Preventing transcriptional gene silencing by active DNA demethylation

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Received 8 July 2005; revised 23 August 2005; accepted 23 August 2005

Available online 31 August 2005

Edited by Shou-Wei Ding

Abstract DNA methylation is important for stable transcriptional gene silencing. DNA methyltransferases for de novo as well as maintenance methylation have been well characterized. However, enzymes responsible for active DNA demethylation have been elusive and several reported mechanisms of active demethylation have been controversial. There has been a critical need for genetic analysis in order to firmly establish an in vivo role for putative DNA demethylases. Mutations in the bifunctional DNA glycosylase/lyase ROS1 in *Arabidopsis* cause DNA hypermethylation and transcriptional silencing of specific genes. Recombinant ROS1 protein has DNA glycosylase/lyase activity on methylated but not unmethylated DNA substrates. Therefore, there is now strong genetic evidence supporting a base excision repair mechanism for active DNA demethylation. DNA demethylases may be critical factors for genome wide hypomethylation seen in cancers and possibly important for epigenetic reprogramming during somatic cell cloning and stem cell function.

Keywords: Arabidopsis; DNA methylation; DNA demethylation; ROS1; DNA glycosylase; Transcriptional gene silencing

1. Introduction

Much has been written about how genes are silenced. We discuss here how genes are kept from being silenced. This mini-review begins with an overview of transcriptional gene silencing. We then survey the roles of DNA methylation and demethylation. Our focus is on the mechanism of active DNA demethylation, with a particular emphasis on the genetic system available in *Arabidopsis* to study active DNA demethylation.

2. Transcriptional gene silencing and heterochromatin

Epigenetic silencing is important for gene regulation during development and for the inactivation of viruses, transposons or transgenes [1–5]. Alongside DNA methylation and histone modifications which are considered as classical epigenetic marks, research over the past few years have shown that non-coding RNA also has a central role in chromatin silencing. RNA silencing regulates the level of gene transcripts, either by causing hypermethylation of gene promoters thereby suppressing gene transcription (i.e., transcriptional gene silencing (TGS)) or by triggering sequence specific mRNA degradation (i.e., post transcriptional gene silencing (PTGS)) [6–13]. The best studied trigger for both of these processes is double-stranded RNA (dsRNA) [14,15]. The dsRNA is cleaved into small sense and antisense RNAs (21–25 nt) by a dsRNA specific ribonuclease III, Dicer [16]. In several systems, these small interfering RNAs (siRNAs) have been shown to interact with other proteins such as Argonaute to form an RNA-induced silencing complex (RISC) and target homologous mRNAs for degradation [17,18].

Several well-known epigenetic phenomena such as genomic imprinting [19–21], X chromosome inactivation [22,23] and paramutation in plants [24,25] are caused by TGS. The expression of some transgenes can also be affected by TGS. In fact, the study of the TGS of transgenes has contributed greatly to our understanding of TGS [4]. TGS of transgenes is often associated with a high copy number of the transgenes arranged in complex repeat structures, or insertion of the transgenes in certain genomic regions [4,12,26]. These transgenes as well as endogenous repetitive genes, transposable elements and imprinted genes show some or all of the characteristics of heterochromatin [5,9,11,27–29]. DNA methylation and chromatin remodeling play important roles in TGS and the assembly of heterochromatin [29]. Mutations in DNA methylation enzymes have been shown to release the TGS of a number of genes [11,29]. Mutations in DNA remodeling factors such as DRD1, DDM1, MOM1, histone H3 methyltransferase and histone deacetylase also can release TGS in *Arabidopsis* [5,10,30,31].

DNA methylation, histone modifications and chromatin remodeling factors are interconnected [5,32]. Methyl CpG-binding proteins are found in transcription repression complexes with histone deacetylases and chromatin remodeling factors such as Mi-2 [33–35]. Both DNA methylation and histone H3 lysine-9 methylation are epigenetic marks of heterochromatin. In animals, fungi and plants, it has been shown that at least certain types of DNA methylation acts downstream of H3-K9 methylation [23,36,37]. H3-K9 methylation is upstream of DNA methylation in *Neurospora crassa* [36], but this is true only for CNG methylation mediated by the plant-specific methyltransferase CMT3 in *Arabidopsis* [37]. In *Arabidopsis*, DNA methylation is sufficient for gene silencing at most loci but H3-K9 methylation is not [38]. The *Arabidopsis* DDM1, a homolog of mouse Lsh, has been shown to be required for the maintenance of DNA methylation, H3-K9

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.08.039

methylation and heterochromatin [39]. However DNA methylation-independent mechanisms of suppression of TGS also exist. For example, in the *Arabidopsis mom1* mutant, transcriptional silencing of a hygromycin-resistance gene was relieved without changes in DNA methylation [40]. Recently, we found that mutations in the second subunit of replication protein A *(rpa2)* also suppress the transcriptional silencing of an *nptII* transgene and reactivated certain transposons without altering DNA methylation [41].

The initial trigger for DNA methylation and TGS appears to be siRNAs. Double-stranded RNA constructs corresponding to transgene promoter sequences were shown to produce small RNAs 21–24 nt in length, and the small RNAs or dsRNA itself caused promoter DNA methylation and TGS of the transgenes [42]. Promoter siRNAs generated from a transgene repeat were the likely trigger of DNA hypermethylation and TGS of a homologous endogenous *Arabidopsis* gene [43]. RNAs may serve as the guide of sequence-specific DNA methylation [44–46]. A unique feature of RNA-directed DNA methylation is that it leads to de novo methylation of almost all cytosine residues (including non-CG methylation) within the region of sequence identity between the triggering RNA and the target DNA. RNA-directed DNA methylation may also be the cause of methylation in protein coding regions during PTGS [47]. The DRM genes encode de novo DNA methyltransferases essential for RNA-directed DNA methylation [48–50] in *Arabidopsis*. Besides the DRMs, plants also encode the CG methyltransferase MET1 [51,52] and a plant-specific CNG methyltransferase CMT3 [53,54].

Recently, RNA-directed DNA methylation and TGS has also been reported in animals [55,56]. Interestingly, siRNAs were not able to trigger DNA methylation or TGS in some cases [57–59]. Although this has not been investigated, the variation in the efficacy of siRNAs in causing TGS could be due to different activities of active DNA demethylation in different cells or for different target genes. Even in organisms without DNA methylation, RNA signals could cause chromatin modifications [8,60,61]. In these organisms such as the fission yeast, histone modifications appear to be sufficient to mark and perpetuate silent chromatin domains.

3. DNA methylation

DNA methylation is a conserved epigenetic modification of the genome. The methyl moiety on the base generally contributes to transcriptional repression by preventing activators from binding to their target, or by favoring the formation of inactive chromatin [27,61]. In prokaryotes, DNA methylation is important for DNA repair and replication, and in recognition and protection of self DNA [62]. In eukaryotes, DNA methylation plays important roles in gene repression, genome organization and stability, genomic imprinting, X chromosome inactivation and other developmental aspects [61,63]. Aberrant methylation patterns of tumor suppressor genes and their subsequent silencing constitute a common feature of many cancers [64]. In mammals, most methylation occurs at the sites of CpG dinucleotides, which often cluster together in genic regions and thus are referred to as CpG islands. In most vertebrates, 60–90% of the cytosines at CpG dinucleotides are methylated [65]. CpG islands are not common in plant genomes. The majority of methylated residues in plants are found in repetitive DNA associated with heterochromatin. Several genes in euchromatic regions have also been shown to be methylated in *Arabidopsis* [66]. Although methylation at symmetric cytosines CpG and CpNpG is most common in plants, it can occur in any sequence context, particularly for RNA-directed DNA methylation [8,66,67].

DNA methylation patterns are established by two different DNA methyltransferase activities: de novo activity that transfers a methyl group to completely unmethylated double-stranded DNA, and maintenance activity that methylates cytosine in proximity with methylcytosine on the complementary strand [68]. On the basis of sequence and structural similarities, four groups of DNA methyltransferases have been recognized [69]: Dnmt1, pmt1/Dnmt2, Dnmt3, CMT (chromomethyltransferases). The mammalian Dnmt3 [70], fungal Masc1 [71] and plant DRMs [48–50] have been shown to encode for de novo methyltransferases. The *Arabidopsis* genome encodes for two related de novo cytosine methyltransferase genes, *DRM1* and *DMR2*. *drm1drm2* double mutants are blocked in TGS at some loci and completely abolish de novo methylation at CpG, CpNpG and asymmetric sites [48–50,72]. Members of the mammalian Dnmt1 [73] and plant MET1 [51,52] class of enzymes serve primarily as maintenance methyltransferases. Mutations in the *Arabidopsis MET1* gene cause a global reduction of cytosine methylation throughout the genome and a number of developmental abnormalities [51,74–76]. Loss of CpG methylation in *met1* has also been shown to abolish the heterochromatic mark H3K9 at loci that remain transcriptionally silent [77].

The CMT class of enzymes appears to be specific to plants [54]. Methylation profiling of CMT3 mutants suggested that CMT3 preferentially methylates transposon-related sequences [78]. Interestingly many of these targets are shared between CMT3 and MET1 suggesting that CG and non-CG methylation systems might function redundantly for regulation of certain transposon sequences. CMT3 and DRMs also act in a partially redundant and locus-specific manner to control asymmetric and CNG methylation [48,54,72].

The initial signals for DNA methylation and how DNA methyltransferases are targeted to specific genomic regions are important unresolved questions. Double-stranded RNA or their derivative small RNAs are possible initial signals for DNA methylation [11,15,42]. The RNA signals may direct chromatin remodeling factors and histone modification enzymes to the target genomic regions, creating a chromatin state that attracts DNA methyltransferases. The DNA is then methylated, which locks the chromatin in a stable silent mode. DDM1, a member of the SNF2/SWI2 family of chromatin remodeling proteins, is required for maintenance of DNA methylation in *Arabidopsis* [39,79]. DDM1 is also required to maintain histone H3 methylation patterns. In wild-type heterochromatin, transposons and silent genes are associated with histone H3 methylated at lysine 9, whereas active genes are preferentially associated with methylated lysine 4 [38]. In a *ddm1* mutant there is loss of DNA methylation and histone H3 methylation at lysine 9 is replaced by histone H3 methylation at lysine 4 [39]. In several systems, it has been shown that DNA methylation depends on histone H3 lysine-9 methylation [23,36–38]. Additionally, DNA methyltransferases are found in protein complexes with histone deacetylases and other proteins [80,81]. In plants, there is a complex interplay between DNA and histone modifications [38,39,77,82–84].

4. DNA demethylation

In contrast to the large amount of information that has accumulated on DNA methylation, relatively little is known about DNA demethylation. Current understanding of the mechanisms responsible for the maintenance of DNA methylation patterns in somatic tissues are based on the supposition that DNA methyltransferases are exclusively responsible for maintaining the methylation state [85]. However, most biological processes such as phosphorylation and acetylation are reversible. Recently methylation of histones at lysine residues has also been shown to be reversible [86]. Histone de-methylation is carried out by the protein LSD1 that has sequence similarity to amine oxidases.

The demethylation of DNA can be either passive or active, or a combination of both. Passive DNA demethylation occurs by inhibition or lack of maintenance DNA methyltransferases throughout cycles of replication, whereas active DNA demethylation requires specific enzymatic reactions [61,87]. In mice, global demethylation of the zygotic genome after fertilization appears to occur by an active mechanism, which is then followed by passive demethylation during cleavage stages [88]. DNA demethylation has been shown to be necessary for the epigenetic reprogramming of somatic cell nuclei in *Xenopus* oocytes [89]. This demethylation has some interesting features: it is selective, i.e., it occurs only in a limited fraction of the genome and operates independently of DNA, RNA and protein synthesis. Local specific gene demethylation also occurs throughout embryonic development and in terminally differentiated cells.

Three active demethylation mechanisms have been proposed, none of which has gained wide acceptance [87]. The first mechanism is direct replacement of the methyl moiety by a hydrogen atom. The human MBD2 (Methylated DNA binding protein 2) was reported to demethylate DNA by this mechanism, i.e., the thermodynamically unfavorable breakage of the carbon–carbon bond that links the pyrimidine to its methyl group [85,90]. This claim was contested and could not be reproduced in other laboratories [36,61,87,91].

The other two proposed mechanisms both involve DNA repair processes. The second mechanism implicates a role for DNA glycosylases, which cleave the bond between the 5-methylcytosine base and the deoxyribose moiety in DNA. The abasic site is then repaired by resident repair activity resulting in replacement of a 5-methylcytosine with an unmethylated cytosine [92,93]. The third mechanism proposed that the methylated nucleotide was removed by nucleotide excision and was then replaced by an unmethylated cytosine [94,95].

Using extracts from tissue culture cells, Weiss et al. [94] observed an in vitro demethylation activity through excision of the methylated dinucleotide CpG. Several key experimental observations supported the suggestion that this reaction is mediated through the participation of RNA molecules. This proposed active role of RNA in the nucleotide excision repair reaction was later re-evaluated [96]. The demethylase involved in the nucleotide excision repair (third mechanism) has not yet been cloned.

There is experimental data to support the second mechanism that a specific DNA glycosylase(s) participates in the demethylation reaction. Two mismatch-repair glycosylases, the G/T mismatch repair enzyme [97,98] and the methylated binding protein MBD4 [99] were shown to possess 5′-methylcytosine DNA-glycosylase (5-MCDG) activity that results in demethylation in vitro. Using chicken embryo nuclear extracts that can promote active demethylation, Jost and colleagues [93,98,100] purified a demethylase. The enzyme is a DNA glycosylase that acts preferentially on hemimethylated CpGs and initiates demethylation by breaking the glycosidic bond of 5-methylcytosine, thus leaving an abasic site that can be further processed by an AP-endonuclease and other DNA repair enzymes. Cloning of the enzyme showed that the gene encodes a G/T mismatch repair DNA glycosylase [98]. MBD4, a human homolog of the chicken enzyme, also has 5-methylcytosine DNA glycosylase activity [97]. The authors also reported evidence that RNAs and an RNA helicase (i.e., p68) are part of the enzyme complex and are involved in the demethylation activity [98,101]. Overexpression of a human 5-MCDG in human embryonic kidney cells led to demethylation of the promoter of a hormone-regulated reporter gene [99]. The specific demethylation of the transgene promoter but not genome-wide demethylation was attributed to the physical association of the 5-MCDG with retinoid X receptor that has binding sites in the transgene promoter [99]. A major concern about the function of MBD4 and other G/T mismatch repair DNA glycosylase as demethylases is that the activities of these enzymes towards 5-methylcytosine DNA substrates are very weak, compared to their activities towards G/T mismatch DNA substrates [97,98]. It is possible that these enzymes have strong 5-methylcytosine DNA glycosylase activity in vivo, which may require other co-factors. Alternatively, a genuine animal DNA demethylase may not have been identified yet.

Also in support of a DNA glycosylase mechanism of active demethylation, Vairapandi and colleagues independently found that HeLa nuclear extracts can demethylate DNA through a DNA glycosylase mechanism and partially purified this demethylase activity [102,103]. However, the identity of the protein responsible for the activity has not been identified.

5. Genetic evidence for a base excision repair mechanism of active DNA demethylation

A number of genetic screens have identified mutations that re-activate previously silenced genes and these mutations define important factors that are required for the establishment or maintenance of gene silencing. For example, in *Arabidopsis*, several DNA methyltransferases, histone methylation or deacetylation enzymes, and other chromatin remodeling factors have been identified by screening for mutations that suppress the TGS of endogenous or transgenes [30,31,37,40,48,50,52–54,72,76,84,104,105]. These and other studies have provided important insights into how silenced genes and transposons are kept inactive. In contrast, how active genes are kept from being silenced has not been well investigated.

We hypothesized that the transcription level of some genes may be balanced by the opposing activities of epigenetic silencing and anti-silencing systems, in addition to regulation by conventional transcriptional activators and repressors. We have developed a novel genetic system in *Arabidopsis* and used it to screen for mutations in repressors of silencing (ROS) [43]. The system consists of the *RD29A-LUC* (*RD29A* promoter driving the firefly luciferase reporter) transgene inserted in

chromosome III as a complex repeat, and the endogenous *RD29A* gene on chromosome V (Fig. 1). In wild-type genetic background, the *RD29A-LUC* transgene and the endogenous *RD29A* gene are transcribed in response to the phytohormone abscisic acid (ABA) or environmental stress signals such as drought, high salt and cold (0–4 °C) that activate the *RD29A* promoter [106]. These genes have been extremely stable and are robustly activated for numerous generations over many years. This is so despite the fact that the transgene repeat generates siRNAs corresponding to the *RD29A* promoter, presumably cleavage products of dsRNA from the promoter due to read through. So, in the wild-type genetic background, there are siRNAs from the *RD29A* promoter, but they do not cause methylation of the promoter (Fig. 1) and thus the promoter can be activated by ABA or other stresses [43].

Using high throughput luminescence imaging [107], we screened for and recovered four mutants in which the *RD29A-LUC* and the endogenous *RD29A* gene are silenced. Pair-wise crosses showed that the mutants fall into three complementation groups, i.e., *ros1*, *ros2* and *ros3*, with two alleles for *ros1* [43] and a single allele for *ros2* and *ros3* (our unpublished results). The *ros* mutations are all recessive based on luminescence analysis of F1 and F2 progenies from their respective backcrosses. That gene silencing and not impaired stress signaling caused the defective gene expression comes from the observation that all of the *ros* mutants are sensitive to kanamycin due to the inactivation of the *NPTII* antibiotic-resistance gene [43]. The gene silencing appears to spread from the *RD29A-LUC* transgene to the linked *NPTII* gene in the same T-DNA construct. Nuclear run-on assays indicate that the silencing at the *RD29A-LUC* and endogenous *RD29A* loci occurs at the transcriptional level. The gene silencing phenotypes together with the recessive nature of the *ros* mutants suggest that the mutants are defective in mechanisms required for the prevention of transcriptional gene silencing.

In *ros1* mutants, the *RD29A* promoter on both the transgene and the endogenous gene is heavily methylated [43]. Promoter methylation and silencing of the endogenous gene is dependent on the presence of the transgene [43] and in fact, dependent on promoter siRNAs (our unpublished results). The transcriptional silencing in *ros1* mutants can be released by the *ddm1* mutation or mutations in DNA methyltransferases (our unpublished results), or by the application of the DNA methylation inhibitor 5-aza-2′-deoxycytidine. We isolated the *ROS1* gene and showed that it encodes a nuclear protein containing an endonuclease III domain similar to DNA base excision repair proteins in the HhH-GPD superfamily. In vitro assays showed that recombinant ROS1 protein has bifunctional DNA glycosylase/lyase activity against methylated but not unmethylated DNA. These results suggest that ROS1 prevents DNA hypermethylation and TGS by demethylating the target promoter DNA [43]. The work thus provides strong genetic evidence supporting a base excision repair mechanism for DNA demethylation.

6. ROS1 is a DNA demethylase

There are at least two possible mechanisms to explain the anti-methylation and anti-silencing function of ROS1. One is that ROS1 may prevent promoter siRNAs from causing DNA methylation. Another possibility is that ROS1 may inhi-

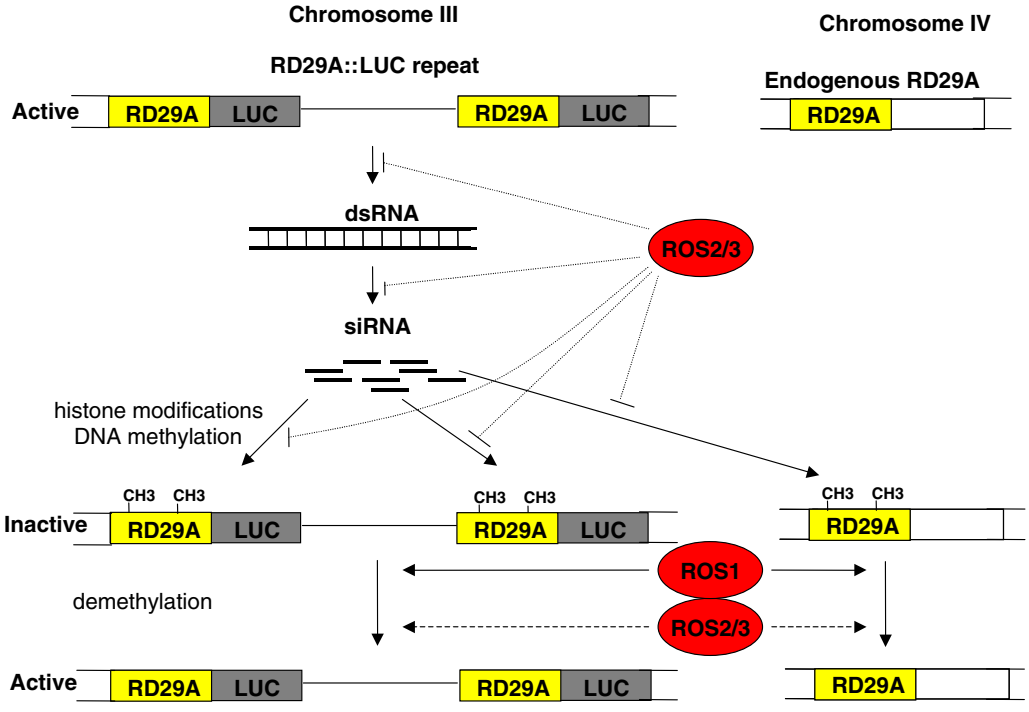

Fig. 1. Suppression of transcriptional gene silencing by ROS proteins. The *RD29A-LUC* transgene repeat is hypothesized to generate aberrant read through transcripts that enter the NRPD1a-RDR2-DCL3 pathway, producing siRNAs that are likely the diffusible signal for triggering the hypermethylation of the *RD29A* promoter at both the transgene and endogenous loci on two different chromosomes. ROS1 counters the silencing by active DNA demethylation. ROS2 and ROS3 have not been cloned but are proposed to prevent siRNA action or heterochromatin formation, or to function together with ROS1 in the demethylation process.

bit the hypermethylation of specific DNA sequences targeted by siRNAs through participation in the demethylation of the DNA. Existing data support the latter hypothesis, although other unforeseen mechanisms cannot be ruled out.

ROS1 encodes a nuclear protein of 1393 amino acids with an HhH-GPD domain found in DNA glycosylases (Fig. 2). The N-terminal region has a basic domain with a weak similarity to histone H1 (Fig. 2). A MBP (maltose binding protein) fusion with the C-terminal 1099 residues of ROS1 was produced in *Escherichia coli*, and the fusion protein was shown to be capable of incising plasmid DNA methylated with the MspI methylase [43]. It did not incise unmethylated plasmid DNA. Full length recombinant ROS1 protein is also capable of incising methylated but not unmethylated plasmid DNA or oligonucleotides (our unpublished data).

DNA glycosylases are typically low molecular weight (200–300 aa) monomeric enzymes responsible for recognizing base lesions in the genome and initiating the DNA base excision repair pathway (BER). These enzymes in most organisms remove common base modification (oxidation, deamination, alkylation) as well as normal bases in a mispair context, caused by endogenous or environmental agents [108–110]. DNA glycosylases recognize the presence of damaged base or mismatched base, and catalyze the breakage of the glycosyl bond between the target base and DNA-sugar phosphate backbone, releasing the free damaged base and leaving an apurinic/apyrimidinic (AP) site that must be further processed. According to their catalytic activity DNA glycosylases can be classified into two subgroups: monofunctional DNA glycosylases which catalyze only hydrolysis of the glycosylic bond or bifunctional DNA glycosylase/lyase with associated AP lyase activity that cleaves the DNA backbone at the site where the base has been removed [109,111]. The bifunctional DNA glycosylase/AP lyases belong to two broad classes, based on their reaction mechanisms: (1) *E. coli* Nth is the prototype of one class that utilizes internal lysine as the active site nucleophile and cleaves the DNA strand at the AP site by β elimination, generating a 3α,β-phospho-unsaturated aldehyde (3′ dRP) at the strand break [112]; (2) *E. coli* Fpg and Nei belong to another class that catalyzes βδ elimination at the AP site and removes the deoxyribose to produce a 3′ phosphate terminus at the DNA strand break [113]. ROS1 belongs to this second class because recombinant ROS1 protein can catalyze βδ elimination (Fig. 3) (our unpublished results).

Structural studies have revealed that all DNA glycosylases fall into two main structural families. The best characterized is the HhH-GPD family, which includes EndoIII, AlkA, MutY and hOGG1 [114]. A lysine residue located at the HhH domain is conserved in all of the bifunctional enzymes of this family [115], and is also present in ROS1 (Lys-953).

The ability of recombinant ROS1 protein to induce strand breaks in DNA containing 5-methylcytosine [43] suggests that ROS1 may be directly involved in DNA demethylation through a base excision repair mechanism. The results thus

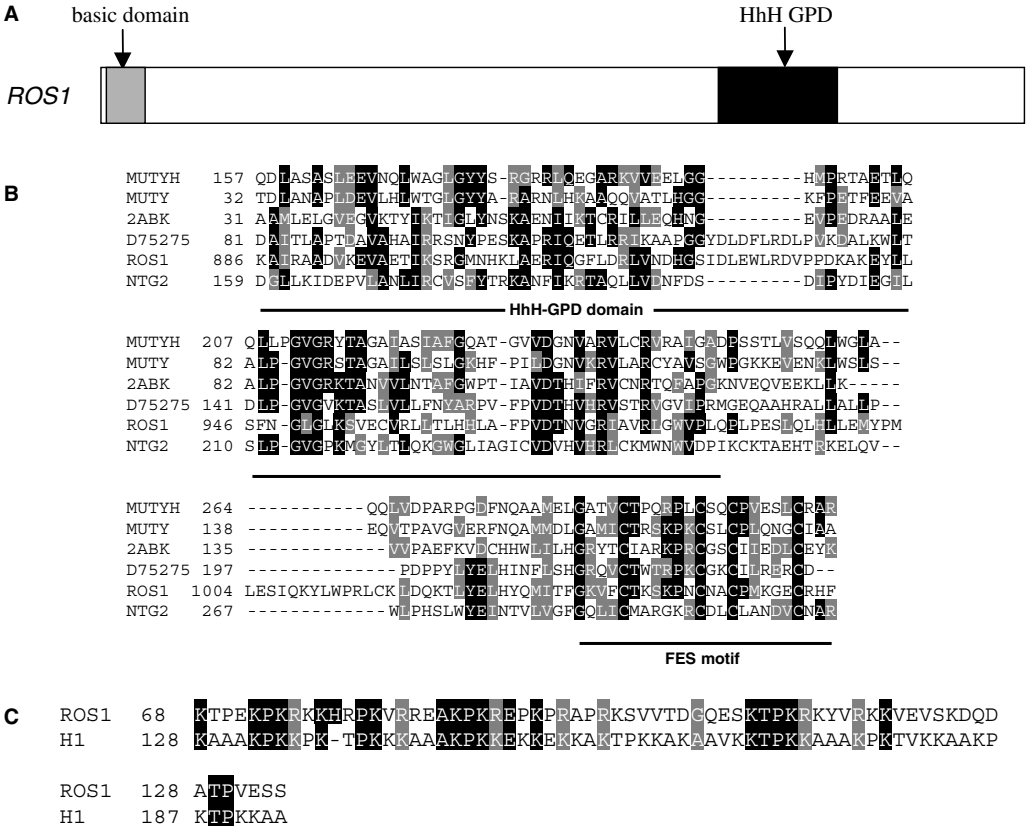

Fig. 2. ROS1 is an atypical DNA glycosylase/lyase with an N-terminal basic domain and an HhH-GPD domain at the C-terminal side. (A) Diagramatic representation of ROS1. (B) Alignment of the HhH-GPD domain of ROS1 with those of DNA glycosylases. The sequences used for the alignment are: MUTY, P17802 (*E. coli*); MUTYH, NP_036354 (human); 2ABK, 1311214 (*E. coli*); D75275 (*Deinococcus radiodurans*); and NTG2, Q08214 (yeast). (C) Alignment of the basic domain of ROS1 with histone H1 from *Chaetopterus variopedatus*.

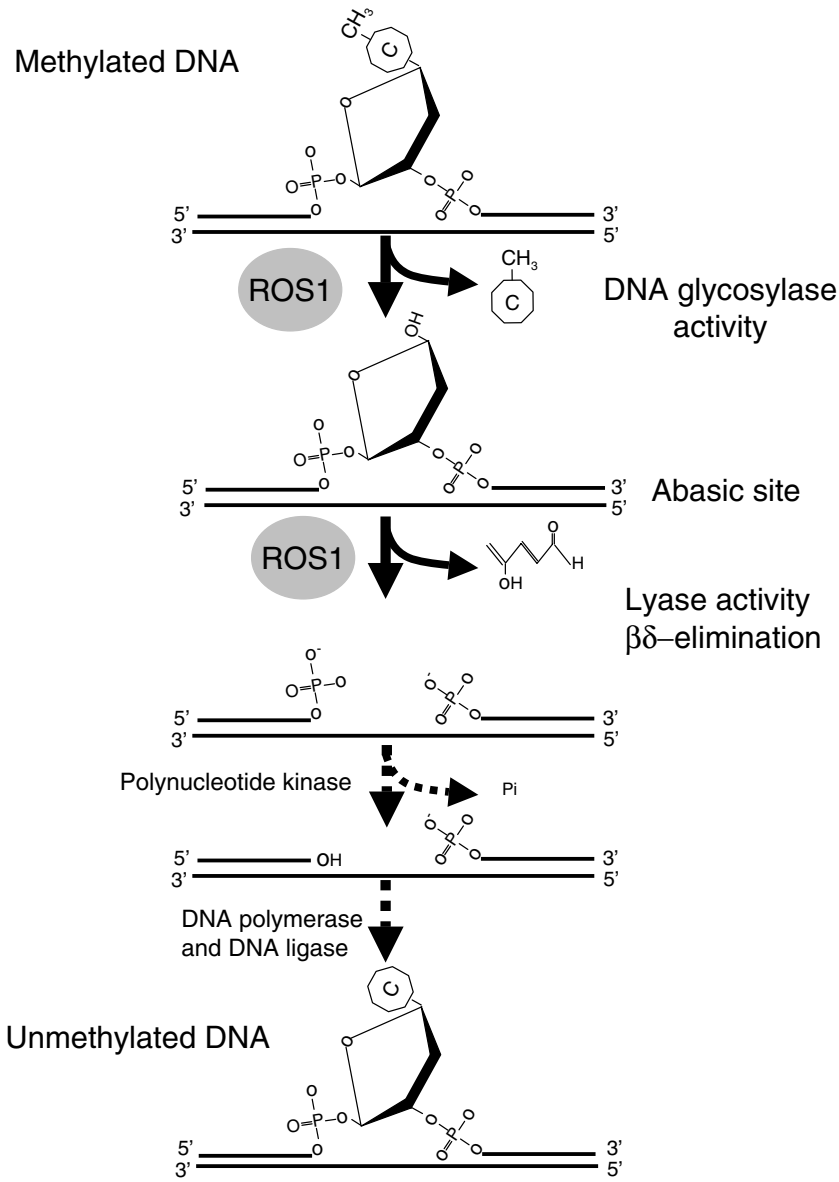

Fig. 3. Proposed biochemical mechanism of DNA demethylation by ROS1. The diagram shows that the DNA glycolyase activity of ROS1 removes methylated cytosine base from the DNA backbone. The AP lyase activity of ROS1 then cleaves the DNA backbone at the site of cytosine removal by βδ-elimination mechanism (our unpublished data). This DNA backbone is subsequently repaired by an unknown mechanism which might involve a putative polynucleotide kinase, a DNA polymerase and a DNA ligase.

provide the first genetic evidence for the functional role of base excision repair enzymes in DNA demethylation. Although the observed strand breaks might reflect excision of mispaired thymine residues that arose by spontaneous 5-methylcytosine deamination, the absence of nicking activity on a heavily methylated plasmid at CpG sequences seems to rule out this possibility. The significance of this strong sequence preference for the in vivo activity of the protein remains to be determined, and will require a complete characterization of the substrate specificity of the enzyme. It should be pointed out that the *RD29A* promoter hypermethylation pattern observed in *ros1* mutant plants also includes CpG sequences. The sequence specificity of ROS1 in vivo may be affected by its potential interaction with siRNAs and other proteins.

The genome of *Arabidopsis* encodes several other proteins belonging to the HhH family of DNA glycosylases, all of them with similar DNA repair activities to homologs found in bacteria, fungi or animals [116–119]. However, there are several characteristics that make ROS1 an atypical DNA glycosylase. It is much bigger (1393 amino acids) than typical DNA glycosylases, which are in the 200–400 amino acids range. The similarity to DNA glycosylases is limited to the HhH-GPD domain, and the only recognizable feature in the rest of the sequence is a region rich in basic residues which displays a weak similarity to H1 histones. A database search revealed three other large *Arabidopsis* proteins that are similar to ROS1 in the HhH-GPD domain and also with the N-terminal basic region. One of them is Demeter (DME) [120]. *DME* is required for endosperm gene imprinting and its ectopic expression induces *Medea* (*MEA*) expression and nicks the *MEA* promoter in vivo. Although DME was originally proposed to function by a mechanism other than to demethylate the *MEA* promoter

since no 5-methylcytosine residues were found in the promoter [120], recent work on the suppression of *dme* mutant phenotype by mutations in the DNA methyltransferase MET1 [121] indicates that DME probably also functions by DNA demethylation.

7. Role of ROS1 in development and DNA repair

ROS1 in *Arabidopsis* may function as a regulator of siRNAs-triggered TGS and of development. After inbreeding for three or more generations, some *ros1* plants began to display a range of developmental phenotypes [43]. The accumulated abnormal phenotypes in the later generations of *ros1* mutants indicate that some genes important in development must be affected by the loss of *ROS1* function. The *Arabidopsis ddm1* and *ddm2*/*met1* mutations also lead to developmental abnormalities in later generations. Although the *ddm1* and *ddm2*/*met1* mutations clearly reduce overall levels of genome methylation, the developmental phenotypes in these mutants are associated with the accumulation of both DNA hypomethylation and hyper-methylation epialleles [75,122]. It is likely that the aberrant phenotypes in *ros1* mutant plants are caused by the accumulation of DNA hyper-methylation epialleles and possibly also hypo-methylation ep-ialleles in specific development regulatory genes. We have found recently that the methylation levels of several endogenous genes are elevated in the *ros1* mutant, supporting a role of ROS1 in the demethylation of endogenous genes (unpublished data).

We also found that *ros1* mutant plants are hypersensitive to genotoxic chemicals such as methyl methanesulfonate and hydrogen peroxide [43]. The fact that *ros1* mutants were hypersensitive to DNA base damage reagents indicates one of the in vivo functions of ROS1 is to repair damaged DNA. The repair of DNA damage is an important step during chromatin assembly and requires both the recognition of altered DNA structures and the recruitment of repair proteins to the damage sites [108,123]. After repair, the chromatin structure of repaired DNA must be re-assembled in order to faithfully restore pre-existing structures, especially in transcribed regions. Other studies have also implicated a mechanistic connection between gene silencing or chromatin remodeling factors and DNA repair proteins. For example, the mammalian TIP60 histone acetylase complex [124] and the *Drosophila* RCAF complex [125] are involved in chromatin remodeling as well as in DNA repair. Recently, Takeda et al. [126] reported that mutations in a novel nuclear protein BRU1 reactivate expression of *TSI* without altering its methylation levels in Arabidopsis. *bru1* plants are highly sensitive to genotoxic stress indicating that BRU1 provides a novel link between DNA damage and transcriptional gene silencing.

8. Perspectives

Our results with ROS1 suggest that a DNA repair factor can serve as a repressor of siRNA-triggered DNA hypermethylation and TGS. Future experiments should be aimed at identifying other endogenous targets of ROS1 by a genome wide expression and methylation analysis. Efforts are also needed to investigate the mechanism of targeting the demethylase to specific loci. It is possible that the demethylase enzyme functions in a complex containing siRNAs and/or chromatin-related proteins that help target the enzyme to specific methylated genes. The discovery of ROS1 and its role in repression of TGS provides the missing genetic evidence for the existence of an active DNA demethylase and its importance in keeping active genes from being silenced. Active DNA demethylases likely have critical roles in epigenetic reprogramming during somatic cell cloning and in maintaining stem cells in an undifferentiated state, and in causing the DNA hypomethylation seen in most cancers [127]. Therefore, the identification of functional homologs of ROS1 in mammals will be of great interest.

Acknowledgments: Research in our laboratory is supported by NIH, NSF and USDA. We thank Becky Stevenson for excellent technical assistance.

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