activity. The partial DNA demethylation detected in the recovered plasmid is the final outcome of a dynamic process in which targeted dCas9-ROS1 actively competes with the gene silencing machinery, including MBD proteins, recruited by methylated CpG sites. Furthermore, BER-dependent demethylation involves a DNA synthesis step, which, in turn, might facilitate access to transcription factors.

No demethylation was detectable in cells expressing targeted dCas9-ROS1Mut, although DNA methylation at several CpG sites was increased when dCas9-ROS1Mut was cotransfected with sgRNA9, but not sgRNA3. The possibility exists that targeting of an inactive DNA glycosylase to specific locations may signal for recruitment of the *de novo* DNA methylation machinery.

Interestingly, the location of sgRNA binding sites influenced the range of DNA demethylation. Thus,

when dCas9-ROS1 was targeted by a single sgRNA, most demethylated CpG sites were located in a region spanning about 40 bp downstream of its binding location. However, the demethylated area widened and covered both regions when targeted by both sgRNAs simultaneously. This result may explain the cooperative effect between sgRNAs observed in luciferase activity reactivation. On the other hand, since some demethylated CpG sites were located upstream of the sgRNA binding location (e.g., CpG site 1 with sgRNA3), it is also possible that some CpG sites are more prone to DNA demethylation than others. In any case, these results show that transcriptional reactivation induced by dCas9-ROS1 is concomitant with a targeted, partial DNA demethylation that is dependent on ROS1 catalytic activity.

We also analyzed the effect of other dCas9-effectors on DNA methylation levels (Fig. S5). No significant changes were detected in cells expressing either dCas9-VP160 or dCas9-TET1, but, surprisingly, we detected decreased DNA methylation in cells transfected with dCas9-p300.

Discussion

Targeted active DNA demethylation based on the CRISPR/Cas technology may shed light on the roles of DNA methylation and its function in regulating gene expression. However, mammalian demethylation mechanisms are complex and generate 5-meC derivatives that may be epigenetic marks on their own. In comparison, plants possess DNA glycosylases that directly excise 5-meC and may become useful tools for epigenetic editing [44–46]. In this work, we have fused the catalytic domain of *Arabidopsis* ROS1 5-meC DNA glycosylase to dCas9. We have found that the resultant fusion protein specifically reactivates transcription of methylation-silenced genes in nonreplicating DNA and that reactivation is accompanied by decreased methylation levels at several CpG sites of the targeted sequence. Our data show that reactivation induced by dCas9-ROS1 requires catalytic DNA glycosylase/lyase activity, thus suggesting that is associated to BER-mediated replacement of 5-meC with unmethylated cytosine.

In contrast to our findings with ROS1, we did not detect significant reactivation when using the 5-meC dioxygenase TET1. Although previous reports have reported TET1-mediated reactivation of methylation-silenced genes [41,54–58], evidence supporting the involvement of an active DNA demethylation process is scarce, since in most cases the experimental conditions used did not prevent DNA replication. Few studies have specifically addressed TET-mediated reactivation of methylated genes in non-replicating DNA. One of such studies reported that

expression of a methylated reporter gene is induced about 10-fold if the harboring plasmid is TET1-oxidized *in vitro* before transfection in ESCs, and showed that such reactivation is TGD-dependent [57]. On the other hand, cotransfection of TET2CD did not significantly increase activity an *in vitro* methylated reporter plasmid, although it increased about 13-fold when cells additionally overexpressed TDG [58]. Thus, targeted active DNA demethylation using TET proteins might require the simultaneous delivery of TDG DNA glycosylase.

Previous studies have shown reactivation of methylation-repressed chromosomal loci using dCas9-TET1. It was found that dCas9-TET1 induced BDNF expression by about 6-fold in embryonic postmitotic neurons [33]. Although a 2-fold reduction of *BDNF* reactivation upon inhibition of the BER factor PARP was taken as evidence of active DNA demethylation, it is important to remember that PARP plays important additional roles in transcription [59]. On the other hand, targeted dCas9-TET1-mediated demethylation of *FMR1* in FX52 iPSCs was only detected 9 days after infection [60], arguing against an active DNA demethylation mechanism. Other reports have failed to find transcriptional reactivation of chromosomal loci by dCas9-TET1, although it enhanced the effect induced by other effectors, such as dCas9-VP64 [61]. Interestingly, we found that dCas9-TET1 increased levels of the luciferase gene reporter mRNA, but such changes were not accompanied by gains in luciferase activity (Fig. 4). In this respect, it is worth noting that TET1 can catalyze the formation of 5-hydroxymethylcytidine (5-hmrC) in RNA [62], which may function as an epitranscriptomic mark modulating mRNA stability and translation [63].

In addition to ROS1 and TET1, in our study we used two chromatin modifiers (VP160 and p300) that have different action mechanisms and are not directly involved in DNA demethylation pathways. VP160, which contains ten tandem copies of herpes simplex viral protein 16 (VP16) [49], belongs to a group of acidic activators with chromatin decondensation activity [64]. They contain short acidic-hydrophobic peptide motifs that recruit chromatin-remodeling complexes and histone modifiers, including endogenous p300 [65]. Therefore, the effects induced by dCas9-p300 in chromatin may be a subset of those induced by dCas9-VP160, which may explain their different efficiencies in restoring transcription of a methylation-silenced gene.

Our results also show that p300 and, particularly, VP160 are more efficient than ROS1 in releasing methylation-induced gene silencing. DNA methylation indirectly promotes the formation of a condensed chromatin environment by recruiting MBPs, which, in turn, recruit histone deacetylases and chromatin remodeling complexes [66]. We speculate that p300 and VP160, but not ROS1, can directly counteract the final effects of DNA methylation on chromatin structure and composition. In fact, it has been previously reported that GAL4−VP16 can prevent the assembly of a repressive chromatin structure on ectopically methylated DNA injected in *Xenopus* oocyte nuclei [67]. Thus, dCas9 protein, which is insensitive to DNA methylation [68], may deliver effectors directly acting on chromatin structure and/or composition to transiently revert or prevent methylation-induced gene silencing. In contrast, methylation removal will necessarily exert a more indirect, slower effect on chromatin decondensation and, eventually, transcriptional activity. However, it remains to be determined whether the effects of DNA demethylation will be less transient than those caused by VP160 and/or p300.

Regardless of their differences, all three effectors (ROS1, p300, and VP160) showed decreased reactivation capacities on DNA containing high DNA methylation levels. For example, reactivation by dCas9-ROS1 was detected when DNA methylation was 50% and 75%, but not 100%. Previous studies carried out with *in vitro* methylated plasmids have reported that transcriptional repression increases as a function of CpG methylation density [67,69,70]. It has been suggested that dense methylation leads to a more stable binding of MBPs, such as MECP1, which would become resistant to displacement by activating factors [69].

It has been previously described that some effectors display enhanced activity when targeted by additional sgRNAs and/or when coexpressed with different effectors [71−73]. In agreement with previous reports [48], we did not detect such a cooperative effect with dCas9-p300. However, we detected a cooperative effect when either dCas9-ROS1 or VP160 were targeted by more than one sgRNA. Since our results suggest that the range of DNA demethylation induced by dCas9-ROS1 is partially limited by the sgRNA binding site, we hypothesize that additional sgRNAs may improve dCa9-ROS1 efficiency by broadening the demethylated region.

In contrast, we could not detect synergy between dCas9-ROS1 and other effectors. It is possible that cooperative effects are more likely among chromatin effectors acting through similar mechanisms. Thus, p300 displays a cooperative effect with UTX (H3K27 demethylase) and MLL4 (H3K4 methyltransferase), both of which are also histone modifiers [73], but not with the acidic transactivator VP64 [48]. On the other hand, we found that reactivation induced by dCas9-ROS1 was not enhanced when coexpressed with downstream BER factors ZDP and/or APE1L. These results suggest that the processing of DNA repair intermediates by the endogenous BER machinery is not a rate-limiting step during targeted ROS1-induced demethylation.

In agreement with previous reports, we found that dCas9-VP160-induced reactivation is not accompanied by methylation changes. Thus, it has been previously reported that the transient expression of TALE-VP64 induced the transcriptional activation of *OCT4* in NIH3T3 cells, but it did not alter its methylation status [74]. However, we found that dCas9-p300-mediated transcriptional activation was accompanied of decreased methylation levels. To our knowledge, no methylation analyses have been performed in previous works reporting targeted p300-induced gene activation [48,73,75,76]. However, there are some indications that histone acetylation may facilitate DNA demethylation. Thus, the demethylation of an ectopically methylated reporter gene in HEK293 cells is increased by the histone deacetylase inhibitor TSA [77] and decreased by overexpression of an acetyltransferase inhibitor [78]. Since TSA effects are abolished by transcription inhibitors, it has been proposed that acetylation-induced transcription facilitates DNA demethylation [79]. The fact that no cooperative effect was detected between dCas9-ROS1 and dCas9-p300 suggests that ROS1 and p300 may function in the same pathway, which is consistent with the notion that histone acetylation facilitates DNA demethylation.

In conclusion, our results suggest that dCas9-ROS1 may be a helpful tool to induce targeted DNA methylation changes in a replication-independent manner.

Materials and Methods

Plasmid construction for expression of dCas9-effector proteins

The constructs pAC93-pmax-dCas9VP160 (#48225 Addgene) [49], pcDNA-dCas9-p300 Core (#61357 Addgene) [48] and pcDNA-dCas9-p300 Core (D1399Y) (#61358 Addgene) [48] were purchased from Addgene. They encode the catalytically inactive Cas9 (dCas9) fused to the VP160 activation domain, the catalytic core of the human acetyltransferase p300, and a mutant version of p300, respectively. The catalytic domain of human TET1 and its mutant version were amplified from construct MLM3727 (#49961 Addgene) [47] and MLM3743 (#49962 Addgene) [47], respectively using Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen) and primers with *Fse*I and *Pac*I sites (Table S2). Amplification products were digested and subcloned into pAC93-pmax-dCas9VP160, replacing VP160. The catalytic domain of *Arabidopsis* ROS1 5-meC DNA glycosylase was synthesized by codon-optimized for expression in human cells (GenScript) and subcloned into pAC93-pmax-dCas9VP160 by fol-

lowing the same strategy described above for the TET1 constructs (Table S2). The catalytically inactive mutant version dCas9-ROS1Mut was generated by site-directed mutagenesis using the Quick-Change II XL kit (Stratagene) and specific oligonucleotides (Table S2). *Arabidopsis* 3′ DNA phosphatase ZDP and APE1L cDNAs were also amplified by PCR primers with *Fse*I and *Pac*I sites (Table S2) and subcloned into pAC93-pmax-dCas9VP160, replacing VP160.

Reporter plasmids

The reporter plasmid TK-Luc was previously described [46]. This plasmid contains the minimal human herpes virus 1 thymidylate kinase (TK) promoter (156 bp) upstream the firefly luciferase reporter gene. As an internal control, *Renilla* luciferase expression under CMV control (Promega) was used to normalize transfection efficiency in firefly luciferase reporter gene assays. To construct the reporter plasmid TK-eGFP, a fragment containing the eGFP reporter gene was obtained by *Hind*III-*Xba*I digestion of pCDNA3-eGFP plasmid (#13031 Addgene) and ligated into *Hind*III-*Xba*I digested TK-Luc plasmid, replacing the luciferase reporter gene. *TdTomato* expression under CMV control (plasmid #54642 Addgene), was used to normalize transfection efficiency in TK-eGFP reporter gene assays.

Reporter plasmids were *in vitro* methylated with CpG methylase M.*Sss*I (New England Biolabs) according to the manufacturer's instructions. Incubation at 37 °C was carried out at different periods of time to obtain different levels of DNA methylation, as indicated. DNA methylation was verified by digestion with methylation sensitive enzyme *Hpa*II (New England Biolabs).

sgRNAs design and expression

sgRNA sequences to target the TK promoter (sgRNAs TK 3 to 12) were designed using *Feng Zhang lab's Target Finder* software (http://crispr.mit.edu) or *CHOPCHOP* software (http://chopchop.cbu.uib.no/index.php). Best guides provided by both tools, scored by the inversed likelihood of off-target binding, were selected. Expression plasmids for TK sgRNAs were constructed by cloning annealed oligos (Table S1) into pMLM3636 (#43860 Addgene) at *Bsm*BI digestion sites.

Cell culture and transfection

Human embryonic kidney HEK293 cells were cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM, 4,5 g/L D-glucose) (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin.

Cells were seeded in 24-well or 6-well plates at a density of 1×10^5 or 6×10^5 cells/well, respectively, 24 h before transfection. For 24-well plates, each well was transfected using 1.5 μl of Lipofectamine LTX™ Reagent (Invitrogen) in 500 μl Opti-MEM® I Reduced Serum Media (Invitrogen) with 500 ng of total plasmid DNA. The DNA cotransfection mix contained 250 ng of reporter plasmid, 5 ng internal control plasmid, 125 ng of the dCas9-effector expression plasmid, and 125 ng of equimolar pooled or individual sgRNAs expression plasmids. All values were scaled up by a factor of 5 when using 6-well plates. Cells were harvested either 24 h (TK-eGFP) or 48 h (TK-Luc) after transfection.

Reporter assays

Firefly and *Renilla* luciferase activities were measured 48 h after cotransfection using the Dual-Luciferase® Reporter Assay System (Promega). All readings were carried out in a TECAN infinite F200 PRO microplate reader and using the i-Control 1.7 software. *Renilla* luciferase was used as a reference gene for normalization. GFP expression was determined by flow cytometry. Cells were harvested 24 h after cotransfection, washed, and resuspended in PBS. The number of GFP$^+$ cells was quantified using a LSR Fortessa SORP (BD Biosciences) flow cytometer and BD FACSDiva software. Data were analyzed with FlowJo software. *TdTomato* was used as a reference gene for normalization.

Western blot analysis

The expression of dCas9-effector fusion proteins was analyzed by standard Western blotting 48 h after transfection. Cells were lysed in RIPA buffer (Sigma) containing 1% protease inhibitor cocktail set III, EDTA-free (Calbiochem). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Monoclonal anti-CRISPR/Cas9-4G10 (1:2000, Diagenode) and anti-actin AC-40 (1:2.000, Sigma-Aldrich) antibodies were used.

DNA methylation analysis

For DNA methylation analysis of reporter genes, plasmid DNA was extracted as previously described [46] and bisulfite-converted with EZ DNA Methylation-Gold Kit (Zymo Research). Bisulfite-converted DNA was amplified with Immolase DNA Polymerase (Bioline) using specific primers (Table S3). DNA pyrosequencing was performed in a PyroMark Q24 instrument (Qiagen) according to the manufacturer's guidelines, and methylation analysis was determined using PyroMark Q24 Software (Qiagen).

Quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). 1 μg RNA was treated DNAse I, RNase-free (Thermo Scientific), and used for cDNA synthesis using the qSCRIPT™ cDNA Synthesis Kit (Quanta Biosciences). cDNA was used as a template in quantitative PCR reactions with the NZY qPCR Green master mix (NZYTech) and specific primers (Table S3). All reactions were carried out on a CFX Connect™ Real-Time System (Bio-Rad), and data were analyzed using the CFX Manager Software (Bio-Rad). Data were normalized using *GAPDH* and fold-increase in gene expression compared with controls was calculated with the formula $2^{-\Delta\Delta Ct}$ [80].

Author contributions

Iván Devesa-Guerra: Investigation, Writing; Teresa Morales-Ruiz: Investigation, Writing; Juan Pérez-Roldán: Investigation; Jara Teresa Parrilla-Doblas: Investigation, Resources; Macarena Dorado-León: Investigation, Resources; María Victoria García-Ortiz: Investigation, Resources; Rafael R. Ariza: Conceptualization, Supervision, Writing; Teresa Roldán-Arjona: Conceptualization, Supervision, Writing, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2020.02.007.

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