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Monomethyl Histone H3 Lysine 4 as an Epigenetic Mark for Silenced Euchromatin in *Chlamydomonas* [W]

Karin van Dijk,[a] Katherine E. Marley,[b] Byeong-ryool Jeong,[a] Jianping Xu,[c] Jennifer Hesson,[c] Ronald L. Cerny,[d] Jakob H. Waterborg,[e] and Heriberto Cerutti[c,1]

[a] Department of Plant Pathology and Plant Science Initiative, University of Nebraska, Lincoln, Nebraska, 68588-0666
[b] Biology Department, Doane College, Crete, Nebraska, 68333
[c] School of Biological Sciences and Plant Science Initiative, University of Nebraska, Lincoln, Nebraska, 68588-0666
[d] Department of Chemistry, University of Nebraska, Lincoln, Nebraska, 68588-0362
[e] School of Biological Sciences, University of Missouri, Kansas City, Missouri, 64110-2499

Histone Lys methylation plays an important role in determining chromatin states and is mostly catalyzed by SET domain–containing proteins. The outcome, transcriptional repression or activation, depends on the methylated histone residue, the degree of methylation, and the chromatin context. Dimethylation or trimethylation of histone H3 Lys 4 (H3K4me2 or H3K4me3) has been correlated with transcriptionally competent/active genes. However, H3K4 methylation has also been implicated in gene silencing. This dualistic nature of the H3K4 methyl mark has thus far remained unresolved. In the green alga *Chlamydomonas reinhardtii*, Mut11p, related to a subunit of trithorax-like methyltransferase complexes, is required for transcriptional silencing. Here, we show that Mut11p interacts with conserved components of H3K4 methyltransferase machineries, and an affinity-purified Mut11p complex(es) methylates histones H3, H2A, and H4. Moreover, a *Mut11* mutant showed global loss of monomethylated H3K4 (H3K4me1) and an increase in dimethylated H3K4. By chromatin immunoprecipitation analysis, this strain also displayed substantial reduction in H3K4me1 and enrichment in H3K4me2 associated with transcriptionally derepressed genes, transgenes, and retrotransposons. RNA interference–mediated suppression of *Set1*, encoding an H3K4 methyltransferase, induced similar phenotypes, but of lower magnitude, and no detectable increase in H3K4me2. Together, our results suggest functional differentiation between dimethyl H3K4 and monomethyl H3K4, with the latter operating as an epigenetic mark for repressed euchromatin.

INTRODUCTION

Transcriptional regulation of gene expression often entails modifying chromatin, a complex structure consisting of DNA wrapped around an octamer of histones (the nucleosome) as the basic repeating unit. Chromatin states are highly dynamic and influenced by postsynthetic modifications of both histones and DNA (Richards and Elgin, 2002; Loidl, 2004). Histone residues can be covalently altered by enzymatic complexes that include, among others, methylation, acetylation, phosphorylation, or ubiquitination activities (Fischle et al., 2003; Loidl, 2004), whereas the main postsynthetic DNA change usually involves cytosine methylation (Richards and Elgin, 2002; Tariq and Paszkowski, 2004). These modifications can affect chromatin states by directly modulating nucleosome structure and/or by creating binding surfaces for chromatin structural/regulatory factors (Richards and Elgin, 2002; Fischle et al., 2003).

[1]To whom correspondence should be addressed. E-mail hcerutti1@unl.edu; fax 402-472-8722.
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Posttranslational histone modifications can function as epigenetic marks for either active or repressed chromatin (Kouzarides, 2002; Fischle et al., 2003; Lachner et al., 2003). In most eukaryotes, methylated histone H3 Lys 4 (H3K4) correlates with transcriptionally competent chromatin, whereas methylated H3K9 occurs preferentially in transcriptionally silent chromatin (Bernstein et al., 2002; Alvarez-Venegas et al., 2003; Fischle et al., 2003; Lachner et al., 2003; Schneider et al., 2004; Tariq and Paszkowski, 2004). However, methylatable Lys residues can exist in monomethylated, dimethylated, or trimethylated states, and recent evidence suggests that the degree of methylation has considerable influence on transcriptional stimulation or repression (Santos-Rosa et al., 2002; Tamaru et al., 2003). In addition, histone methylation likely cooperates with DNA methylation in maintaining heterochromatic domains (Richards and Elgin, 2002; Tariq and Paszkowski, 2004). Moreover, underscoring the complexity of these processes, the RNA interference (RNAi) machinery has recently been implicated in the establishment of silent heterochromatin (Matzke et al., 2004; Pal-Bhadra et al., 2004).

Methylation of most histone Lys residues is mediated by proteins containing a conserved SET domain, first identified in the *Drosophila melanogaster* chromatin factors Su(var)3-9, Enhancer-of-zeste, and Trithorax (Trx) (Rea et al., 2000). Trx displays histone methyltransferase (HMTase) activity specific for H3K4 (Smith et al., 2004), and complexes with similar enzymatic

capacity participate in transcriptional activation in several eukaryotes (Roguev et al., 2001; Nakamura et al., 2002; Hughes et al., 2004). In *Arabidopsis thaliana*, ATX1, a Trx homolog, acts as an activator of homeotic genes (Alvarez-Venegas et al., 2003). However, there is also unexplained evidence linking the H3K4 methylation machinery to chromatin repression. In mammals, the H3K4 HMTase Mixed-Lineage Leukemia (MLL) is part of a supercomplex with both repressive and activating functions (Nakamura et al., 2002; Xia et al., 2003). Moreover, in *Saccharomyces cerevisiae*, subunits of a H3K4 methylating complex are required for silencing of certain chromosomal loci (Bryk et al., 2002; Carvin and Kladde, 2004).

We have previously characterized a *Chlamydomonas reinhardtii* mutant, Mut-11, defective in the transcriptional silencing of *RbcS2*:*aadA*:*RbcS2* transgenes (conferring spectinomycin resistance), *TOC1*, a retrotransposon-like element, and *Gulliver*, a cut-and-paste DNA transposon (Jeong et al., 2002; Zhang et al., 2002). *Mut11* encodes a WD40-repeat protein (Mut11p) homologous to *S. cerevisiae* Swd3 and human WDR5, conserved subunits of activating H3K4 HMTase complexes (Roguev et al., 2001, 2004; Hughes et al., 2004; Dou et al., 2005). WDR5 has also been implicated in the transcriptional repression mediated by the clock protein PERIOD1 (Brown et al., 2005). Here, we show that Mut11p copurifies with histone methylating activities. Deletion of *Mut11* or RNAi-mediated suppression of *Set1* (encoding a H3K4 methyltransferase) results in defects in H3K4 monomethylation and transcriptional derepression of certain genes, transgenes, and transposons. Our findings suggest that monomethyl H3K4 is associated with silenced euchromatin and that certain Trx-like complexes may function in gene repression.

RESULTS

Mut11p Associates with Homologs of Trx HMTase Complex Subunits

To elucidate the molecular role of Mut11p, we sought to identify interacting protein partners using a tandem affinity purification (TAP) approach (Rigaut et al., 1999) as well as yeast two-hybrid screens. For affinity purification, the *Mut11* coding sequence was fused to the TAP tag, placed under the control of a constitutive promoter, and transformed into Mut-11. Expression of Mut11-TAPp partly rescued the mutant phenotype, evidenced by resilencing of *RbcS2*:*aadA*:*RbcS2* (Figure 1A). Mut11-TAPp–associated proteins were isolated by affinity purification, resolved by SDS-PAGE, and identified by tandem mass spectrometry (Figure 1B, Table 1). Three of the purified polypeptides were similar to HMTase complex subunits (Roguev et al., 2001, 2004; Hughes et al., 2004). The 83-kD protein (band 1) is related to yeast Swd1/human Rbbp5 (for Retinoblastoma binding protein 5), whereas the 42-kD polypeptide (band 8) is homologous to yeast Bre2/human Ash2L (for Absent, small or homeotic discs 2-like) (Figure 1B, Table 1). The protein represented by band 9, named Set4p, contains a plant homeodomain zinc finger and a C-terminal SET domain with similarity to the Trx class of HMTases (Figures 2C and 3) (Kouzarides, 2002). Control purifications using a TAP-tagged Ble fusion, expressed from an integrated transgene that confers bleomycin resistance, did not identify any of the Mut11-TAPp–associated proteins (Figure 1B).

The remaining Mut11-TAPp–associated polypeptides corresponded to protein chaperones, namely HSP90A, HSP70A, and

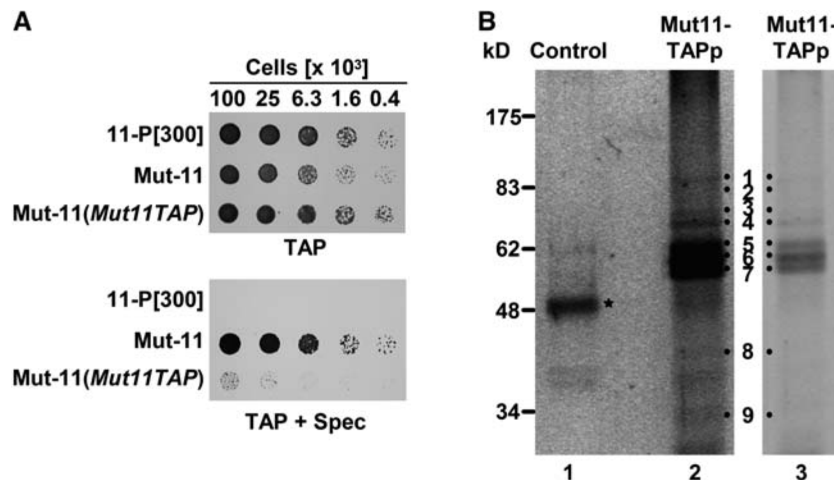

Figure 1. An Affinity-Purified Mut11-TAPp Complex(es) Includes Subunits of H3K4 Methyltransferases.

(A) Growth and survival on TAP medium or on TAP medium containing spectinomycin (TAP + Spec) of the indicated strains. 11-P[300], strain containing a single-copy, transcriptionally silenced *RbcS2*:*aadA*:*RbcS2* transgene. Mut-11(*Mut11TAP*), Mut-11 mutant complemented with a transgene expressing TAP-tagged Mut11p. Reactivation of expression of *RbcS2*:*aadA*:*RbcS2* allows cell survival on TAP + Spec.

(B) Mut11-TAPp–associated proteins, isolated by affinity purification, were separated by SDS-PAGE and visualized by Sypro Ruby staining. Lanes 2 and 3 correspond to different exposures of the same image. Numbered bands were identified by mass spectrometry analyses: 1, Rbbp5 homolog; 2, HSP90A; 3, HSP70A; 4, CCT subunit ϵ; 5, CCT subunits γ, η, and ζ; 6, Mut11-TAPp; 7, CCT subunits α, β, δ, and θ; 8, Ash2L homolog; 9, Set4p. Lane 1 corresponds to a control purification with a TAP-tagged Ble protein (indicated by the asterisk).

Table 1. Peptide Identities for Mut11-TAPp Complex(es) Subunits

Band No.	Protein	Number of Peptides	Peptide Sequence	Annotated Gene
1	Rbbp5	4	MFELAPAVAGR, LQLGSAVLR, VTDLVLNR, GALTVVDTATR	C_30021
2	HSP90A	2	ADLINNLGTIAR, GIVDSEDLPLNISR	C_730014
3	HSP70A	5	TLSSAAQTTIELDSLFEGVDFATSITR, VQQLLQDFFNGK, AVVTVPAYFNDSQR, IINEPTAAAIAYGLDKK, RTLSSAAQT TIELDSLFEGVDFATSITR	C_1340012
4	CCT ε-subunit	4	AVLAVADLER, VATENLDNIK, FDFSKENIEPLVK, FQELSADKLGHAGSVK	C_110180
5	CCT γ-subunit	4	IIDELAFPIDTNDR, GLSDLAAHYLTK, MQLPVTVLNANTK, HMMNVVNSCIGTK	C_150024
5	CCT η-subunit	2	VQTTVNNLDTK, CVASGANIVLSR	C_410079
5	CCT ζ-subunit	6	TLNANAEVMNR, FLSEGTHPR, GIDPISLDALAK, AAALFMNINAAK, LAEPLADQLTDIVTDSVLMVR, KATLEFLETFK	C_330064
6	MUT11-TAPp	3	FSPDGSLLASGSADR, DSPPVSFAAFTPNAK, GPGDTDMDEASADAAIPSSTPNPTVAFR	C_400025
7	CCT δ-subunit	4	DAVTDLALHYLAK, IALIQFQISPPK, IDDIVPVR, DIERDEIELISK	C_1490029
7	CCT α-subunit	7	ILASGANVVLTTK, MAIPTESLGTETLLNTAR, ILVELAELQDSEVGDGTTSVVILAAELLK, SSLGPVGLDK, LLEVEHPAAK, GANDYMLDEMDR, NNVEAGVLEPAMSK	C_150176
7	CCT θ-subunit	11	YAEAFEVVPR, NPNNFNVDNVR, MLPGGGAPELELAR, DLSGAPDSILDLFSTK, AVNDAINTYK, GSTDGFLDDVER, MPSKFELAR, QTGLEQYAIVK, ATFGAPTTDELGFAK, DGLQTSEVVDGYQR, LFVTSDASTIVSELEVQHPAAK	C_340025
7	CCT β-subunit	4	VQDDEVGDGTTSVVVFAGELLR, AVEELASR, GASTHILDEAER, GQEVTITNDGATILK	C_1660022
8	Ash2L	2	TLEPQQNEYAK, EGDVVGMYIHLPPGGR	C_920027
9	Set4p	1	VDIPQDAVVTEFR	C_160112

the eight subunits of the cytosolic chaperonin TriC/CCT (Figure 1B). HSP70/90 and CCT assist in the folding of WD40-repeat proteins (Siegers et al., 2003) and regulate the formation of certain repressive complexes with histone deacetylase activity (Guenther et al., 2002). Thus, it is tempting to speculate that these chaperones are required for the proper folding of Mut11p and, possibly, its assembly into a protein complex(es). Intriguingly, a Mut11p-β-glucuronidase fusion protein, although predominantly localized in the nucleus, can also be detected at lower abundance in the cytosol (Zhang et al., 2002), the likely subcellular location of a HSP70/90/CCT-mediated step.

In yeast two-hybrid screens with Mut11p as a bait, two types of clones were repeatedly isolated from two *Chlamydomonas* cDNA expression libraries (Figure 2A). One class of cDNAs encoded a SET domain–containing protein, named Set1p. Based on the similarity of aligned SET motifs (Figures 2B, 2C, and 3), Set1p, a predicted polypeptide of 1795 amino acids, is related to the Trx class of HMTases (Kouzarides, 2002). This protein is encoded by *C_1710009* in the draft *Chlamydomonas* genome sequence (http://genome.jgi-psf.org/chlre2/chlre2.home.html). The second type of cDNAs coded for a polypeptide named QAp, for a long stretch of alternating Gln/Ala residues. QAp is encoded by *C_410063* and corresponds to a predicted protein of 1472 amino acids containing a Leu zipper domain (Figure 2B), typical of DNA binding proteins. The QAp C-terminal region is weakly similar to *S. cerevisiae* Ssn6, which forms a global transcriptional corepressor with Tup1. Intriguingly, Mut11p, the interacting partner of QAp, has similarity to the carboxyl end of Tup1 (Zhang et al., 2002).

We have tried to verify the interaction between Mut11p and Set1p by coimmunoprecipitation assays and by in vivo protein cross-linking with formaldehyde (Rohila et al., 2004) prior to Mut11-TAPp complex purification. However, all of our attempts have been unsuccessful (data not shown). Thus, we cannot rule out that the yeast two-hybrid interaction between Mut11p and Set1p is artifactual. Alternatively, the failure to detect Set1p in association with Mut11-TAPp could be due to technical limitations, such as low affinity interactions, compounded by the low abundance of the proteins under study. Interestingly, in animals and fungi, the Mut11p, Ash2L, and Rbbp5 homologs are conserved components of virtually all affinity-purified Trx-like HMTase complexes, suggesting that they represent strongly interacting core subunits. By contrast, other proteins, including several HMTases, appear to associate more transiently and vary in different complexes

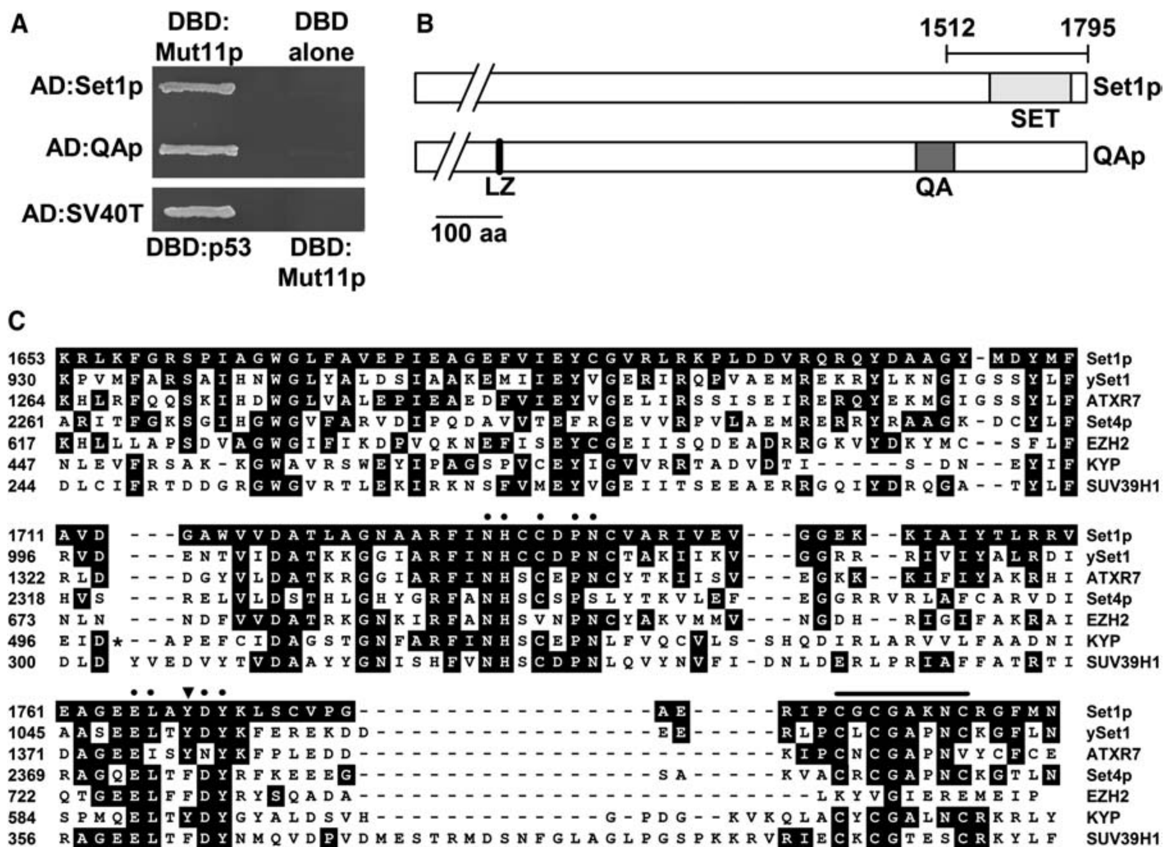

Figure 2. Mut11p Interacts in Yeast Two-Hybrid Assays with a SET Domain–Containing Protein, Set1p, and with a Putative Transcriptional Corepressor, QAp.

(A) Auxotrophic yeast cells were cotransformed with the pGBKT7 plasmid encoding the GAL4 DNA binding domain alone (DBD alone) or fused to Mut11p (DBD:Mut11p) as well as with the pGADT7-Rec vector encoding the GAL4 activation domain (AD) fused to the indicated proteins. Constructs of murine p53 (DBD:p53) and the SV40 large T-antigen (AD:SV40T) were included as a positive control. Positive protein–protein interactions were detected as growth on medium lacking His and Ade (shown) or as α-galactosidase activity (data not shown).

(B) Schematic representation of Mut11p interacting proteins. Protein lengths and position of conserved domains are shown to scale except when indicated by slashes. The Set1p fragment (Set1p*, amino acids 1512 to 1795), used in in vitro HMTase assays, is also indicated. LZ, Leu zipper motif; QA, stretch of alternating Gln/Ala residues. aa, amino acids.

(C) Sequence alignment of the SET domains of *Chlamydomonas* Set1p, Set4p, and five related HMTases: *S. cerevisiae* Set1 (ySet1), *A. thaliana* TRITHORAX RELATED 7 (ATXR7), *H. sapiens* Enhancer of Zeste Homolog 2 (EZH2), *A. thaliana* KRYPTONITE (KYP), and *H. sapiens* Suppressor of Variegation 3-9 Homolog 1 (SUV39H1). The alignment was done with ClustalX and adjusted manually. The SET domain of KYP is interrupted by 33 amino acids, which were removed to improve the alignment (denoted by the asterisk). Residues important for the HMTase catalytic core (Zhang et al., 2003) are indicated by closed circles, and the residue implicated in the Phe/Tyr switch hypothesis (Collins et al., 2005) is indicated by an arrowhead. The post-SET motif is denoted by a black bar. The Set1p sequence and invariant residues in other proteins are highlighted in black. Set1p is most similar to ySet1 (52% identity).

depending on the purification conditions (Roguev et al., 2001, 2004; Nakamura et al., 2002; Goo et al., 2003; Wysocka et al., 2003; Hughes et al., 2004; Yokoyama et al., 2004; Popovic and Zeleznik-Le, 2005). Nevertheless, our findings, taken together, clearly support an association of Mut11p with conserved HMTase complex subunits.

The Mut11p Complex(es) Possesses Histone Methylating Activity and Recombinant Set1p Methylates H3K4

Given the relatedness of Mut11-TAPp copurifying polypeptides to H3K4 HMTase complex subunits, we examined whether the Mut11-TAPp complex(es) had HMTase activity. Purified proteins were incubated with core histones and the methyl donor *S*-adenosyl-L-(methyl-^{14}C)methionine (^{14}C-SAM). In this assay, the Mut11-TAPp complex(es) displayed several activities, methylating not only H3 but also H2A and H4 (Figure 4A), even though by affinity purification we had only identified Set4p interacting with Mut11-TAPp. By contrast, no HMTase activity was detected in association with the TAP-tagged Ble control. Since SET domain-containing HMTases are quite specific in terms of the histone and Lys residue that they modify, because of the knot-like structure of the active site (Lachner et al., 2003; Zhang et al., 2003; Collins et al., 2005), these observations

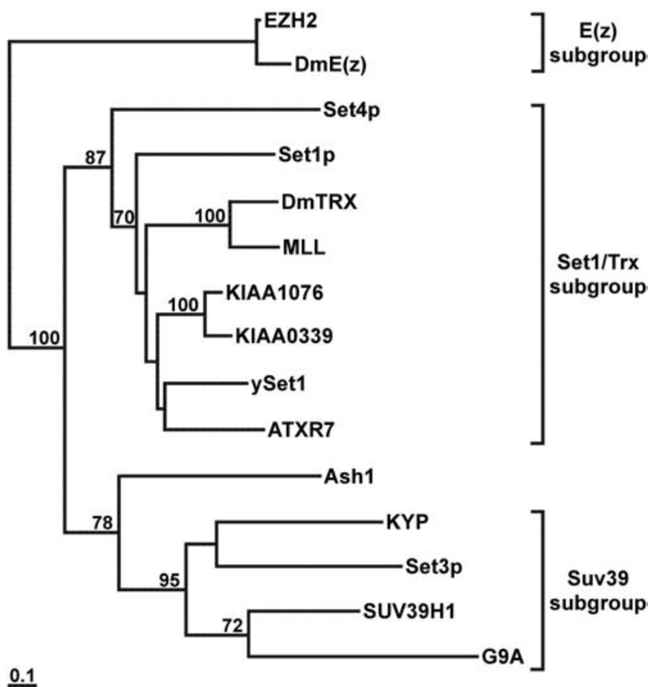

Figure 3. Unrooted Phylogenetic Tree Indicating the Relationship of *Chlamydomonas* Set1p and Set4p to SET Domain–Containing HMTases from Other Organisms.

Figures indicate bootstrap values in percentage (100% = 1000 replications).

suggest that Mut11p likely interacts with several HMTases, either as part of one supercomplex or, as described in mammals, of distinct complexes. Of the SET domain HMTases with known substrate specificity (Kouzarides, 2002; Lachner et al., 2003; Collins et al., 2005), Set4p is more similar to the Trx class but relatively weakly (Figures 2C and 3), and, thus far, we have not been able to detect any HMTase activity associated with a partly truncated recombinant protein (data not shown).

We also tested the methylating activity of *Chlamydomonas* Set1p, which clearly belongs (by sequence comparisons) to the Trx family of HMTases (Figure 3). When a C-terminal fragment of Set1p (Figure 2B) was incubated with core histones and with ^{14}C-SAM, only histone H3 was specifically methylated, albeit at a relatively low level (Figure 4B). The addition of Mut11p to the reaction mixture did not enhance the methylating activity of Set1p (Figure 4B). By N-terminal Edman sequencing of the radioactively methylated H3 we also found that Set1p specifically methylates Lys 4 of histone H3 (Figure 4C). However, the in vitro activity was too low to assess whether Set1p functions as a monomethyltransferase, dimethyltransferase, and/or trimethyltransferase by mass spectrometry analysis of modified peptides. Interestingly, a recent study of SET-containing HMTases with known product specificity suggests that a key residue in the active site determines how many methyl groups they add (Collins et al., 2005). HMTases containing Phe at the position corresponding to Y1768 of Set1p (Figure 2C, arrowhead) function preferentially as dimethyltransferases and/or trimethyltransferases, whereas those containing Tyr at this position work predominantly as monomethyltransferases and/or dimethyltransferases (Collins et al., 2005). The only known exception to this Phe/Tyr switch hypothesis is *S. cerevisiae* Set1 that has a Tyr residue at the comparable position and yet can monomethylate, dimethylate, and trimethylate H3K4 (Santos-Rosa et al., 2002; Boa et al., 2003; Collins et al., 2005). However, recent findings have indicated that an N-terminal RNA-recognition motif (RRM), perhaps regulated by interaction with additional proteins, is required for the H3K4 trimethylation activity of the yeast enzyme (Fingerman et al., 2005; Morillon et al., 2005; Schlichter and Cairns, 2005). In its absence or when mutated, *S. cerevisiae* Set1 behaves mostly as a monomethyltransferase and dimethyltransferase. A corresponding RRM domain is missing from both *Chlamydomonas* Set1p and Set4p (Figure 2B; data not shown). Thus, based on their active site sequences (Figure 2C), Set1p would be predicted to function as a monomethyltransferase and/or dimethyltransferase, while Set4p would work as a dimethyltransferase and/or trimethyltransferase.

Deletion of *Mut11* Causes Global Changes in H3K4 Methylation

Because our results suggested that gene silencing in *Chlamydomonas* requires a H3K4 HMTase complex subunit, Mut11p, we examined whether the mutant strain showed defects in H3K4 methylation. In immunoblot analyses with specific antibodies, Mut-11 displayed almost undetectable levels of H3K4me1 but significantly increased amounts of H3K4me2, in comparison with the parental strain (Figure 4D). Although the anti-H3K4me3 antibody cross-reacted with several nonhistone proteins in *Chlamydomonas*, it revealed that Mut-11 was also defective in H3K4 trimethylation (Figure 4E). We also tested for additional alterations in histone methylation since, given the multiple HMTase activities associated with Mut11-TAPp, we expected that Mut-11 would be deficient in several modifications. However, using antibodies specific for H3K9me2 or H4K20me2, we were unable to detect a signal corresponding to histones H3 or H4, respectively (data not shown). This is in agreement with previous data indicating that *Chlamydomonas* may not contain significant amounts of dimethyl H3K9 (Waterborg et al., 1995). When we examined the global levels of H3K9me1, all strains behaved similarly to the wild type (Figure 4D).

In agreement with our findings in *Chlamydomonas*, recent experiments demonstrated that small interfering RNA (siRNA)-mediated downregulation of the mammalian Mut11p homolog, WDR5, results in defects in both H3K4me1 and H3K4me3, but not in H3K4me2 (Wysocka et al., 2005). Furthermore, WDR5 was reported to play a role in the transcriptional activation of homeotic *Hox* genes, coincidental with the conversion of H3K4me2 to H3K4me3 by an associated HMTase (Wysocka et al., 2005). However, the function of H3K4 monomethylation was not examined in this study. Interestingly, lack of WDR5 has also been implicated in defects in H3K9me2 and in gene repression mediated by the clock protein PERIOD1 (Brown et al., 2005). These apparently contradictory observations could be explained if WDR5 is a core component of distinct HMTase complexes with either activating or repressing function(s) (or of a supercomplex with distinctly regulated activities), and the observed outcome (activation or

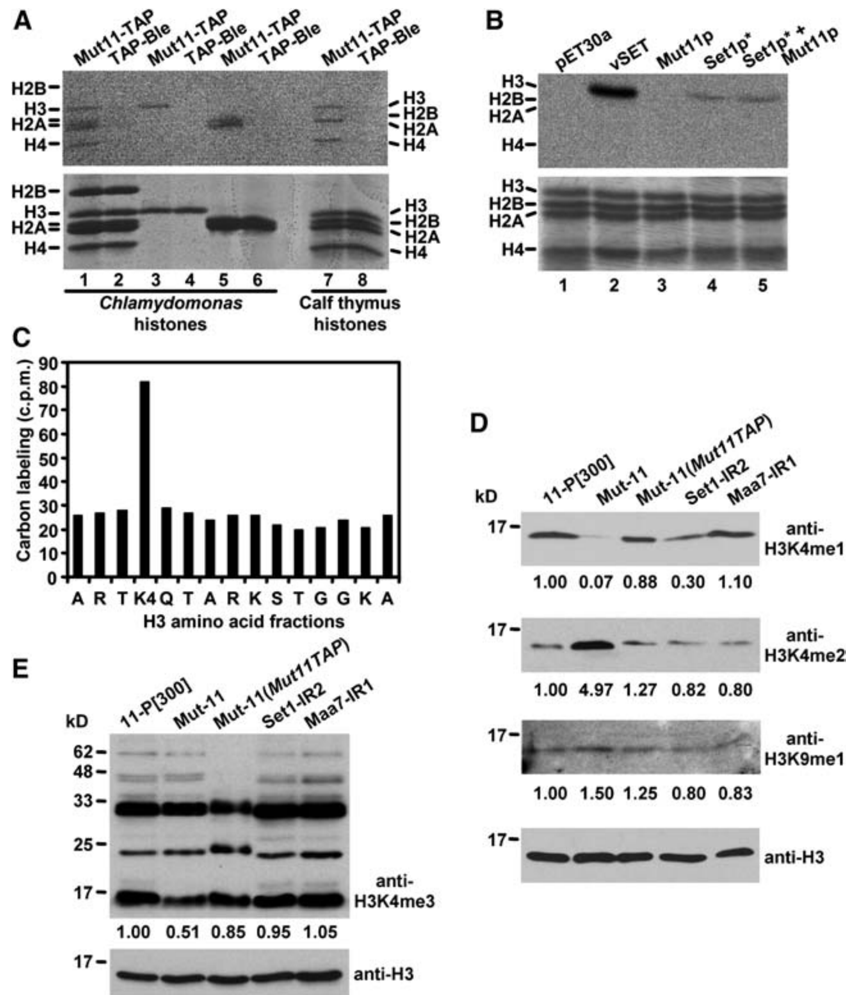

Figure 4. The Mut11-TAPp Complex(es) Methylates Histones H3, H2A, and H4, and Set1p Is an H3K4 HMTase.

(A) Proteins isolated by affinity purification with Mut11-TAPp or the TAP-Ble control were incubated with the methyl donor ^{14}C-SAM and with *Chlamydomonas* core histones (lanes 1 and 2), histone H3 (lanes 3 and 4), or histone H2A (lanes 5 and 6). Samples were resolved by SDS-PAGE and stained with Coomassie blue (bottom panel). Incorporation of the radiolabel was detected using a phosphor imager (top panel). Similar reactions were performed with calf thymus histones (lanes 7 and 8).

(B) Recombinant Set1p methylates histone H3 in vitro. A C-terminal fragment of Set1p (Set1p*, amino acids 1512 to 1795), Mut11p, and an empty vector extract (pET30a) (a negative control) were purified and used in HMTase assays with calf thymus core histones. As a positive control, we used an active SET domain protein (vSET) encoded by *Paramecium bursaria* chlorella virus 1 (Manzur et al., 2003). Samples were analyzed by Coomassie blue staining (bottom panel) followed by phosphor imager detection (top panel). Coincubation of Mut11p with truncated Set1p does not enhance the HMTase activity (cf. lanes 4 and 5).

(C) Set1p is an H3K4 HMTase. Recombinant Set1p* was purified and used in an HMTase assay with calf thymus H3 in the presence of ^{14}C-SAM. After SDS-PAGE, radioactively labeled H3 was transferred to a polyvinylidene difluoride membrane and sequenced by sequential Edman degradation. The radioactivity incorporated into each amino acid fraction, determined by liquid scintillation counting, is indicated.

(D) Immunoblot analyses of in vivo H3K4 and H3K9 methylation states. Total protein extracts from the indicated strains were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-H3K4 (top panels) or anti-H3K9 (middle panel) methylation-specific antibodies. Sample loading was calibrated based on immunoblots with an anti-H3 modification-insensitive antibody (bottom panel). Note that Mut-11(*Mut11TAP*) does not show complete reversion of the H3K4 monomethylation defect, whereas H3K4 dimethylation is very similar to wild-type levels. A control RNAi strain (Maa7-IR1) did not exhibit alterations in histone methylation. Numbers below the panels indicate relative levels of the specific histone modifications normalized to the histone H3 amount.

(E) Immunoblot analysis of H3K4 trimethylation as described in **(D)**. The anti-H3K4me3 polyclonal antibody also cross-reacts with nonhistone *Chlamydomonas* proteins of unknown identity.

silencing) depends on the gene(s) examined (see Discussion). This may also be the case with *Chlamydomonas* Mut11p. Trimethylation of H3K4 has been strongly linked to transcriptional activity in virtually all examined eukaryotes (Lachner and Jenuwein, 2002; Schneider et al., 2004). Thus, we hypothesize that Mut11p and Set4p (based on its active site sequence) may be involved in H3K4me3 and gene activation (although this awaits further experimental support). However, since we have been exploring the role of Mut11p in gene silencing, we decided to examine the function of H3K4 monomethylation, perhaps performed by Set1p and/or an equivalent HMTase.

Suppression of *Set1* Expression by RNAi Causes Gene Silencing Defects and a Global Deficiency in H3K4 Monomethylation

We tested whether RNAi-mediated *Set1* suppression induced defects in gene repression similar to those of Mut-11, potentially supporting a role for this H3K4 HMTase in silencing as well as an in vivo interaction between Mut11p and Set1p. However, since Mut11p appears to associate with several HMTase activities in *Chlamydomonas* (Figure 4A), a defect in *Mut11* may result in stronger phenotypic abnormalities than the lack of a single SET domain protein. *Chlamydomonas* 11-P[300], containing a silenced *RbcS2*:*aadA*:*RbcS2* transgene, was transformed with an inverted repeat construct (Rohr et al., 2004) designed to produce double-stranded RNA homologous to *Set1* (Set1-IR). We recovered several transformants displaying strong downregulation of *Set1* expression (Figure 5A; data not shown). These Set1-IR strains grew on spectinomycin-containing media, albeit to a different extent than Mut-11, indicating transcriptional derepression of *RbcS2*:*aadA*:*RbcS2* (Figure 5B; data not shown). Moreover, similar to our previous observations with Mut-11 (Zhang et al., 2002), Set1-IR strains also showed enhanced expression of the *TOC1* retrotransposon-like element (Figure 5C), whose transcripts are heterogeneous in size and produce a smeary signal in RNA gel blot analyses (Jeong et al., 2002). Expression of *Trxh2*, encoding a cytosolic thioredoxin isoform that is typically transcribed at low levels (Sarkar et al., 2005), was also enhanced in Mut-11 and Set1-IR2 (Figure 7A). On the contrary, transcription of a highly active gene, encoding the 40S ribosomal protein S3 (Rps3), did not change in the RNAi strains or the mutant (Figure 7A).

The RNAi-suppressed strains also showed global defects in H3K4 monomethylation. In immunoblot analyses, Set1-IR2 displayed decreased amounts of H3K4me1, whereas H3K4me2 and H3K4me3 remained at wild-type levels (Figures 4D and 4E). Likewise, other tested histone modifications were not altered in the Set1-IR strains (Figure 4D; data not shown). Taken together, these results suggested that monomethyl H3K4 may be associated with transcriptional repression, since suppression of the H3K4 HMTase Set1p caused defects in this epigenetic mark accompanied by reactivation of *RbcS2*:*aadA*:*RbcS2*, *Trxh2*, and the *TOC1* retrotransposon (Figures 5B, 5C, and 7A). However, the Mut-11 strain exhibited more severe silencing defects compared with those of Set1-IR2 and more drastic changes in histone methylation status (Figure 4D). Likewise, RNAi-mediated downregulation of expression of a *Mut11* homolog (*At3g49660*) in *Arabidopsis* caused defects not only in H3K4me1 but also a

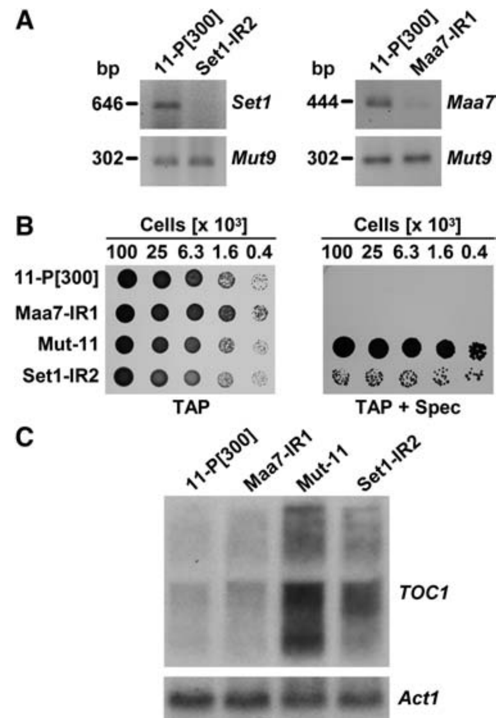

Figure 5. RNAi-Mediated Suppression of *Set1* Causes Gene Silencing Defects.

(A) RT-PCR analyses of Set1-IR2 and Maa7-IR1. These strains are transformants of 11-P[300] expressing inverted repeat (IR) transgenes designed to induce RNAi of the indicated genes. Transcript levels corresponding to *Set1* or *Maa7* (encoding Trp synthase β-subunit) are compared in 11-P[300] and each of the RNAi strains. Amplification of *Mut9* (encoding a Ser/Thr protein kinase) transcripts was used as a loading control.

(B) Growth and survival of the indicated strains on TAP or TAP + Spec. Maa7-IR1 was used as a negative control to demonstrate that expression of any inverted repeat transgene does not result in derepression of *RbcS2*:*aadA*:*RbcS2*.

(C) Reactivation of the *TOC1* retrotransposon in Set1-IR strains. The panels show an RNA gel blot of total RNA of the indicated strains probed sequentially for *TOC1* (top panel) and for *Actin1* (*Act1*, bottom panel) to test for similar loading of the lanes.

significant reduction in H3K9me2 and relaxation of heterochromatic chromocenters (J. Xu, K. van Dijk, S. Sato, T. Clemente, and H. Cerutti, unpublished data). A deficiency in H3K4me1, H3K4me3, and H3K9me2 was also reported for siRNA-mediated suppression of WDR5 in mammalian cells (Brown et al., 2005; Wysocka et al., 2005). Consequently, Mut11p-dependent silencing in *Chlamydomonas* may be mediated by more than one histone modification, although thus far we have only detected alterations in H3K4 methylation in the mutant background (see Discussion).

Monomethylated H3K4 Is Associated with Transcriptionally Repressed Loci

To determine if changes in H3K4 monomethylation were directly involved in the transcriptional reactivation of *TOC1*, *RbcS2*:

aadA:*RbcS2*, and *Trxh2*, we analyzed their chromatin environment by chromatin immunoprecipitation (ChIP) assays (Figures 6 and 7). Using primers specific for the promoter or transcribed sequences of the *TOC1* transposon, we detected a strong decrease in monomethylated H3K4 associated with these elements in both Mut-11 and Set1-IR2 (Figure 6), as compared with the parental strain (11-P[300]) where *TOC1* is repressed. Consistent with the global changes in histone H3 methylation (Figure 4D), we also found a dramatic enrichment in H3K4me2 associated with *TOC1* in Mut-11. On the other hand, no differences in H3K4 dimethylation were observed between Set1-IR2 and 11-P[300] (Figure 6). Similar patterns of H3K4me1 and H3K4me2 were identified for the *RbcS2*:*aadA*:*RbcS2* transgene (Figure 6) and the weakly expressed *Trxh2* gene (Figure 7B). We also tested by ChIP whether H3K9me2 was associated with the chromatin of the silenced loci, since this epigenetic mark has been shown to correlate with transcriptional repression in a number of eukaryotes (Richards and Elgin, 2002; Tachibana et al., 2002; Tariq and Paszkowski, 2004). However, in agreement with our immunoblotting results (see above), we could not detect H3K9me2 associated with *TOC1* in any of the strains (data not shown). Lastly, as the available antibodies for H3K4me3 exhibited cross-reactivity with additional (unknown) *Chlamydomonas* proteins (Figure 4E), we did not examine this histone modification by ChIP.

We also explored the degree of H3K4 methylation associated with the chromatin of a transcriptionally active gene, *Rps3*, encoding the highly expressed ribosomal protein S3. By real-time RT-PCR analysis, the steady state level of the *Rps3* transcript was similar in all strains (Figure 7A). ChIP assays revealed very low amounts of H3K4me1, nearly identical in all strains, in association with this gene (Figure 7C). By contrast, H3K4me2 was readily detectable, although somewhat enriched in Mut-11 (Figure 7C). Interestingly, in the wild-type strain, transcriptionally repressed chromatin (for all the genes analyzed) showed

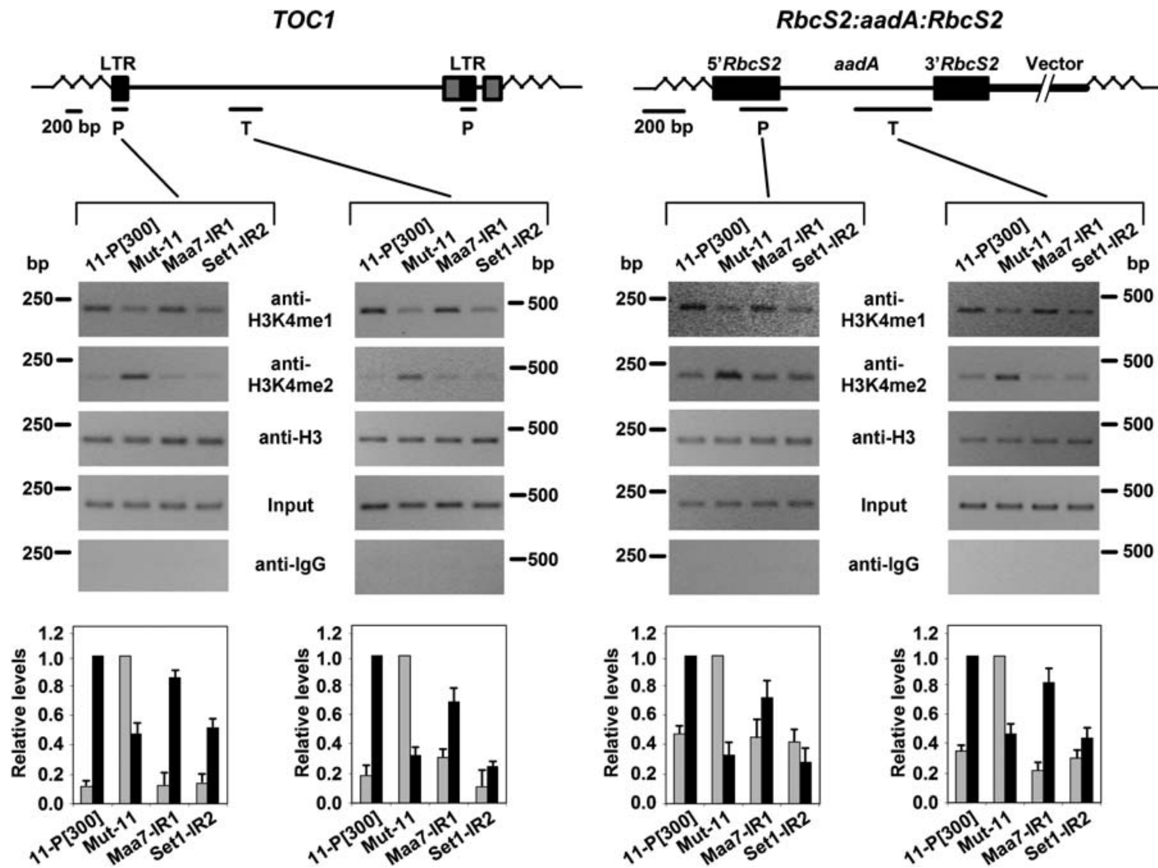

Figure 6. Monomethylated H3K4 Is Associated with the Transcriptionally Silenced *RbcS2*:*aadA*:*RbcS2* Transgene and the *TOC1* Retrotransposon.

The *TOC1* and *RbcS2*:*aadA*:*RbcS2* transcription units and the regions examined by ChIP are represented in the diagram at the top. P corresponds to the promoter or long terminal repeat (LTR) regions, whereas T indicates transcribed (coding) sequences. ChIP assays were performed with the indicated antibodies. A nonspecific anti-human IgG antibody (anti-IgG) was used as a negative control. Coimmunoprecipitated DNA was examined by PCR with specific primers. PCR reactions using 1:20 diluted input chromatin DNA are also shown (input). Signal intensities were quantified with Quantity One software (Bio-Rad) and normalized relative to the anti-H3 control reactions. Relative levels of monomethylated H3K4 (black) or dimethylated H3K4 (gray) are presented in the bar graphs, which correspond to the average of three independent experiments (± SD). For illustration purposes, the level of monomethylated H3K4 in the 11-P[300] parental strain and the level of dimethylated H3K4 in the Mut-11 mutant were set to 1.0 and the remaining samples adjusted accordingly in the bar graphs.

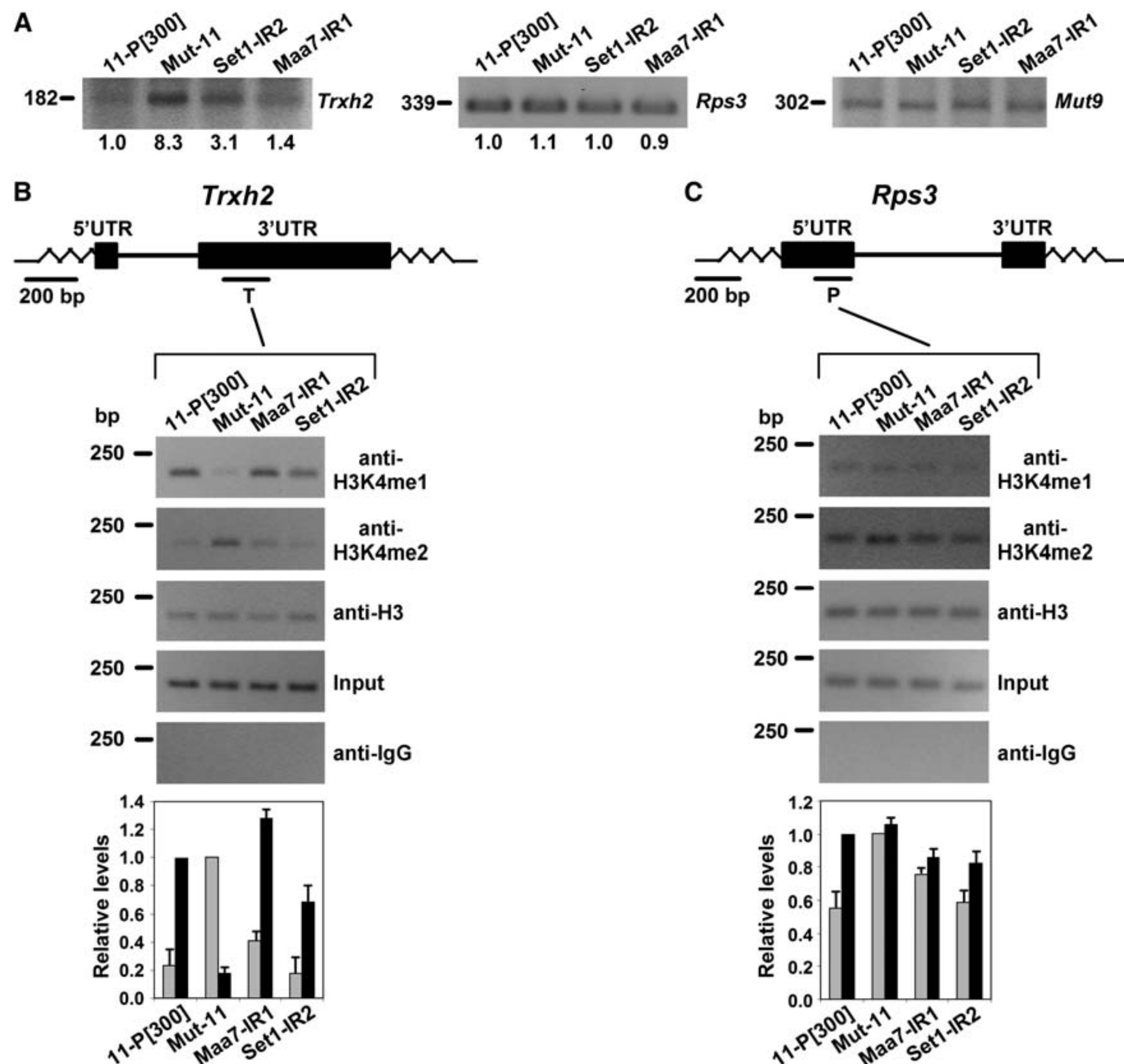

Figure 7. Monomethylated H3K4 Is Preferentially Associated with the Transcriptionally Repressed *Trxh2* Gene, whereas Dimethylated H3K4 Is Preferentially Associated with the Transcriptionally Active *Rps3* Gene.

(A) Expression of *Trxh2* (encoding cytosolic thioredoxin h2) is upregulated in the Mut-11 and Set1-IR2 strains, whereas transcript levels for the highly transcribed *Rps3* gene (encoding 40S ribosomal protein S3) remain unchanged. RNA levels corresponding to *Trxh2* or *Rps3* were analyzed in the indicated strains by real-time RT-PCR amplification. The amount of amplifiable *Mut9* (encoding a Ser/Thr protein kinase) mRNA was used as a control for similar inputs of template RNA. The numbers below the *Trxh2* and *Rps3* panels indicate relative transcript levels, normalized to the *Mut9* control, determined by real-time RT-PCR.

(B) Monomethylated H3K4 is associated with the chromatin of the weakly expressed *Trxh2* gene. The *Trxh2* transcription unit and the region examined by ChIP are represented in the diagram at the top. T indicates a transcribed but untranslated (3′UTR) sequence. The ChIP assays were performed on the indicated strains as described in the legend to Figure 6.

(C) Dimethylated H3K4 is preferentially associated with the highly active *Rps3* gene. The *Rps3* transcription unit and the region examined by ChIP are represented in the diagram at the top. P corresponds to the promoter and 5′UTR sequence. The ChIP assays were performed as described in the legend to Figure 6.

relatively high levels of monomethyl H3K4 in comparison with dimethyl H3K4 (Figures 6 and 7B). On the other hand, the highly active *Rps3* gene displayed the opposite methylation pattern with H3K4me2 being more abundant than H3K4me1 (Figure 7C). Thus, in *Chlamydomonas*, monomethyl H3K4 is clearly associated with repressed euchromatic regions, and its decrease correlates with transcriptional activation.

DISCUSSION

Chlamydomonas Mut11p is related to a subunit of H3K4 HMTase complexes, and our results clearly implicate this protein in histone methylation effects. Affinity-purified Mut11-TAPp interacts with a set of core components, including the Ash2L and Rbbp5 homologs, that are shared by almost all purified Trx-like

complexes (Roguev et al., 2001, 2004; Nakamura et al., 2002; Hughes et al., 2004; Dou et al., 2005). The isolated Mut11-TAPp complex(es) displays several HMTase activities and includes at least one detectable SET domain–containing protein, Set4p. Moreover, deletion of *Mut11* causes defects in the H3K4 monomethylation associated with derepressed transcription units and a corresponding increase in H3K4 dimethylation. The mutant strain also shows a global deficiency in H3K4 trimethylation. Although the Mut11p machinery could have an indirect effect on H3K4me1 and H3K4me3, perhaps by modifying other histone residues that stimulate the activity of a H3K4 HMTase(s), a direct role seems much more likely given the evolutionarily conserved function of Mut11p homologs as components of Trx-like complexes in fungi and animals (Roguev et al., 2001; Nakamura et al., 2002; Goo et al., 2003; Wysocka et al., 2003; Hughes et al., 2004; Yokoyama et al., 2004; Popovic and Zeleznik-Le, 2005). In addition, similar defects in H3K4me1 and H3K4me3 have recently been reported for siRNA-mediated suppression of WDR5 in mammalian cells (Wysocka et al., 2005). However, Set4p is unlikely to be the enzyme responsible for H3K4 monomethylation since, according to the Phe/Tyr switch model (Cheng et al., 2005; Collins et al., 2005), it would be expected to act predominantly as a dimethyltransferase and/or trimethyltransferase. As already mentioned, we speculate that Mut11p homologs may be components of HMTase complexes with either activating or silencing functions (see below). In this context, Set4p could conceivably be involved in H3K4 trimethylation and gene activation, or alternatively, it could modify other histone residue(s). We expect to address the Set4p substrate specificity in the future by the phenotypic and molecular characterization of strains showing RNAi-mediated suppression of *Set4*.

Several Trx-like HMTase complexes, which include the putative core subunits (Mut11p, Ash2L, and Rbbp5 homologs) but differ in the associated H3K4 methyltransferases, have been isolated from mammalian cells (Nakamura et al., 2002; Goo et al., 2003; Wysocka et al., 2003; Hughes et al., 2004; Yokoyama et al., 2004; Dou et al., 2005; Popovic and Zeleznik-Le, 2005). Likewise, the multiple activities displayed by affinity-purified Mut11-TAPp also suggest that Mut11p may associate with several methyltransferases. Indeed, it is tempting to speculate that a variable range of proteins, including potential transcription corepressors or activators and different HMTases, may interact with Mut11p homologs and confer specific histone-modifying activity(ies) to the resulting complexes. In yeast two-hybrid assays, Mut11p associates with Set1p, a Trx-like H3K4 HMTase. However, we have been unable to verify this interaction, despite repeated attempts by a variety of approaches. Thus, we cannot rule out that Set1p functions independently from Mut11p. Yet, it is also possible that the interaction between these proteins is relatively weak precluding its detection due to technical limitations. As already discussed, there is some precedence for this in the characterization of similar complexes from mammalian cells, where different components are isolated depending on the purification conditions (Nakamura et al., 2002; Hughes et al., 2004; Yokoyama et al., 2004; Popovic and Zeleznik-Le, 2005).

Recombinant Set1p methylates H3K4, and based on its active site sequence (Collins et al., 2005), it is expected to function predominantly as a monomethyltransferase and/or dimethyltransferase. Consistent with this interpretation, suppression of *Set1* by RNAi resulted in reduction of monomethylated H3K4 associated with transcriptionally repressed loci as well as gene silencing defects. These phenotypes were similar to those of the *Mut11* deletion mutant but of lower magnitude. The Set1-IR strains also failed to display the increase in H3K4me2 and the reduction in H3K4me3 observed in Mut-11. Thus, these results are ambiguous with respect to a potential interaction between Set1p and Mut11p. These proteins could be components of independent molecular machineries capable of modifying H3K4. Alternatively, they could work together, but Mut11p likely associates with additional HMTases, and its depletion results in further defects than those caused by *Set1* suppression. Interestingly, deletion of *Mut11* did not entirely abolish H3K4 monomethylation (Figures 6 and 7B), suggesting that other WD40-repeat containing proteins may partly compensate for the loss of Mut11p, a H3K4 monomethyltransferase normally associated with (or stimulated by) Mut11p may be able to act with reduced efficacy in its absence, and/or an alternative H3K4 monomethylating machinery is still functional in the *Chlamydomonas* mutant. Systematic suppression by RNAi of the putative H3K4 HMTases encoded in the *Chlamydomonas* genome (http://genome.jgi-psf.org/chlre2/chlre2.home.html) will help address some of these issues. However, independent of the specific molecular machinery(ies) responsible for H3K4 monomethylation, our results strongly suggest that this epigenetic mark is associated with transcriptionally repressed euchromatin in *Chlamydomonas*.

H3K4 Monomethylation and Euchromatin Silencing

In all eukaryotes examined to date, transcriptionally competent chromatin has been firmly linked to histone H3K4 dimethylation and/or trimethylation (Lachner and Jenuwein, 2002; Alvarez-Venegas et al., 2003; Schneider et al., 2004; Tariq and Paszkowski, 2004). Although the specific role(s) of these modifications remains poorly understood, *S. cerevisiae* H3K4me3 appears to be associated with the transition from transcript initiation to elongation and requires members of the Paf1 elongation complex, phosphorylation of Ser 5 of the C-terminal domain of RNA polymerase II, and ubiquitination of K123 on H2B (Briggs et al., 2002; Krogan et al., 2003; Ng et al., 2003; Morillon et al., 2005; Peters and Schubeler, 2005). Moreover, in metazoans, sites of H3K4me2/H3K4me3 correlate with the transcription starts of active genes, and the degree of modification correlates positively with the transcriptional rate (Schneider et al., 2004; Schubeler et al., 2004; Bernstein et al., 2005). However, in *D. melanogaster*, the H3K4 HMTase Trx is only required for transcriptional activation in the presence of repressive activity from Polycomb group proteins (Klymenko and Muller, 2004; Peters and Schubeler, 2005). This suggests that Trx-mediated H3K4 di/trimethylation functions as an antirepressor, as a mark that maintains the active state, rather than as a transcriptional activator (Peters and Schubeler, 2005). In this context, if repression is weakened, transcription can likely occur (although perhaps at relatively low rates) without an increase in H3K4me2/H3K4me3, as detected for some metazoan genes (Perkins et al., 2004; Schubeler et al., 2004) and suggested by our results with the Set1-IR strains. In more active genes, H3K4 di/trimethylation may facilitate higher

transcription rates through the maintenance of greater chromatin accessibility (Nishioka et al., 2002; Zegerman et al., 2002; Santos-Rosa et al., 2003; Pray-Grant et al., 2005). Thus, H3K4me2/H3K4me3 may be part of a positive feedback loop facilitating transcription and providing a molecular memory of transcriptional activity (Gerber and Shilatifard, 2003; Ng et al., 2003). In contrast with this wealth of data supporting a correlation between H3K4 di/trimethylation and gene activity, the role of H3K4 monomethylation has remained virtually unexplored.

In *Chlamydomonas*, Mut11p and Set1p are required for H3K4 monomethylation and the maintenance of transcriptional repression of certain genes, transgenes, and transposons. The silenced transcription units that we have examined likely correspond to euchromatic domains. The *TOC1* and *Gulliver* transposons are dispersed throughout the genome as single insertions in intergenic regions (http://genome.jgi-psf.org/chlre2/chlre2.home.html). These elements, as well as repressed single copy *RbcS2*:*aadA*:*RbcS2* transgenes, lack the heavy DNA methylation typical of heterochromatic repeats (Cerutti et al., 1997; Jeong et al., 2002; Zhang et al., 2002), and silent transgenes do not show the restricted accessibility to nucleases typical of heterochromatin (Cerutti et al., 1997). Moreover, *Mut11* and *Set1* are necessary for the repression of some euchromatic genes, such as *Trxh2*. Interestingly, when grown under normal laboratory conditions, the majority of *Chlamydomonas* chromatin appears to be in a silenced state since only ~20% of histone H3 is dynamically multiacetylated (Waterborg, 1998), an indication of transcriptional activity (Zhang et al., 2004). Moreover, recent microarray experiments have demonstrated that 60% of the *Chlamydomonas* predicted genes are not expressed during flagellar regeneration (Stolc et al., 2005).

Under normal *Chlamydomonas* growth conditions, 81.2% of histone H3 is monomethylated at K4, and 15.6% shows K9 monomethylation (Waterborg et al., 1995). Consistent with our ChIP results, the extensive amount of H3K4me1 (equivalent to the silenced chromatin fraction) strongly indicates that this modification is associated with repressed chromatin. Moreover, our findings also suggest that monomethyl H3K4 is necessary for gene silencing since a decrease in H3K4me1 in the Set1-IR strains resulted in reactivation of transcriptionally repressed loci. In the Mut-11 mutant, we observed more dramatic changes, as loss of H3K4me1 was accompanied by a substantial increase in H3K4me2, reduction in H3K4me3, and more extensive defects in gene silencing (Jeong et al., 2002; Zhang et al., 2002). In this case, we speculate that lack or diminished H3, H2A, and/or H4 HMTase activities likely results in more marked deficiencies in repressive chromatin (although potential alterations in H2A and H4 methylation remain to be explored). In addition, if Mut11p is also a component of activating HMTase complexes (as suggested by the Mut-11 defect in H3K4me3), the mutant strain may also be deficient in the activation of certain genes. Substantial alterations in chromatin states were also observed in *Arabidopsis* after RNAi-mediated suppression of a *Mut11* homolog (J. Xu, K. van Dijk, S. Sato, T. Clemente, and H. Cerutti, unpublished data), suggesting that at least some of the Mut11p-mediated processes have been evolutionarily conserved in the plant lineage.

Links between H3K4 methyltransferase subunits and gene repression have also been observed in yeast and mammals, but the actual mechanism has remained unexplained (Spada et al., 2005). In mammalian cells, the DNA methyltransferase Dnmt3a, involved in gene silencing, copurifies with both H3K9 and H3K4 HMTase activities (Fuks et al., 2003). Some mammalian H3K4 methyltransferases occur in supercomplexes containing transcriptional repressors such as Sin3 and NuRD (Nakamura et al., 2002; Wysocka et al., 2003; Xia et al., 2003). Moreover, knockdown of WDR5 has recently been demonstrated to result in several histone H3 methylation defects (including reduction in H3K4me1, H3K4me3, and H3K9me2) and alterations in both gene activation (Wysocka et al., 2005) and gene silencing (Brown et al., 2005). In budding yeast, loss of H3K4 HMTase subunits results in derepression of several genes (Carvin and Kladde, 2004) and defects in heterochromatic silencing (Bryk et al., 2002). In the latter case, H3K4 methylation has been postulated to play an indirect role in gene repression (Bernstein et al., 2002; van Leeuwen and Gottschling, 2002; Santos-Rosa et al., 2004). Indeed, H3K4me2 is depleted from silenced, heterochromatic domains and marks genome-wide active chromatin in *S. cerevisiae* (Bernstein et al., 2002). Therefore, loss of H3K4me2/H3K4me3 has been proposed to allow the nonspecific binding of silencing factors throughout the genome, resulting in reduced concentration of these proteins at normally repressed heterochromatic loci and corresponding defects in silencing (van Leeuwen and Gottschling, 2002; Santos-Rosa et al., 2004). However, this hypothesis cannot explain our results regarding euchromatic silencing in *Chlamydomonas* since monomethylated H3K4 is predominantly associated with repressed transcriptional units, and its loss correlates with gene reactivation, without a change (Set1-IR2 strain) or with an increase (Mut-11 strain) in H3K4me2. Conversely, H3K4me1 is barely detectable at constitutively active genes that, by comparison, show relatively high levels of H3K4me2.

The dual (activating and silencing) nature of the H3K4 methyl mark may be alternatively explained if monomethyl H3K4 is one of the epigenetic marks associated with euchromatic gene silencing, perhaps as part of a combinatorial set of posttranslational histone modifications (Fischle et al., 2003). In this respect, H3K4me1 has recently been found associated with the repressed *MET16* gene in *S. cerevisiae*, and its levels drop rapidly upon gene reactivation, particularly at the promoter region, coincidental with the appearance of H3K4me3 (Morillon et al., 2005). Moreover, consistent with the presence of H3K4me1 on inactive genes, this modification is independent of H2B ubiquitination or Paf1, which are required for H3K4me2 and H3K4me3 (Morillon et al., 2005). Furthermore, in mouse embryonic stem cells, initiation of X chromosome inactivation correlates with a reduction in H3K4me2/H3K4me3, but no change in H3K4 monomethylation, suggesting that this mark is compatible with a state of transcriptional repression (Kohlmaier et al., 2004). In *Chlamydomonas*, as already described, loss of monomethyl H3K4 mimics some of the molecular defects caused in mammalian and plant cells by reduced H3K9me2, a well established epigenetic silencing mark (Richards and Elgin, 2002; Tachibana et al., 2002; Tariq and Paszkowski, 2004). Thus, our results suggest functional differentiation between dimethyl H3K4 (and most likely trimethyl H3K4, although we have been unable to study this modification by ChIP due to the nonspecific cross-reaction of available

antibodies with nonhistone proteins) and monomethyl H3K4, with the latter correlating with transcriptional repression in euchromatic domains. We also hypothesize that in some eukaryotes there may be at least two kinds of Trx-like complexes containing Mut11p homologs: a repressive one that monomethylates H3K4 (and likely modifies other histone residues) and an activating one that trimethylates H3K4. In other eukaryotes, such as *S. cerevisiae*, a single complex may be able to perform monomethylation, dimethylation, or trimethylation of H3K4, but the degree of methylation is likely modulated by interacting proteins (Morillon et al., 2005).

METHODS

Chlamydomonas reinhardtii Strains and Growth Conditions

Chlamydomonas cells were routinely grown in Tris-acetate-phosphate (TAP) medium (Cerutti et al., 1997; Zhang et al., 2002). Strains 11-P[300] and Mut-11 have been described previously (Cerutti et al., 1997; Zhang et al., 2002). To suppress the expression of *Set1* or *Maa7* (encoding Trp synthase β-subunit), we used an RNAi approach explained in detail elsewhere (Rohr et al., 2004). Briefly, 11-P[300] was transformed by the glass bead method (Cerutti et al., 1997; Zhang et al., 2002) with inverted repeat transgenes designed to produce hairpin double-stranded RNA and induce the degradation of homologous transcripts. For *Set1*, a 628-bp *Sma*I-*Hinc*II fragment spanning 60 bp of coding sequence and 568 bp of the 3′ untranslated region (UTR) was inserted in sense and antisense orientation flanking a spacer sequence in vector *Maa7/X* IR (Rohr et al., 2004). The *Maa7* construct, employed as a negative control in our experiments, has already been described (Rohr et al., 2004). To test for expression of the *RbcS2*:*aadA*:*RbcS2* transgene, serially diluted cells were spotted onto selective (containing spectinomycin) and nonselective TAP medium and allowed to grow for 7 to 10 d (Cerutti et al., 1997; Zhang et al., 2002).

DNA and RNA Analyses

Standard procedures were used for DNA and RNA manipulations (Sambrook and Russell, 2001). For RT-PCR analyses, total cell RNA was isolated with TRI reagent, according to the manufacturer's directions (Molecular Research Center), treated with DNase-I (Ambion), and amplified as previously described (Rohr et al., 2004). Five-microliter aliquots of each RT-PCR reaction were resolved on 2.0% agarose gels and visualized by ethidium bromide staining. The following primer sets were used for specific amplifications: *Set1*, Set1-1 (5′-ACCGCAGCATATCCCCTTCA-3′) and Set1-2 (5′-CCGTCCACAGCGAACATATAG-3′); *Maa7*, Maa7-F (5′-TGAACATCACTGCCCCTACTC-3′) and Maa7-R (5′-CCCAGCGAGTTGTTGATCTTA-3′); *Trxh2,* Trxh2-1 (5′-TGTGCGCTAACTCGAACAAC-3′) and Trxh2-2 (5′-CCTCAATAGCAGCGAGCAT-3′); *Rps3*, C_20102codL1, (5′-GAGATTATCATCCGCGCTACTC-3′) and C_20102codR1 (5′-AAGACACCATGTAGCCATCCTT-3′); *Mut9*, Mut9-5 (5′-GCTGTACATCTCGTGCGTGT-3′) and Mut9-2 (5′-ATGGCGGTCACGTAGAAGC-3′). Real-time RT-PCR analysis was performed on the Bio-Rad iCycler iQ using SYBR Green. PCR was performed in triplicate, and the experiments were repeated twice starting with independently isolated RNA samples. After each run, a melting curve was performed to ensure that no primer dimer contaminated the quantification. Relative mRNA levels were determined using the method developed by Pfaffl (2001).

Yeast Two-Hybrid Screens

We generated two *Chlamydomonas* cDNA libraries in pGADT7-Rec, using the Matchmaker Library Construction and Screening kit (BD Biosciences). These prey plasmids contain unidirectionally cloned cDNAs expressed as fusion proteins with the GAL4 activation domain. As bait, we used a fusion protein consisting of the *Mut11* coding sequence (accession number AF443204) and the GAL4 DNA binding domain cloned into pGBKT7. The libraries were screened by interaction mating for activation of two GAL4 responsive selectable markers, *HIS3* and *ADE2*, on medium lacking His and Ade. Isolated clones were retransformed, together with the bait, into the auxotrophic yeast strain AH109 and retested for the activation of *HIS3*, *ADE2*, as well as the GAL4-regulated *MEL1* gene, encoding α-galactosidase.

Protein Expression, Purification, and Identification by Mass Spectrometry

For the isolation of a Mut11p protein complex from *Chlamydomonas*, we fused the TAP tag (Rigaut et al., 1999), modified by Rohila et al. (2004), and the coding sequence of the bleomycin gene (*ble*) (Rohr et al., 2004) to the N-terminal end of Mut11p. This construct was placed under the control of *RbcS2* regulatory sequences (Cerutti et al., 1997), allowing constitutive expression, and transformed into Mut-11. Transformants were selected on TAP medium containing 4 μg/mL of bleomycin and verified by DNA gel blot hybridization. Protein expression was examined by protein gel blotting with a rabbit anti-Ble antibody (Cayla) using standard procedures (Sambrook and Russell, 2001). To purify Mut11-TAPp–associated polypeptides, transgenic Mut-11(*Mut11TAP*) strains were grown to midlogarithmic phase in TAP medium containing 0.7 μg/mL of bleomycin and collected by centrifugation. For each experiment, 4×10^{10} cells were resuspended in IPP150 lysis buffer (Rigaut et al., 1999), containing 2 μL/mL of plant protease inhibitor cocktail (Sigma-Aldrich), and broken by two passages through a French-press at 5000 psi. Subsequent purification steps were performed as previously described by Rigaut et al. (1999). Isolated proteins were fractionated by SDS-PAGE, stained with Sypro Ruby (Bio-Rad), trypsin digested in gel, and identified by tandem mass spectrometry (Rohila et al., 2004). For the expression of recombinant proteins in *Escherichia coli*, we used the pET system, according to the manufacturer's protocol (Novagen). DNA fragments encoding the C-terminal 1512 to 1795 amino acids of Set1p (amplified by RT-PCR and verified by sequencing) and the full-length Mut11p coding sequence were subcloned in pET30a, expressed in Rosetta DE3, and purified on nickel-nitrilotriacetic acid agarose His binding columns following standard recommendations (Novagen).

HMTase Assays

The histone methylating activity of the affinity-purified Mut11-TAPp complex or of recombinant proteins, isolated from *E. coli*, was tested as described (Rea et al., 2000). Briefly, Mut-11(*Mut11TAP*) protein lysates were directly incubated with 200 μL Calmodulin-Sepharose (Stratagene). These beads were subsequently washed with 25 mL Calmodulin binding buffer (Rigaut et al., 1999) followed by 5 mL of methylase activity buffer (Rea et al., 2000). Proteins retained on the sepharose beads were incubated, in 40 μL of methylase activity buffer, with 250 nCi of the methyl donor ^{14}C-SAM (Amersham) and 10 μg of *Chlamydomonas* core histones (Waterborg et al., 1995) or 10 μg of calf thymus histones (Roche) (Rea et al., 2000). After 60 min at 30°C, samples were resolved by SDS-PAGE on 15% gels, stained with Coomassie Brilliant Blue, and dried onto filter paper. The incorporated radioactivity was detected with a phosphor imager (Amersham). A similar protocol was used to examine the activity of purified recombinant proteins.

N-Terminal Edman Sequencing

Calf thymus histone H3 (Roche) was methylated as described above using ^{14}C-SAM (Amersham) as the methyl donor. After separation by

SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and stained with Coomassie Brilliant Blue, and the histone band was excised and sequenced by sequential Edman degradation at the University of Nebraska Medical Center Protein Structure Core Facility. The residue containing the incorporated radiolabel was identified by scintillation counting of the amino acid fractions.

Protein Gel Blot Analysis

To examine the histone methylation status in vivo, 2.5×10^6 cells from each strain were pelleted and frozen in liquid nitrogen. Cell pellets were directly boiled in SDS gel loading buffer, and the total protein extracts were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Specific methylated states of histone H3 Lys residues were detected with antibodies against monomethyl H3K4 (Abcam, ab8895), dimethyl H3K4 (Upstate, 07-030), trimethyl H3K4 (Abcam, ab8580), monomethyl H3K9 (Upstate, 07-395), or dimethyl H3K9 (Upstate, 07-212). A modification-insensitive anti-H3 antibody (Abcam, ab1791) was used to adjust sample loading. The methylation status of histone H4 was examined with a rabbit antiserum that recognizes dimethylated H4 Lys 20 (Upstate, 07-367). Reacting proteins were detected by chemiluminescence with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma-Aldrich).

ChIP Assays

To determine the methylation status of histone H3 at specific chromosomal loci, 2×10^7 TAP-grown cells were treated with 1% formaldehyde for 10 min followed by 0.1 M Gly for 5 min. Cells were then pelleted, washed, and frozen in liquid nitrogen. The cell pellet was resuspended in 2 mL ChIP lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 2 μL/mL of plant protease inhibitor cocktail [Sigma-Aldrich], 0.5 mM PMSF, and 2 mM benzamidine), 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 (determined experimentally) and subjected to immunoprecipitation using a ChIP assay kit (Upstate). Immunoprecipitations were performed with antibodies against H3 (5 μL, Abcam ab1791), monomethylated H3K4 (20 μL, Abcam ab8895), dimethylated H3K9 (15 μL, Upstate 07-212), dimethylated H3K4 (5 μL, Upstate 07-030), or human IgG (2 μL at 6 μg/μL, Sigma-Aldrich I-2511). Immunoprecipitations, histone/DNA complex elutions, and reversions of cross-linking were performed according to the manufacturer's instructions (Upstate). DNA was recovered by phenol/chloroform extraction and ethanol precipitation and resuspended in 30 μL of TE buffer. Standard PCR analysis (Sambrook and Russell, 2001) was performed on 1:10, 1:20, and 1:50 dilutions of the input and antibody precipitated DNA (using samples precipitated with human IgG as negative controls). The amount of immunoprecipitated DNA used in each reaction was adjusted empirically based on the quantity needed to amplify equal amounts of each specific PCR fragment from the anti-H3 antibody samples. The number of PCR cycles was also optimized to show a linear relationship between input DNA and band intensity of the final products. PCR fragments were resolved by electrophoresis on 2% agarose gels, images acquired with the Bio-Rad Gel Doc system, and quantitation performed with the Quantity One software (Bio-Rad). The primer pairs used were as follows: *aadA* promoter (aadA-ProL, 5′-TGATGTTTGATGGGGTATT-TGA-3′; aadA-ProR, 5′- GAGTCGATACTTCGGCGATAAC-3′); *aadA* coding region (aadA-CodL, 5′-TCTGGCTATCTTGCTGACAAAA-3′; aadA-CodR, 5′-TAGTGATCTCGCCTTTCACGTA-3′); *Trxh2* 3′UTR (Trxh2-1, 5′-TGTGCGCTAACTCGAACAAC-3′; Trxh2-2 5′-CCTCAATAGCAGCGAG-CAT-3′); *TOC1* LTR (TOC1-LTR-5′, 5′-ATCGCAGCGCAGTGGAGAT-3′; TOC1-LTR-3′, 5′-GTCCCGGCAGAGGGACGAT-3′); *TOC1* internally transcribed sequence (TOC1-5′, 5′-TTCGTTGTACATGCCTTTGC-3′; TOC1-3′, 5′-TCA-CTGAGGGCGAAATATCC-3′); *Rps3* promoter (C_20102proL1, 5′-AAGG-GCGCTGCTAGTATAACCA-3′; C_20102proR1, 5′-CCTTTGTTCCCGA-GAGAGAGAA-3′).

Phylogenetic Analysis

SET domain sequences were aligned using ClustalX (Thompson et al., 1994) with default gap penalties (see Supplemental Table 1 online). An unrooted phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) from the PHYLIP version 3.6b package (kindly distributed by J. Felsenstein, University of Washington, Seattle, WA).

Accession Numbers

Sequence data for this article can be found in the EMBL/GenBank data libraries under the following accession numbers: human EZH2, gi:21361095; *D. melanogaster* DmE(z), gi:29429136; *D. melanogaster* DmTRX, gi:12644002; human MLL, gi:5174569; human KIAA1076, gi: 41149776; human KIAA0339, gi:27500172; *S. cerevisiae* ySet1, gi: 6321911; *A. thaliana* ATXR7 (At5g42400), gi:30694058; *D. melanogaster* Ash1, gi:17737643; *A. thaliana* KYP (At5g13960), gi:30580520; human SUV39H1, gi:4507321; human G9a, gi:18375637; and *Chlamydomonas* Set3p, AY702654.

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