SET3p monomethylates histone H3 on lysine 9 and is required for the silencing of tandemly repeated transgenes in *Chlamydomonas*

Juan Casas-Mollano
*University of Nebraska - Lincoln*, jcasasmollano2@unl.edu

Karin V. van Dijk
*University of Nebraska - Lincoln*, kvandijk2@unl.edu

John Eisenhart
*University of Nebraska - Lincoln*

Heriberto D. Cerutti
*University of Nebraska - Lincoln*, hcerutti1@unl.edu

Follow this and additional works at: https://digitalcommons.unl.edu/plantscifacpub

Part of the Plant Sciences Commons

Casas-Mollano, Juan; van Dijk, Karin V.; Eisenhart, John; and Cerutti, Heriberto D., "SET3p monomethylates histone H3 on lysine 9 and is required for the silencing of tandemly repeated transgenes in *Chlamydomonas*" (2007). *Faculty Publications from the Center for Plant Science Innovation*. 2.

https://digitalcommons.unl.edu/plantscifacpub/2

This Article is brought to you for free and open access by the Plant Science Innovation, Center for at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Faculty Publications from the Center for Plant Science Innovation by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
SET3p monomethylates histone H3 on lysine 9 and is required for the silencing of tandemly repeated transgenes in *Chlamydomonas*

J. Armando Casas-Mollano, Karin van Dijk, John Eisenhart and Heriberto Cerutti*

School of Biological Sciences and Plant Science Initiative, University of Nebraska, Lincoln, NE 68588-0666, USA

Received October 24, 2006; Revised December 9, 2006; Accepted December 15, 2006

ABSTRACT

SET domain-containing proteins of the SU(VAR)3-9 class are major regulators of heterochromatin in several eukaryotes, including mammals, insects, plants and fungi. The function of these polypeptides is mediated, at least in part, by their ability to methylate histone H3 on lysine 9 (H3K9). Indeed, mutants defective in SU(VAR)3-9 proteins have implicated di- and/or trimethyl H3K9 in the formation and/or maintenance of heterochromatin across the eukaryotic spectrum. Yet, the biological significance of monomethyl H3K9 has remained unclear because of the lack of mutants exclusively defective in this modification. Interestingly, a SU(VAR)3-9 homolog in the unicellular green alga *Chlamydomonas reinhardtii*, SET3p, functions in vitro as a specific H3K9 monomethyltransferase. RNAi-mediated suppression of *SET3* reactivated the expression of repetitive transgenic arrays and reduced global monomethyl H3K9 levels. Moreover, chromatin immunoprecipitation (ChIP) assays demonstrated that transgene reactivation correlated with the partial loss of monomethyl H3K9 from their chromatin. In contrast, the levels of trimethyl H3K9 or the repression of euchromatic sequences were not affected by *SET3* down-regulation; whereas dimethyl H3K9 was undetectable in *Chlamydomonas*. Thus, our observations are consistent with a role for monomethyl H3K9 as an epigenetic mark of repressed chromatin and raise questions as to the functional distinctiveness of different H3K9 methylation states.

INTRODUCTION

Transcriptional gene expression in eukaryotes is regulated at two major levels: the operation of the transcription machinery and the modulation of chromatin structure (1). Indeed, the regulation of chromatin packaging has recently emerged as an important mechanism for maintaining gene expression patterns. Eukaryotic genomes are commonly organized into two main types of chromatin: euchromatin, consisting of transcriptionally permissive or active domains, and heterochromatin, characterized by densely packed silent regions (2). These functionally and structurally distinct chromatin states are marked by distinctive covalent modifications on the DNA and on the nucleosomal histones. For instance, active euchromatin is usually characterized by the presence of histone H3 methylated on lysines 4 and 36; whereas silenced heterochromatin is often distinguished by the presence of histone H3 methylated on lysines 9 and 27, and histone H4 methylated on lysine 20 (3,4). This organization of chromosomes into domains with distinct histone methylation patterns appears to be common across the eukaryotic spectrum (5–12) and the histone modifications are proposed to influence directly chromatin structure and/or bring about the recruitment of chromatin modifiers (3,4). However, it is becoming apparent that the specific modifications associated with functionally equivalent regions (such as pericentric heterochromatin) can be quite variable among different species.

Histone H3 on lysine 9 (H3K9) methylation is carried out by the SET domain of the SU(VAR)3-9-related proteins, which belong to the histone methyltransferase (HMTase) superfamily (13,14). The first SU(VAR)3-9-encoding gene was identified in a genetic screen for suppressors of position effect variegation in *Drosophila melanogaster* (15), providing clues as to its function as a key regulator of repressive heterochromatic organization. Indeed, *Drosophila* SU(VAR)3-9 and its mammalian homologs, SUV39H1 and SUV39H2, were latter found to be enriched in interphase heterochromatin and to accumulate transiently at centromeric loci during mitosis (16–18). SU(VAR)3-9-related proteins are now known to be widely distributed among eukaryotes. For instance, the model plant *Arabidopsis thaliana* contains 29 genes encoding SET domain proteins (19). Ten of these polypeptides have been classified as SU(VAR)3-9...
homologs (SUVHs), based on the degree of similarity between their SET domains and those of animal SU(VAR)3-9 proteins. SUVH genes have also been identified in a number of other plant species such as tobacco (20), maize (21) and rice (http://www.chromdb.org) as well as in several fungi (22,23). However, they have not been described as yet in algal systems.

Seven plant SUVH genes have been characterized in some detail: tobacco NtSET1 (20,24,25), and Arabidopsis SUVH1 (8), SUVH2 (8), SUVH3 (26), SUVH4 (KRYPTONITE) (27,28), SUVH5 (29) and SUVH6 (30,31). Several of the corresponding proteins have been shown to possess HMTase activity targeting H3K9 (and H4K20 or H2A in the cases of SUVH2 and SUVH5, respectively) and to associate preferentially with putative heterochromatic regions (8,24,29). Indeed, SUVH2 appears to play a major role in heterochromatin formation and/or maintenance in Arabidopsis since loss-of-function mutants display defects in gene silencing and a reduction in multiple heterochromatin-specific histone methylation marks (8). In contrast, SUVH4, SUVH5 and SUVH6 seem to have partly overlapping functions and control H3K9 methylation and gene silencing at specific loci (29,31).

Methylatable lysine residues can exist in monomethylated, dimethylated or trimethylated states, increasing the coding potential of modified histone lysines as epigenetic marks (4). Interestingly, in mammals, pericentric heterochromatin is enriched in trimethyl H3K9 (H3K9me3), a modification carried out predominantly by the SUV39H1 and SUV39H2 HMTases (4–6,9). Conversely, mono- and dimethyl H3K9 (H3K9me1 and H3K9me2) are enriched in certain euchromatic domains, which have been postulated to be transcriptionally silent (4–6,9,32). The latter modifications are mediated in part by the G9a HMTase, which plays an essential role in developmental regulation of gene expression (5,6,9,33). In Arabidopsis, in contrast to mammals, heterochromatinic chromocenters are marked by H3K9me1 and H3K9me2, whereas H3K9me3 is predominately found in euchromatic regions (7,8,27). Yet in another plant species, maize; only H3K9me1 was observed in heterochromatinic domains (as well as in some euchromatinic regions) while H3K9me2 and H3K9me3 were restricted to euchromatin and the centromeres (11).

These observations suggest that H3K9 mono-, di- and trimethylation states may serve distinct functions since they reside at least partly in separate chromosomal domains. Moreover, in mammals and Arabidopsis, different HMTases are responsible for specific degrees of H3K9 methylation at certain locations (5,6,9,29,34) and different HMTase mutants have distinct phenotypes (5,33), implying that the HMTases (and the histone modifications that they catalyze) are differentially targeted to diverse genomic regions for regulatory purposes. Yet, seemingly equivalent domains (such as pericentric heterochromatin) are characterized by species-specific H3K9 methylation states, raising questions as to the functional significance of different degrees of H3K9 methylation. In addition, genetic and biochemical analyses in a variety of eukaryotes have established a role for H3K9me2 and H3K9me3 in the formation and/or maintenance of transcriptionally repressive chromatin (3,4,8), whereas H3K9me1 lacks a defined functional assignment at present, partly because a HMTase exclusively responsible for this modification has not been identified thus far.

To gain further insight into the role of H3K9 methylation in chromatin organization, we undertook a reverse genetics approach to the study of SUVHs in the green alga Chlamydomonas reinhardtii. BLAST searches of the almost fully sequenced genome (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) revealed the existence of two SU(VAR)3-9-related proteins in Chlamydomonas. We report here on the characterization of one of these homologs, named SET3p. This polypeptide showed the same domain organization as plant SUVH proteins and its suppression by RNA interference (RNAi) released the transcriptional silencing of tandem transgenes. In contrast, repressed single-copy euchromatic sequences and dispersed transposons were not reactivated. Recombinant SET3p behaved, in vitro, as an exclusive monomethyl H3K9 HMTase. Moreover, chromatin immunoprecipitation (ChIP) assays demonstrated that, in vivo, H3K9me1 was dependent on the SET3p activity and was associated with silent multiple-copy transgenes. Conversely, H3K9me3 was mainly detected in a euchromatic gene and its intensity was not altered by SET3 suppression. Thus, our results provide direct evidence for a functional role of monomethyl H3K9 in the maintenance of repressed chromatin and add to the growing body of evidence suggesting that seemingly equivalent chromatin states may be characterized by species-specific combinations of histone modifications.

**MATERIALS AND METHODS**

*Chlamydomonas reinhardtii* strains and culture conditions

*Chlamydomonas* cells were routinely grown in Tris-acetate-phosphate (TAP) medium under moderate light conditions (35,36). The 1-P[300] strain, containing over 100 integrated copies of the *RbcS2:aadA:RbcS2* transgene, has been previously described (35). The Set3-IR and Maa7-IR strains are derivatives of 1-P[300] in which the expression of *SET3* or *MAA7* (encoding tryptophan synthase β subunit) has been suppressed by RNAi, by using the approach described by Rohr et al. (37). Briefly, 1-P[300] was transformed by the glass bead method (35) with transgenes that produce double-stranded RNA (dsRNA) capable of inducing the degradation of homologous transcripts. For *SET3*, a 630-bp fragment corresponding to part of the coding sequence and the 3′ UTR of the transcript was amplified by PCR with primers Set3-F1 (5′-GGGACGGCAACCTGACCATCC-3′) and Set3-R1 (5′-CTGACCCACACCAAGCTCTGAC-3′). This segment was then inserted in sense and antisense orientations, flanking a spacer sequence, in vector Maa7/X IR (37). The Maa7-IR3 construct, utilized as a negative control, has already been described (37). Reactivation of the *RbcS2:aadA:RbcS2* transgenes was tested by spotting serial dilutions of cells on TAPagar plates with or without 50 mg/l of spectinomycin.
Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses

Total RNA was isolated using the TRIZOL reagent, according to the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH, USA), and contaminant DNA was removed by DNase-I treatment (Ambion, Austin, TX, USA). First-strand cDNA synthesis and PCR reactions were performed as previously described by Rohr et al. (37). PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. The numbers of cycles showing a linear relationship between input RNA and the final product were determined in preliminary experiments. Controls included the use as template of reactions without RT and verification of PCR products by hybridization with specific probes (data not shown). The primer sequences were as follows: for SET3, Set3-RF6 (5'-GGTGTGC AAGTTCCTGATGCAC-3') and Set3-RF7 (5'-TGAA CTGCAGCATCTCCCTCGTC-3'); for aadA, aadA-CodL, (5'-TCTGGCTATCTTGGTCAACAAA-3'); and aadA-CodR, (5'-TAGTGATCTCGCCTTTACAGTA-3'); and for MUT9, Mut9-5 (5'-GGTGTA CATCTCGTGGCT GTG-3') and Mut9-2 (5'-ATGCCGGTCA CGTAGAAGC-3').

Phylogenetic analysis

Individual domains present in SET3p (AAV84356) and other SU(VAR)3-9-related proteins were identified using the SMART database (38). The SET domain amino acid sequences were aligned using CLUSTAL X version 1.81 (39) and manually corrected with the GENEDOC program (http://www.psc.edu/biomed/genedoc). Phylogenetic relationships between these sequences were inferred by the neighbor-joining (NJ) method (40). The MEGA program version 3.1 (41) was used to obtain the NJ trees, using Poisson-corrected amino acid distances, and the bootstrap support values for 1000 pseudoreplicates.

Partial protein purification and immunoblotting

Chromatin-associated proteins were partially purified by differential centrifugation prior to immunoblotting. Approximately 5 × 10⁸ TAP-grown cells were resuspended in nuclear isolation buffer (20 mM PIPES, pH 7.0, 0.25 M sucrose, 10 mM MgCl₂, 2.0 mM spermidine, 100 mM sodium butyrate, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 2 mM benzamide and 0.1 mM PMSF) and broken by two passages through a French press. Lysed cells were then centrifuged for 10 min at 30 000 g. The supernatant was discarded and proteins in the pellet (including nuclear chromatin) were solubilized in high salt buffer (20 mM HEPES, pH 7.5, 2 M NaCl, 1 mM EDTA, 1 mM DTT, 2 mM benzamide and 2 μl/ml of plant protease inhibitor cocktail [Sigma-Aldrich, Saint Louis, MO, USA]). Soluble proteins were then separated from insoluble debris by centrifugation for 10 min at 100 000 g, resolved by SDS-PAGE and transferred to nitrocellulose membranes. Specific methylated states of histone H3 lysine 9 were detected with antibodies against monomethyl H3K9 (Upstate, 07-395; or Abcam, ab9045), dimethyl H3K9 (Upstate, 07-212; or Abcam, ab7312) or trimethyl H3K9 (Abcam, ab1186 or ab8889). A modification-insensitive anti-H3 antibody (Abcam, ab1791) was used to adjust the amount of histone H3 loaded in each lane.

Histone methyltransferase assays

The histone methylating activity of recombinant SET3p was assayed as described earlier (42). Briefly, 10 μg of core histones or purified H3 from calf thymus and 1 μg of recombinant SET3p protein or a protein extract from an empty vector control were incubated with 250 nCi of S-adenosyl-L-(methyl-¹⁴C)methionine (¹⁴C-SAM) (GE Healthcare, Piscataway, NJ, USA) in a final volume of 40 μl for 2 h at 30°C in MAB buffer. Samples were then resolved by SDS-PAGE on 15% polyacrylamide gels, stained with Coomassie Brilliant Blue and dried onto filter paper. The incorporated radioactivity was detected with a phosphor imager (Amersham). The methylation activity of SET3p on wild-type (H3N) and lysine mutated (K4R, K9R, K27R or 3K-R) histone H3 tails fused to GST was assayed in a similar way. For time course experiments, 8 μg of recombinant SET3p protein, 40 μg of recombinant human histone H3 (43) and 20 μM of unlabeled SAM were incubated at 30°C in MAB buffer in a final volume of 80 μl. Ten microliters of aliquots were taken at different times (Figure 3D) and the reactions stopped by the addition of 10 μl of 2× SDS-PAGE loading buffer. Samples were then examined by western blotting with antibodies against specific H3K9 methylation states.

Chromatin immunoprecipitation (ChIP) assays

To examine the methylation status of histone H3 at specific chromosomal loci, 5 × 10⁷ TAP-grown cells were cross-linked for 10 min with 1% formaldehyde and then quenched by adding glycine to 0.1 M. Cells were then pelleted, washed with TBS and frozen in liquid nitrogen (42). The cell pellet was resuspended in 6 ml of ChIP lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 2 μl/ml of plant protease inhibitor cocktail [Sigma-Aldrich]), and cells were lysed by two passages through a French press at 5000 psi. Chromatin was sheared by sonication to an average size of 500 bp and then immunoprecipitated using a ChIP assay kit (Upstate) and a modification-insensitive anti-H3 antibody (15 μl, Abcam ab1791). The precipitated chromatin was eluted by incubation for 10 min at 68°C with 100 μl of ChIP elution buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA and 1% SDS) (44). This procedure was repeated once and 10% of the combined eluate was assayed as described earlier (42). Briefly, 10 μg of core histones or purified H3 from calf thymus and 1 μg of recombinant SET3p protein or a protein extract from an empty vector control were incubated with 250 nCi of S-adenosyl-L-(methyl-¹⁴C)methionine (¹⁴C-SAM) (GE Healthcare, Piscataway, NJ, USA) in a final volume of 40 μl for 2 h at 30°C in MAB buffer. Samples were then resolved by SDS-PAGE on 15% polyacrylamide gels, stained with Coomassie Brilliant Blue and dried onto filter paper. The incorporated radioactivity was detected with a phosphor imager (Amersham). The methylation activity of SET3p on wild-type (H3N) and lysine mutated (K4R, K9R, K27R or 3K-R) histone H3 tails fused to GST was assayed in a similar way. For time course experiments, 8 μg of recombinant SET3p protein, 40 μg of recombinant human histone H3 (43) and 20 μM of unlabeled SAM were incubated at 30°C in MAB buffer in a final volume of 80 μl. Ten microliters of aliquots were taken at different times (Figure 3D) and the reactions stopped by the addition of 10 μl of 2× SDS-PAGE loading buffer. Samples were then examined by western blotting with antibodies against specific H3K9 methylation states.
extractions, the immunoprecipitated DNA was quantified by real-time PCR on a Bio-Rad iCycler iQ using SYBR Green. After each run, a melting curve was performed to ensure that no primer dimers interfered with the quantification. Serial dilutions of the primary input (DNA prior to anti-H3 immunoprecipitation) were used to generate a calibration curve with which the amounts of target DNA in the secondary inputs (DNA co-immunoprecipitated with anti-H3, anti-H3K9me1, anti-H3K9me3 or IgG antibodies) were calculated. The levels of the H3K9 modifications or of the IgG negative control were then normalized relative to the anti-H3 controls for each examined sequence. Primers used for amplification of the target loci were: for RbcS2: aadA-RbcS2, aadA-CodL (5'-TCTGGCTATCTTGCTGA CAAAAC-3') and aadA-CodR (5'-TAGTGATCTCGCCTT TCACGTA-3'); and for RPS3, C_20102proL1 (5'-AAG GGCGCTGCTAGTATAACCA-3') and C_20102proR1 (5'-CCTTTGTTTCCCAGAGAGAA-3').

DNA methylation analysis

Genomic DNA from Chlamydomonas was isolated, digested, resolved in agarose gels, blotted and hybridized following standard procedures (35,45). The aadA and psbA probes used for Southern hybridization correspond to the coding sequence of the RbcS2:aadA:RbcS2 transgene and the 3' end of the chloroplast psbA gene, respectively (35).

RESULTS

Chlamydomonas reinhardtii SET3p is a homolog of plant SUVH proteins

SET3 encodes a SET domain-containing protein with a predicted size of 957 amino acids. The carboxyl terminal region of this polypeptide is well conserved relative to that of SU(VAR)3-9 proteins and consists of the SET domain flanked by two cysteine-rich motifs, the pre-SET and the post-SET domains (Figure 1A). However, SET3p also includes a unique insertion, of approximately 300 amino acids, within the SET domain that separates the conserved sub-motifs II and III (46) (Figure 1A).

DNA methylation analysis

Genomic DNA from Chlamydomonas was isolated, digested, resolved in agarose gels, blotted and hybridized following standard procedures (35,45). The aadA and psbA probes used for Southern hybridization correspond to the coding sequence of the RbcS2:aadA:RbcS2 transgene and the 3' end of the chloroplast psbA gene, respectively (35).

RESULTS

Chlamydomonas reinhardtii SET3p is a homolog of plant SUVH proteins

SET3 encodes a SET domain-containing protein with a predicted size of 957 amino acids. The carboxyl terminal region of this polypeptide is well conserved relative to that of SU(VAR)3-9 proteins and consists of the SET domain flanked by two cysteine-rich motifs, the pre-SET and the post-SET domains (Figure 1A). However, SET3p also includes a unique insertion, of approximately 300 amino acids, within the SET domain that separates the conserved sub-motifs II and III (46) (Figure 1A). At its N-terminal end, SET3p displays the configuration characteristic of plant SUVH proteins (Figure 1A).

Figure 1. SU(VAR)3-9 homologs in Chlamydomonas and several eukaryotes. (A) Domain organization of SU(VAR)3-9-related proteins. (B) Phylogenetic relationship between Chlamydomonas SET3p and SET5p and histone methyltransferases from several species. Numbers indicate bootstrap values, as percentage, based on 1000 pseudoreplicates. At SUVH1 to At SUVH9, Arabidopsis thaliana SU(VAR)3-9 homologs 1 to 9 (AAK28966 to AAK28974, respectively); At SUVR1 to At SUVR8, A. thaliana SU(VAR)3-9-related 1 to 5 (AAK77165, AAK92218, NP_974212, AAL01113 and NP_179954, respectively); Cr SET3p, Chlamydomonas reinhardtii SET3p protein (AAV84356); Cr SET5p, Chlamydomonas reinhardtii SET5p protein (145254 at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html); Dm E[Z], Drosophila melanogaster Enhancer of zeste protein (P42124); Dm SU(VAR)3-9, Drosophila Suppressor of variegation 3-9 protein (P45975); Dm Triithorax, Drosophila Triithorax protein (P20659); Hs G9a, human SET domain protein G9a (NP_000700); Hs SUV39H1, human SU(VAR)3-9 homolog 1 (O43463); Nc Dim-5, Neurospora crassa Dim-5 (Q8X225); Sp Clr4, Schizosaccharomyces pombe Cryptic loci regulator 4 protein (AAC18302).
These polypeptides contain, instead of the characteristic chromodomain of the metazoan enzymes, a conserved motif designated YDG or SRA (SET and RING finger associated) (19,47).

A phylogenetic tree constructed using sequences corresponding to the SET domain of multiple HMTases shows that SET3p clusters with the plant SUVH proteins (Figure 1B). These polypeptides have been reported to fall into four distinct subgroups, suggesting a possible functional differentiation that might have preceded the angiosperm/gymnosperm divergence (8,19,21). However, SET3p behaves as an outgroup to all of the Arabidopsis SUVH proteins, implying that most of the functional diversification of SUVH polypeptides likely occurred within the plant lineage after the evolutionary separation of green algae and land plants. In addition, higher plants also contain SU(VAR)3-9-RELATED (SUVR) proteins, which lack the YDG/SRA domain but are grouped with the SUVHs into the Class V of plant SET domain polypeptides (19,21). The Chlamydomonas genome also encodes a SU(VAR)3-9-related protein, named SET3p (145254 at http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), that lacks the YDG/SRA domain (Figure 1A). Interestingly, whereas Chlamydomonas SET3p is closely related to plant SUVHs, SET3p associates with plant SUVRs in phylogenetic analyses (Figure 1B), suggesting that differentiation of the SUVH and SUVR genes likely occurred prior to the divergence of the green algae and plant lineages.

RNAi-mediated suppression of SET3 results in defective silencing of multiple-copy transgenes

Plant SUVH genes have been implicated in gene silencing, heterochromatin formation and the partial control of non-CpG DNA methylation. For instance, SUVH4 mutants derepress the silenced SUPERMAN and PAI genes (27,28) and loss-of-function of SUVH2 reactivates a LUCIFERASE transgene (8). To examine whether Chlamydomonas SET3 has a similar role in gene silencing, we tested the effect of downregulating its expression on the transcriptional activity of repressed transgenes. Chlamydomonas strain 1-P[300] contains over 100 silenced copies of the RbcS2:aadA:RbcS2 transgene (conferring resistance to spectinomycin), integrated as head-to-head, tail-to-tail and head-to-tail concatamers at two chromosomal loci (35). Based on the repeated conformation of the transgenic arrays, their transcriptionally silenced state (35) and the presence of DNA methylation (see below), it is possible that the chromatin associated with these loci is (hetero)chromatic in nature, as reported for equivalent concatamers in other eukaryotes (8,48–50).

To suppress SET3 expression, the 1-P[300] strain was transformed with an inverted repeat construct that generates dsRNA homologous to the 3′ end of the SET3 mRNA. We recovered several independent transformants showing reduced SET3 transcript levels and two strains, Set3-IR1 and Set3-IR2, were examined in detail for reactivation of the RbcS2:aadA:RbcS2 transgenes as well as for their ability to survive on medium containing spectinomycin (Figure 2 and data not shown). RT-PCR analyses indicated that RNAi-mediated SET3 suppression resulted in increased RbcS2:aadA:RbcS2 mRNA levels (Figure 2A). In addition, the Set3-IR strains were able to grow on medium containing spectinomycin whereas the non-transgenic wild-type (CC-124) and the silenced parental strain (1-P[300]) were not (Figure 2B). These effects were specific for the Set3-IR transformants since RNAi-mediated downregulation of an unrelated gene, MAAT (encoding tryptophan synthase β subunit), did not reactivate RbcS2:aadA:RbcS2 expression in the 1-P[300] strain nor allow survival in the presence of spectinomycin (Figure 2, Maa7-IR3 strain). Yet, SET3 suppression did not release the silencing of an unmethylated, single-copy transgene (in strain 11-P[300]) or of dispersed transposable elements, such as the TOC1 retrotransposon (data not shown). We have reported earlier that the 11-P[300] transgene and the TOC1 loci appear to be repressed by euchromatin-specific epigenetic marks (42).

![Figure 2. Suppression of SET3 expression and reactivation of RbcS2:aadA:RbcS2 transgenes in Set3-IR strains. (A) RT-PCR analysis of SET3 and RbcS2:aadA:RbcS2 (aadA) expression in the indicated strains. Amplification of MUT9 (encoding a Ser/Thr protein kinase) transcripts was used as a control for equivalent RNA input and efficiency of the RT-PCR reactions. (B) Growth and survival of the indicated strains on medium with (TAP + Spec) or without (TAP) spectinomycin. Strains: CC-124, wild-type strain; 1-P[300], silenced parental strain; Maa7-IR3, 1-P[300] transformant expressing dsRNA complementary to the MAA7 gene; Set3-IR1 and Set3-IR2, 1-P[300] transformants expressing dsRNA complementary to SET3.](image-url)
Thus, our results suggest that downregulation of SET3 expression leads to defects in the transcriptional silencing of multiple-copy cis-tandem transgenes, presumably associated with a (hetero)chromatic conformation (see discussion).

**SET3p possesses H3K9 monomethyltransferase activity in vitro**

In order to investigate whether SET3p might be a determinant of repressed chromatin structure, we first tested its ability to methylate core histones in vitro. Recombinant, His-tagged SET3p was purified from *Escherichia coli* and used in a methylation assay with a mix of calf thymus histones or purified histone H3 as the substrates. When SET3p was incubated with histones in the presence of L-[methyl-14C]-SAM, as the methyl donor, a 17-kDa band corresponding to methylated histone H3 was readily detected by phosphor imager scanning (Figure 3A). On the contrary, no signal was observed when core histones were incubated with an empty vector protein extract.

We next examined the histone H3 lysine residue that was targeted by the SET3p activity. We carried out in vitro HMTase assays using as substrates the histone H3 N-terminal tail (amino acids 1–52) with the wild-type sequence (H3N) or with mutations to arginine in specific lysines (K4R, K9R or K27R) or in the three lysine residues (3K-R = K4R, K9R and K27R). These polypeptides were expressed as GST fusions in *E. coli* and purified as recombinant proteins. When incubated in the presence of SET3p and L-[methyl-14C]-SAM, H3N was readily methylated, whereas recombinant GST alone did not serve as a substrate (Figure 3B, lanes 1 and 2). The K4R and K27R fusions, carrying mutations in H3 lysine 4 or 27, were methylated as well as H3N (Figure 3B, lanes 3 and 5). Conversely, mutation of H3 lysine 9 (K9R) or of the three lysine residues (3K-R) completely eliminated methylation by SET3p (Figure 3B, lanes 4 and 6). Thus, SET3p appears to have HMTase activity specific for lysine 9 of histone H3.

Several residues within the SET domain have been shown to be important for the degree of methylation carried out by an HMTase, with the Phe/Tyr switch being the most extensively characterized (51). The SET domains of proteins with di- and/or trimethylating activity generally possess a Phe residue, corresponding to positions 281 in *Neurospora crassa* Dim-5 and 943 in human G9a (Figure 3C). In contrast, a Tyr residue at this position inhibits trimethylation and Phe-to-Tyr mutants of both Dim-5 and G9a show altered specificity behaving as mono- or dimethyltransferases (51,52). SET3p contains a Tyr in the position corresponding to the Phe/Tyr switch (Figure 3C), suggesting that it might be restricted in its capacity to carry out trimethylation. To address this question, we incubated SET3p with recombinant...
(unmethylated) histone H3 in the presence of unlabeled SAM and proceeded to detect the degree of SET3p-catalyzed H3K9 methylation by immunoblotting with antibodies raised specifically against mono-, di- or trimethyl H3K9. Interestingly, SET3p carried out monomethylation of H3K9, which reached a plateau after 2–3 h of incubation (Figure 3D). In contrast, neither di- nor trimethylation of H3K9 could be detected, even after 21 h of incubation. These observations, taken together, indicated that SET3p behaves in vitro as an exclusive H3K9 monomethyltransferase.

SET3p is responsible for H3K9 monomethylation, but not H3K9 trimethylation, in vivo

Given its in vitro role as a H3K9 HMTase, we next examined whether SET3p affected this modification in vivo. Immunoblots of extracts enriched in chromatin proteins, probed with antibodies raised against the different forms of methylated H3K9, revealed the presence of H3K9me1 and H3K9me3 in Chlamydomonas (Figure 4A). In contrast, as reported earlier (42), dimethyl H3K9 was undetectable even though the anti-H3K9me2 antibody was capable of recognizing this modification in calf thymus histone H3 (Figure 3D, control). Interestingly, significantly lower levels of monomethyl H3K9 were observed in the strains undergoing suppression of SET3 expression, whereas trimethyl H3K9 remained unchanged, in comparison with the parental 1-P[300] strain (Figure 4A). Global H3K9me1 and H3K9me3 levels in the control Maa7-IR3 strain were similar to those in the 1-P[300] strain (Figure 4A). Hence, in agreement with the in vitro data, Chlamydomonas SET3p seems to be at least partly responsible for global H3K9 monomethylation in vivo, whereas H3K9me3 depends on another yet unidentified HMTase.

Monomethyl H3K9 is associated with the chromatin of silenced, multiple-copy transgenes

To determine if the loss of H3K9me1 in the Set3-IR strains was directly associated with reactivation of the silenced RbcS2:aadA:RbcS2 transgenes, we analyzed their chromatin environment by sequential ChIP assays. By using primers specific for the 5' end of the coding sequence of the RbcS2:aadA:RbcS2 transgene and real-time PCR, we found that monomethyl H3K9 was highly enriched from an equivalent region in the constitutively expressed RPS3 gene (encoding ribosomal protein S3) (Figure 4B). ChIP analysis also revealed a substantial decrease in monomethyl H3K9 associated with the RbcS2:aadA:RbcS2 transgenes in the Set3-IR2 strain, as compared with the parental 1-P[300] strain (Figure 4B). Unexpectedly, trimethyl H3K9 was almost undetectable in the RbcS2:aadA:RbcS2 transgenes (independently of their transcriptional state), whereas low levels of H3K9me3 were observed in the transcriptionally active RPS3 gene. Furthermore, no differences in H3K9me3 signals were discernable between the Set3-IR2 and the 1-P[300] strains at the tested loci. Thus, in
Chlamydomonas, monomethyl H3K9 may function as an epigenetic mark for the silenced chromatin, presumably heterochromatin-like, typical of tandem transgenes. Conversely, trimethyl H3K9 was not associated with the repressed RbcS2:aadA:RbcS2 transgenes and the low levels detected on the active RPS3 gene suggest that this modification may be present in euchromatic domains.

CpG DNA methylation of the RbcS2:aadA:RbcS2 transgenes is increased in strains experiencing RNAi-mediated suppression of SET3

Since in some species cytosine methylation appears to be dependent on H3K9 methylation (4,23,27,29) and direct interaction between H3K9 HMTases and DNA methyltransferases has been demonstrated in mammals (53), we also studied the effect of SET3 downregulation on the DNA methylation of the RbcS2:aadA:RbcS2 transgenes. The methylation-sensitive isoschizomers HpaII and MspI recognize the same DNA sequence (5'-CCGG-3'), but HpaII is inhibited by methylation of either cytosine, whereas MspI is only sensitive to methylation of the outer cytosine residue. Thus, digestion of an unmethylated RbcS2:aadA:RbcS2 transgene with these enzymes and HindIII generates a fragment of 530 bp and three fragments smaller than 160 bp that can be detected by hybridization with a probe encompassing the aadA coding sequence (Figure 5A). In contrast, if some of the HpaII/MspI sites become methylated, the inability of the enzymes to cleave will result in the appearance of DNA fragments of higher molecular weight. By using this approach, we observed that the multiple copies of the RbcS2:aadA:RbcS2 transgene were nearly fully digested with MspI but only partly cleaved with HpaII in the 1-P[300] parental strain (Figure 5B), suggesting the presence of CpG DNA methylation in the aadA coding sequence and, possibly, in the upstream RbcS2 promoter. The almost complete digestion with MspI indicated that CpNpG methylation (at least in a CCG context) is virtually absent from the RbcS2:aadA:RbcS2 transgenes. Interestingly, in the Set3-IR strains we detected a noticeable decrease in the cleaving capability of HpaII, indicative of a higher degree of CpG DNA methylation in comparison to the 1-P[300] strain, whereas the patterns of MspI digestion remained unchanged (Figure 5B). As a control, Southern hybridization analysis of the same blots with a psbA (encoding a Photosystem II component) probe showed that the chloroplast DNA was equally digested in all lanes, substantiating that differences in aadA restriction patterns were not due to defective enzymatic activity in some samples. Similar results were observed with other methylation sensitive restriction enzymes, such as HhaI and ScrFI (data not shown). Thus, RNAi-mediated downregulation of SET3 appears to result in increased levels of CpG DNA methylation associated with the RbcS2:aadA:RbcS2 transgenes.

DISCUSSION

SUVHs are conserved proteins directly implicated in chromatin-mediated silencing in a variety of eukaryotes.
also involved in the transcriptional silencing of tandemly repeated transgenes. However, the expression of single-copy transgenes and dispersed endogenous transposons, previously shown to be repressed by euchromatic histone marks (42), was not affected by RNAi-mediated suppression of SET3. Although, we cannot rule out that partial downregulation of SET3 may have not reduced the corresponding protein to a level low enough to compromise the silencing of euchromatic sequences. Nonetheless, in a number of species, transgenic repeat arrays appear to become silenced by condensation into heterochromatin (8,48,49,50), an intrinsic property of tandem arrays not attributable to position effects of nearby sequences (56,57). Indeed, it has been proposed that this form of repression may reflect a genomic defense response against invading foreign sequences such as transposable elements (56). Likewise, we speculate that concatameric transgenes in *Chlamydomonas* may be characterized by a (hetero)chromatic configuration that is at least partly dependent on SET3p activity. Although, the current lack of sequence data on heterochromatic *Chlamydomonas* repeats prevented us from testing more directly whether SET3p is required for the maintenance of natural heterochromatin.

The role of SU(VAR)3-9 proteins in the organization of transcriptionally repressive chromatin is mediated, at least in part, by their specific H3K9 methyltransferase activity (8,10,58). However, the ε-amino group of H3K9 can be mono-, di- or trimethylated, thereby adding to the coding complexity of this particular histone modification. In mammals, as already mentioned, pericentric heterochromatin is characterized by H3K9me3 and this modification is almost entirely eliminated in *suv39h1/suv39h2* double mutants, where H3K9me1 becomes predominant (4–6,9). Based on these and other observations it has been proposed that SUV39H-mediated methylation of heterochromatin is primed by an unidentified H3K9 monomethylating HMTase (59). In wild-type mammalian cells, mono- and dimethyl H3K9 are mostly found in euchromatin (4–6,9,12,32). Monomethylated forms of H3K9 and H4K20 partition together into discrete nuclear compartments that have been proposed to represent silent chromatin states, functionally different from those characterized by the di- and/or trimethylated forms (32). This organization of chromosomes into domains with distinct H3K9 methylation states has also been observed in plants (7,8,11,27). Moreover, isotope-labeling of mammalian cells to detect half lives and histone exchange rates revealed differential stability of the H3K9 methylation states: fast turnover for H3K9me1, intermediate turnover for H3K9me2 and very slow turnover for H3K9me3 (60). Thus, different degrees of H3K9 methylation may be functionally relevant. Yet, the biological significance of monomethyl H3K9 has remained unexplained because of the lack of mutants exclusively defective in this modification.

Interestingly, *Chlamydomonas* SET3p behaved as an exclusive H3K9 monomethyltransferase *in vitro* and RNAi-mediated downregulation of SET3 significantly reduced global H3K9me1 levels *in vivo*. ChIP assays demonstrated that H3K9me1 was specifically associated with silent, multiple-copy transgenes. Moreover, the reactivation of these transgenes in the SET3 RNAi strains correlated with the partial loss of monomethyl H3K9 from their chromatin. Somewhat surprisingly, we were unable to detect dimethyl H3K9, an abundant H3K9 modification in *Arabidopsis* (61), in whole-cell extracts or partially purified histone extracts from *Chlamydomonas*, indicating that this mark is either absent or present at very low levels in this alga. This is in agreement with an earlier report that examined bulk histone H3 modifications in *Chlamydomonas* by protein sequencing (62). On the other hand, trimethyl H3K9 was readily observable but independent of the SET3p activity. In addition, H3K9me3 was virtually absent from the silenced multiple-copy transgenes, although it was associated at low levels with the transcriptionally active *RPS3* gene. In higher plants, reports on the presence and distribution of trimethyl H3K9 have been somewhat contradictory (8,30,61) but recent observations suggest that it may be enriched in euchromatin (8,11). Conversely, monomethyl H3K9 is found preferentially in heterochromatin both in *Arabidopsis* and maize (7,8,11). Our findings in *Chlamydomonas* are also consistent with a role for H3K9me1 in indexing silent, presumably (hetero)chromatic domains, which may be typical of tandemly repeated sequences (56,57). Thus, our results, taken together, implicate SET3p as the first exclusive H3K9 monomethyltransferase and provide direct evidence that H3K9me1 may function as an epigenetic mark for repressive chromatin.

In several organisms, heterochromatin is also characterized by high levels of methylation of the underlying DNA (64,65). In plants, cytosine methylation is found predominantly at symmetric CpG and CpNpG sequences and a lower frequency at asymmetric CpNpN sites (where N = A, T or C) (66). In *Arabidopsis*, CpG methylation appears to direct H3K9me2 to silent chromosomal regions (64,67). Conversely, several SUVH proteins seem to control the deposition of non-CpG methylation, mediated by the CMT3 chromomethylase, at distinct loci (27–29,31). Likewise, in *Neurospora*, the H3K9 HMTase Dim-5 is required for DNA methylation (23). In mammals, H3K9 methylation and CpG methylation show a complex interplay, perhaps as part of a self-reinforcing loop for repressive chromatin (53,68). The *suv39h1/suv39h2* double mutant cells display an altered DNA methylation profile at pericentric satellite repeats, but not at other repetitive sequences (68); whereas mouse embryonic stem cells deficient in maintenance or *de novo* DNA methyltransferases show no alteration in H3K9 methylation at several repeats (69). In green algae, cytosine methylation appears to be restricted to the CpG sequence (35,70) and a possible relationship between DNA methylation and H3K9 methylation had not been previously examined. To our surprise, we detected an increase in DNA methylation of the reactivated *RbcS2:aadA:RbcS2* transgenes in the SET3 RNAi strains. This suggests that CpG methylation in *Chlamydomonas* is not dependent on the H3K9me1 mark. Indeed, we speculate that a reduction in H3K9 monomethylation may result in an opening of the chromatin structure and
increased accessibility of the DNA to CpG methyltransferases and/or to other factors that control DNA methylation. Transgene reactivation in this context is likely the net result of a reduction in (hetero)chromatic structure, due to suppression of SET3p activity, partly counteracted by an increase in DNA-methylation-dependent silencing.

With the present results all H3K9 methylation states have now been implicated in the establishment and/or maintenance of repressive chromatin, yet their precise roles remain unclear since their distribution in nuclear domains is species specific. Moreover, H3K9me3 has also been found associated with active genes in some species (this work and 63). The different H3K9 methylation states are in all likelihood bound by specific chromatin proteins that determine the higher order levels of chromatin organization. For instance, HETEROCHROMATIN PROTEIN1 (H1P), a key factor for heterochromatin formation in Drosophila, Neurospora and mammals, is partly localized to specific nuclear domains by preferential binding to H3K9me3 (and with lower affinity to H3K9me2); however it will not recognize chromatin characterized by H3K9me1 (65,71,72). Interestingly, in Arabidopsis, characterized by H3K9me1 and H3K9me2 in its heterochromatin, the putative H1p homolog (LIKE HETEROCHROMATIN PROTEIN1) appears to be dispensable for heterochromatic silencing (28,73). Thus, there must be differences in the molecular mechanisms of recognition and interpretation of epigenetic histone modifications that ultimately produce the same functional outcome (for instance a silent heterochromatic domain) from varying methylation states in different species. Within this context, how H3K9me1 is recognized and transduced into a repressive chromatin domain, or whether it has other roles, remains to be explored. Furthermore, the cumulative evidence from multiple eukaryotes (10,59) strongly supports the notion that a combination of several histone lysine methylation marks (and probably other histone modifications as well as DNA methylation), rather than a single epigenetic mark, is required to discriminate active from inactive chromatin.

ACKNOWLEDGEMENTS

We are grateful to D. Rokhsar and Joint Genome Institute scientists for allowing access to the Chlamydomonas genome sequence prior to publication and to members of the Cerutti’s lab for helpful discussions. This work was supported in part by a grant from the National Science Foundation (MCB-0544448). Funding to pay the Open Access publication charge was provided by the National Science Foundation.

Conflict of interest statement. None declared.

REFERENCES

1. Kornberg, R.D. and Lorch, Y. (2002) Chromatin and transcription: where do we go from here. Curr. Opin. Genet. Dev., 12, 249–251.
2. Farkas, G., Leibovitch, B.A. and Elgin, S.C. (2000) Chromatin organization and transcriptional control of gene expression in Drosophila, Gene, 253, 117–136.
3. Sims, R.J., III, Nishioka, K. and Reinberg, D. (2003) Histone lysine methylation: a signature for chromatin function. Trends. Genet., 19, 629–639.
4. Lachner, M., Sengupta, R., Schotta, G. and Jenuwein, T. (2004) Trilogies of histone lysine methylation as epigenetic landmarks of the euchromatic genome. Cold Spring Harb. Symp. Quant. Biol., 69, 209–218.
5. Peters, A.H., Kubicek, S., Mechtler, K., O’Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y. et al. (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol. Cell, 12, 1577–1589.
6. Rice, J.C., Briggs, S.D., Ueberheide, B., Barber, C.M., Shabanowitz, J., Hunt, D.F., Shinkai, Y. and Aliis, C.D. (2003) Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol. Cell, 12, 1591–1598.
7. Mathieu, O., Probst, A.V. and Pazsokwski, J. (2005) Distinct regulation of histone H3 methylation at lysines 27 and 9 by CpG methylation in Arabidopsis. EMBO J., 24, 2783–2791.
8. Naumann, K., Fischer, A., Hofmann, I., Krauss, V., Phalke, S., Irmler, K., Hause, G., Aurich, A.C., Dorm, R. et al. (2005) Pivotal role of AtSUHV2 in heterochromatic histone methylation and gene silencing in Arabidopsis. EMBO J., 24, 1418–1429.
9. Wu, R., Terry, A.V., Singh, P.B. and Gilbert, D.M. (2005) Differential subnuclear localization and replication timing of histone H3 lysine 9 methylation states. Mol. Biol. Cell, 16, 2872–2881.
10. Ebert, A., Lein, S., Schotta, G. and Reuter, G. (2006) Histone modification and the control of heterochromatic gene silencing in Drosophila. Chromosome Res., 14, 377–392.
11. Shi, J. and Dawe, R.K. (2006) Partitioning of the maize epigenome by the number of methyl groups on histone H3 lysines 9 and 27. Genetics, 173, 1571–1583.
12. Zinner, R., Albiz, H., Walter, J., Peters, A.H., Cremer, T. and Cremer, M. (2006) Histone lysine methylation patterns in human cell types are arranged in distinct three-dimensional nuclear zones. Histochem. Cell Biol., 125, 3–19.
13. Rea, S., Eisenhaber, F., O’Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P. et al. (2006) Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature, 406, 593–599.
14. Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D. and Grewal, S.I. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science, 292, 110–113.
15. Tschiersch, B., Hofmann, A., Krauss, V., Dorm, R., Korge, G. and Reuter, G. (1994) The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3–9 combines domains of antagonistic regulators of homeotic gene complexes. EMBO J., 13, 3822–3831.
16. Aagaard, L., Laible, G., Selenko, P., Schmid, M., Dorm, R., Schotta, G., Kuhfittig, S., Wolf, A., Lebersorger, A. et al. (1999) Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3–9 encode centromere-associated proteins which complex with the heterochromatin component M31. EMBO J., 18, 1923–1938.
17. Aagaard, L., Schmid, M., Warburton, P. and Jenuwein, T. (2000) Mitotic phosphorylation of Su[V39H1], a novel component of active centromeres, coincides with transient accumulation at mammalian centromeres. J. Cell Sci., 113, 817–829.
18. Schotta, G. and Reuter, G. (2000) Controlled expression of tagged proteins in Drosophila using a new modular P-element vector system. Mol. Gen. Genet., 262, 916–920.
19. Haumbusch, I.O., Thorstensen, T., Krauss, V., Fischer, A., Naumann, K., Assalkhouri, R., Schulz, J., Reuter, G. and Aalen, R.B. (2001) The Arabidopsis thaliana genome contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes. Nucleic Acids Res., 29, 4319–4333.
20. Shen, W.H. (2001) NiSET1, a member of a newly identified subgroup of plant SET-domain-containing proteins, is chromatin-associated and its ectopic overexpression inhibits tobacco plant growth. Plant J., 28, 371–383.
21. Springer, N.M., Napoli, C.A., Selinger, D.A., Pandey, R., Cone, K.C., Chandler, V.L., Kaeppler, H.F. and Kaeppler, S.M. (2003) Comparative analysis of SET domain proteins in maize and Arabidopsis reveals multiple duplications preceding the divergence of monocots and dicots. Plant Physiol., 132, 907–925.

22. Ivanova, A.V., Bonaduce, M.J., Ivanov, S.V. and Klar, A.J. (1998) The chromo and SET domains of the Ctr4 protein are essential for silencing in fission yeast. Nat. Genet., 19, 192–195.

23. Tamura, H. and Selker, E.U. (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature, 414, 277–283.

24. Yu, Y., Dong, A. and Shen, W.H. (2004) Molecular characterization of the tobacco SET domain protein NsSET1 unravels its role in histone methylation, chromatin binding, and segregation. Plant J., 40, 659–671.

25. Shen, W.H. and Meyer, D. (2004) Ectopic expression of the NsSET1 histone methyltransferase inhibits cell expansion, and affects cell division and differentiation in tobacco plants. Plant Cell Physiol., 45, 1715–1719.

26. Casas-Mollano, J.A., Lao, N.T. and Kavanagh, T.A. (2006) Intron-regulated expression of SULF3, an Arabidopsis Su(var)3–9 homologue. J. Exp. Bot., 57, 3301–3311.

27. Jackson, J.P., Polyakov, A.M., Cao, X. and Jacobsen, S.E. (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone methyltransferase. Nature, 416, 556–560.

28. Malagenc, F., Bartee, L. and Bender, J. (2002) An Arabidopsis SET domain protein required for maintenance but not establishment of DNA methylation. EMBO J., 21, 6842–6852.

29. Ebbs, M.L. and Bender, J. (2006) Locus-specific control of DNA methylation by the Arabidopsis SUVH5 histone methyltransferase. Plant Cell, 18, 1166–1176.

30. Jackson, J.P., Johnson, L., Jasencakova, Z., Zhang, X., Perez-Burgos, L., Singh, P.B., Cheng, X., Schubert, I., Jenuwein, T. et al. (2004) Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in Arabidopsis thaliana. Chromosoma., 112, 308–315.

31. Ebbs, M.L., Bartee, L. and Bender, J. (2005) Histone H3 lysine 9 methylation is maintained on a transcribed inverted repeat by combined action of SUVH6 and SUVH4 histone methyltransferases. Mol. Cell. Biol., 25, 10507–10515.

32. Sims, J.K., Houston, S.I., Magazinnik, T. and Rice, J.C. (2006) A trans-tail histone code defined by monomethylated H4 Lys-20 and H3 Lys-9 demarcates distinct regions of silent chromatin. J. Biol. Chem., 281, 12749–12766.

33. Tachibana, M., Sugimoto, K., Nozaki, M., Ueda, J., Ohta, T., Okhi, M., Fukuda, M., Takeda, N., Niida, H. et al. (2002) G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. Genes Dev., 16, 1779–1791.

34. Thorstensen, T., Fischer, A., Sandvik, S.Y., Johnsen, S.S., Grini, P.E., Reuter, G. and Aalen, R.B. (2006) The Arabidopsis SUV4R4 protein is a nucleolar histone methyltransferase with preference for monomethylated H3K9. Nucleic Acids Res., 34, 5461–5470.

35. Cerutti, H., Johnson, A.M., Gillham, N.W. and Boynton, J.E. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., 25, 4876–4882.

36. Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4, 406–425.

37. Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform., 2, 150–163.

38. van Dijk, K., Marley, K.E., Jeong, B.R., Xu, J., Hesson, J., Cerny, R.L., Waterbolk, T.H. and Cerutti, H. (2005) Monomethyl histone H3 lysine 4 as an epigenetic mark for silenced euchromatin in Chlamydomonas. Plant Cell, 17, 2439–2453.

39. Zhang, Y., Griffin, K., Mondal, N. and Parvin, J.D. (2004) Phosphorylation of histone H2A inhibits transcription on chromatin templates. J. Biol. Chem., 279, 21866–21872.

40. Geisberg, J.V. and Struhl, K. (2004) Quantitative sequential chromatin immunoprecipitation, a method for analyzing co-occupancy of proteins at genomic regions in vivo. Nucleic Acids Res., 32, e15110.1093/nar/gnh148.

41. Sambrook, J. and Russell, D.W. (2001) Molecular Cloning – A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

42. Cheng, X., Collins, R.E. and Zhang, X. (2005) Structural and sequence motifs of protein (histone) methylation enzymes. Annu. Rev. Biophys. Biomol. Struct., 34, 267–294.

43. Alvarez-Venegas, R. and Avravomo, Z. (2002) SET-domain proteins of the Su(var)3–9, E(z) and trithorax families. Gene, 285, 25–37.

44. Ye, F. and Signer, E.R. (1996) RIGS (repeat-induced gene silencing) in Arabidopsis is transcriptional and alters chromatin configuration. Proc. Natl. Acad. Sci. U.S.A., 93, 10881–10886.

45. Probst, A.V., Franz, P.F., Paszkowski, J. and Mittelsten Scheid, O. (2003) Two means of transcriptional reactivation within heterochromatin. Plant J., 33, 743–749.

46. Wang, F., Koyama, N., Nishida, H., Haraguchi, T., Reith, W. and Tsukamoto, T. (2006) The assembly and maintenance of heterochromatin initiated by transgene repeats are independent of the RNA interference pathway in mammalian cells. Mol. Cell Biol., 26, 4028–4040.

47. Collins, R.E., Tachibana, M., Tamura, H., Smith, K.M., Jia, D., Zhang, X., Selker, E.U., Shinkai, Y. and Cheng, X. (2005) In vitro and in vivo analyses of a Phe/Tyr switch controlling product specificity of histone lysine methyltransferases. J. Biol. Chem., 280, 5563–5570.

48. Zhang, X., Yang, Z., Khan, S.I., Horton, J.R., Tamura, H., Selker, E.U. and Cheng, X. (2003) Structural basis for the product specificity of histone lysine methyltransferases. Mol. Cell, 12, 177–185.

49. Fuks, F., Hurd, P.J., Deplus, R. and Kouzarides, T. (2003) The DNA methyltransferases associate with H3K14 methyltransferase. Nucleic Acids Res., 31, 2305–2312.

50. Firestein, R., Cui, X., Huie, P. and Cleary, M.L. (2000) Set domain-dependent regulation of transcriptional silencing and growth control by SUV39H1, a mammalian ortholog of Drosophila Su(var)3–9. Mol. Cell. Biol., 20, 4900–4909.

51. Snowden, A.W., Gregory, P.D., Case, C.C. and Pabo, C.O. (2002) Gene-specific targeting of H3K9 methylation is sufficient for initiating repression in vivo. Curr. Biol., 12, 2159–2166.

52. Henikoff, S. (1998) Conspiracy of silence among repeated transgenes. Bioessays, 20, 532–535.

53. Martienssen, R.A. (2003) Maintenance of heterochromatin by RNA interference of tandem repeats. Nat. Genet., 35, 213–214.

54. Ebert, A., Schotta, G., Lein, E., Kubicek, S., Krauss, V., Jenuwein, T. and Reuter, G. (2004) Su(var) genes regulate the balance between euchromatin and heterochromatin in Drosophila. Genes Dev., 18, 2973–2983.

55. Jenuwein, T. (2006) The epigenetic magic of histone lysine methylation. FEBS J., 273, 3121–3135.

56. Zaccai, B.D., Kubicek, S., Yen, M., O’Sullivan, R.J., Sengupta, R., Perez-Burgos, L., Oparil, S., Mechtler, K. and Schotta, G. et al. (2006) Mjmd2h antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. Genes Dev., 20, 1557–1562.

57. Johnson, L., Mollah, S., Garcia, B.A., Muratore, T.L., Shabanowitz, J., Hunt, D.F. and Jacobsen, S.E. (2004) Mass spectrometry analysis of Arabidopsis histone H3 reveals distinct combinations of post-translational modifications. Nucleic Acids Res., 32, 6511–6518.

58. Probst, A.V., Franz, P.F., Paszkowski, J. and Mittelsten Scheid, O. (2003) Two means of transcriptional reactivation within hetero-
62. Waterborg, J.H., Robertson, A.J., Tatar, D.L., Borza, C.M. and Davie, J.R. (1995) Histones of Chlamydomonas reinhardtii: synthesis, acetylation, and methylation. *Plant Physiol.*, **109**, 393–407.

63. Vakoc, C.R., Mandat, S.A., Olenchock, B.A. and Blobel, G.A. (2005) Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol. Cell.*, **19**, 381–391.

64. Soppe, W.J., Jasencakova, Z., Houben, A., Kakutani, T., Meister, A., Huang, M.S., Jacobsen, S.E., Schubert, I. and Fransz, P.F. (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. *EMBO J.*, **21**, 6549–6559.

65. Maison, C. and Almouzni, G. (2004) HP1 and the dynamics of heterochromatin maintenance. *Nat. Rev. Mol. Cell Biol.*, **5**, 296–304.

66. Gruenbaum, Y., Naveh-Many, T., Cedar, H. and Razin, A. (1981) Sequence specificity of methylation in higher plant DNA. *Nature*, **292**, 860–862.

67. Tariq, M., Saze, H., Probst, A.V., Lichota, J., Habu, Y. and Panzowski, J. (2003) Erasure of CpG methylation in *Arabidopsis* alters patterns of histone H3 methylation in heterochromatin. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 8823–8827.

68. Lehnertz, B., Ueda, Y., Derijck, A.A., Braunschweig, U., Perez-Burgos, L., Kubicek, S., Chen, T., Li, E., Jenuwein, T., et al. (2003) Suv39h-mediated histone H3 lysine 9 methylation directly DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr. Biol.*, **13**, 1192–200.

69. Martens, J.H., O’Sullivan, R.J., Braunschweig, U., Opravil, S., Radolf, M., Steinlein, P. and Jenuwein, T. (2005) The profile of repeat-associated histone lysine methylation states in the mouse epigenome. *EMBO J.*, **24**, 800–812.

70. Babinger, P., Kobli, I., Mages, W. and Schmitt, R. (2001) A link between DNA methylation and epigenetic silencing in transgenic *Volvox carteri*. *Nucleic Acids Res.*, **29**, 1261–1271.

71. Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D. and Khorasanizadeh, S. (2003) Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes Dev.*, **17**, 1870–1881.

72. Freitag, M., Hickey, P.C., Khlaflallah, T.K., Read, N.D. and Selker, E.U. (2004) HP1 is essential for DNA methylation in *Neurospora*. *Mol. Cell.*, **13**, 427–434.

73. Lindroth, A.M., Shultis, D., Jasencakova, Z., Fuchs, J., Johnson, L., Schubert, D., Patnaik, D., Pradhan, S., Goodrich, J., et al. (2004) Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3. *EMBO J.*, **23**, 4286–4296.