Mutation of Arabidopsis SMC4 identifies condensin as a corepressor of pericentromeric transposons and conditionally expressed genes

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In eukaryotes, transcriptionally inactive loci are enriched within highly condensed heterochromatin. In plants, as in mammals, the DNA of heterochromatin is densely methylated and wrapped by histones displaying a characteristic subset of post-translational modifications. Growing evidence indicates that these chromatin modifications are not sufficient for silencing. Instead, they are prerequisites for further assembly of higher-order chromatin structures that are refractory to transcription but not fully understood. We show that silencing of transposons in the pericentromeric heterochromatin of Arabidopsis thaliana requires SMC4, a core subunit of condensins I and II, acting in conjunction with CG methylation by MET1 (DNA METHYLTRANSFERASE 1), CHG methylation by CMT3 (CHROMOMETHYLASE 3), the chromatin remodeler DDM1 (DECREASE IN DNA METHYLATION 1), and histone modifications, including histone H3 Lys 27 monomethylation (H3K27me1), imparted by ATXR5 and ATXR6. SMC4/condensin also acts within the mostly euchromatic chromosome arms to suppress conditionally expressed genes involved in flowering or DNA repair, including the DNA glycosylase ROS1, which facilitates DNA demethylation. Collectively, our genome-wide analyses implicate condensin in the suppression of hundreds of loci, acting in both DNA methylation-dependent and methylation-independent pathways.

[Keywords: DNA methylation; SMC proteins; chromosome condensation; epigenetic regulation; gene silencing; heterochromatin formation]

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Condensins are multisubunit protein complexes named for their ability to catalyze ATP-dependent condensation of newly replicated chromosomes (Wood et al. 2010; Hirano 2016; Uhlmann 2016). Two condensin subtypes [I and II] have at their core a heterodimer of the STRUCTURAL MAINTENANCE OF CHROMOSOMES (SMC) ATPases SMC2 and SMC4, which are highly conserved and essential for viability. Three additional subunits, each having paralogs that differ in condensins I and II, interact with the SMC2–SMC4 heterodimers, forming pentameric complexes that can topologically entrap DNA sequences brought together by looping, loop stacking, or other long-range interactions, thereby compacting the DNA. In addition to roles in mitosis, condensins affect genome organization and recombination and DNA repair, with a number of studies also implicating condensin in the repression of specific genes [Lupo et al. 2001; Bhatta et al. 2002; Dej et al. 2004; Machin et al. 2004; Meyer 2010; Wood et al. 2010, Rawlings et al. 2011, Jeppsson et al. 2014, He et al. 2016].

Much of what is known about condensin’s effects on gene regulation stems from studies conducted using yeast,
Drosophila, or Caenorhabditis elegans, whose genomes lack appreciable DNA methylation. However, genomic cytosine methylation is common in eukaryotes, including plants and mammals [Law and Jacobsen 2010]. The majority of cytosine methylation occurs at CG motifs and is accomplished by orthologous enzymes in mammals and plants; namely, the cytosine methyltransferases DNMT1 [DNA METHYLTRANSFERASE 1; mammals] or MET1 [plants]. CG motifs are symmetrical in duplex DNA, and hemimethylated pairs of CG motifs are recognized by UHRF [mammals] or VIM [plants] proteins to facilitate DNMT1 or MET1 recruitment, thereby maintaining methylation on both strands [Bostick et al. 2007; Woo et al. 2007; Hashimoto et al. 2008]. CHG methylation (where H is A, C, or T) is also symmetric and can be maintained in Arabidopsis by CMT3 [CHROMOMETHYLASE 3]. CMT3 has a chromodomain that binds histone H3 Lys9 (H3K9) dimethylated by SUVH4 [or related paralogs], and SUVH4 in turn binds methylated CHG, allowing CHG methylation and H3K9 methylation (H3K9me) to specify and maintain one another [Law and Jacobsen 2010].

Pericentromeric regions account for most of the constitutive heterochromatin in Arabidopsis [Fransz et al. 2002; Zhang et al. 2006]. These regions are transposon-poor and gene-poor, with dense CG maintenance methylation required to keep the transposons inactive [Soppe et al. 2002; Lippman et al. 2004; Simon et al. 2015]. However, transposons located elsewhere, particularly in the mostly euchromatic chromosome arms, require additional methylation by DRM2, the ortholog of mammalian DNMT3 enzymes. DRM2 methylates cytosines in CG, CHG, or CHH motifs in an RNA-directed manner [Cao and Jacobsen 2002, Cao et al. 2003; Zemach et al. 2013; Matzke and Mosher 2014; Wendte and Pikaard 2017]. In some contexts, primarily pericentromeric heterochromatin, CHH methylation can be maintained by the DNA methyltransferase CMT2 [Zemach et al. 2013; Stroud et al. 2014]. CG, CHG, and CHH maintenance methylation has at least one thing in common, namely, the need for the chromatin remodeling ATPase DDM1 [DECREASE IN DNA METHYLATION 1] [Jeddeloh et al. 1999; Brzeski and Jerzmsowski 2003], which enables maintenance methylation within regions of dense heterochromatin enriched for linker histone H1 [Zemach et al. 2013].

Here, we report a hitherto unrecognized role for condensins I and II in methylation-dependent repression of pericentromeric transposons whose silencing depends on MET1, CMT3, DDM1, and the H3K27 monomethylases ATXR5 and ATXR6 [Jacob et al. 2009, 2010]. Cytosine methylation is not appreciably altered in smc4 mutants, suggesting that condensin is not required for DNA methylation but acts in conjunction with DNA methylation to assemble higher-order repressive chromatin complexes. We also show that SMC4/condensin does not act solely at heavily methylated loci of pericentromeric regions but also represses sparsely methylated, conditionally expressed genes throughout the chromosome arms, suggesting a broad role in shaping the Arabidopsis epigenome.

Results

Overexpression of the NRPE1 C-terminal domain (CTD) results in defective RNA-directed DNA methylation (RdDM)

Our finding that SMC4/condensin regulates gene silencing came about unexpectedly through studies of RdDM, which involves two specialized multisubunit RNA polymerases: polymerase IV [Pol IV] and Pol V [Haag and Pikaard 2011; Matzke and Mosher 2014; Zhou and Law 2015; Wendte and Pikaard 2017]. We found that overexpressing the CTD of the Pol V largest subunit, NRPE1, causes a dominant-negative phenotype resembling nrpe1 loss-of-function mutants [Fig. 1]. For instance, at 45S and 5S rRNA gene loci, Pol IV-dependent 24-nucleotide [nt] siRNAs that are diminished in a nrpe1 mutant are similarly reduced in the NRPE1 CTD overexpression line CTD-OX [Fig. 1A]. Likewise, RdDM at AtSN1 and SoloLTR retrotransposons is lost in the nrpe1-11 mutant and greatly reduced in CTD-OX plants, making the DNA of these elements susceptible to HaeIII or AluI digestion such that PCR amplification fails [Fig. 1B].

Knocking out both DRM2-mediated RdDM and CHG methylation by CMT3 causes overexpression of the F-box gene SDC, resulting in plants with elongated twisted leaves [Henderson and Jacobsen 2008]. We found that cmt3 CTD-OX double mutants, like drm1 drm2 cmt3 mutants, display strong SDC expression [Fig. 1C] and the characteristic twisted leaf phenotype [Fig. 1D]. AtSN1 and soloLTR retrotransposons are also highly expressed in CTD-OX plants, as in nrpe1 mutants [Fig. 1C]. We assayed endogenous NRPE1 mRNA expression levels using both RT–PCR and quantitative RT–PCR [qRT–PCR] assays, detecting an increase in the nrpd1-3 [pol IV] mutant but no change in NRPE1 mRNA levels in CTD-OX plants [Fig. 1E, Supplemental Fig. S1A], suggesting CTD-OX interference with RdDM at a step downstream from NRPE1 transcription.

Evidence that the CTD-OX transgene induces RNAi

Using a homozygous cmt3 CTD-OX line in which all progeny displayed the SDC phenotype, we conducted a suppressor screen. Seeds were subjected to EMS mutagenesis, and rare plants with a wild-type phenotype were recovered using a homozygous cmt3 CTD-OX line [Fig. 1A]. Likewise, RdDM at AtSN1 and SoloLTR retrotransposons is lost in the nrpe1-11 mutant and greatly reduced in CTD-OX plants, making the DNA of these elements susceptible to HaeIII or AluI digestion such that PCR amplification fails [Fig. 1B].

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Using a homozygous cmt3 CTD-OX line in which all progeny displayed the SDC phenotype, we conducted a suppressor screen. Seeds were subjected to EMS mutagenesis, and rare plants with a wild-type phenotype were identified in the next generation [M2]. These plants no longer expressed SDC, as illustrated in Figure 1F for four such mutants [m17, m65, m71, and m73]. In the mutants, AtSN1 and soloLTR elements that had been derepressed in the cmt3 CTD-OX parental line [a pol V or RdDM mutant phenotype], were resilenced [Fig. 1F] and remethylated [Fig. 1G], and the CTD transgene remained expressed [Fig. 1F; Supplemental Fig. S1B], ruling out its silencing as the basis for the suppressor phenotype.

Evidence that recovered mutants were affecting more than one activity came from analyses of small RNAs. These included 24-nt siRNAs matching 5S rRNA genes or AtSN1 elements, 21-nt secondary siRNAs generated from TAS1, TAS2, or TAS3 noncoding RNAs [Allen et al. 2005], or a 21-nt microRNA [miR160] [Fig. 2A]. In
Figure 1. Overexpression of the Pol V largest subunit CTD induces a dominant-negative RdDM phenotype suppressed in EMS-induced mutants. (A) RNA blot analysis of small RNA of wild-type (Col-0), pol IV mutant (nrpd1), pol V mutant (nrpe1-11), or CTD-OX plants. The blot was sequentially probed for small RNAs matching the 45S rRNA gene promoter, 5S rRNA gene intergenic spacer (siR1003), or microRNA mir166. An image of the ethidium bromide (EtBr)-stained gel is shown at the bottom. (B) Analysis of AtSN1 and SoloLTR transposon DNA methylation levels using Chop-PCR. Genomic DNA of wild-type Col-0, Pol V mutant (nrpe1-11), or CTD-OX plants was digested [chopped] with the indicated methylation-sensitive endonucleases (the sequence context of queried cytosines are shown in parentheses) or left uncut as a control and then amplified using PCR primers specific for AtSN1 or soloLTR retrotransposons. PCR products were resolved by agarose gel electrophoresis and visualized with EtBr staining. (C) RT–PCR analyses of SDC, CTD, AtSN1, and soloLTR expression levels relative to a ubiquitin (UBQ) control. The genotypes of plants tested are indicated at the top of each lane. Reactions in which reverse transcriptase was omitted (no RT) control for DNA contamination. (D) CHG and CHH methylation-deficient cmt3 CTD-OX plants display the SDC overexpression phenotype. (E) RT–PCR analysis of CTD and native NRPE1 expression. The genotypes of plants tested are indicated at the top of each lane. Reactions lacking reverse transcriptase (no RT) control for DNA contamination. UBQ reactions control for the amount of RNA tested. (F) RT–PCR analysis of SDC, AtSN1, soloLTR, and CTD expression in the cmt3 CTD-OX parental line and in the suppressor mutants m17, m65, m71, and m73. The nrpe1-11 (pol V) mutant served as control for derepression of AtSN1 and SoloLTR elements silenced by RdDM in wild type (Col-0). UBQ served as a loading control. Reactions without reverse transcriptase (no RT) served as controls for DNA contamination. (G) Analysis of AtSN1 and SoloLTR DNA methylation levels using Chop-PCR. Assays were conducted as in B, comparing wild-type Col-0 with the indicated mutants.

Although NRPE1 expression levels are not appreciably affected by the CTD-OX transgene [see Fig. 1E; Supplemental Fig. S1], the fact that rdr6, sgs3, and ago1 mutations prevent SDC, AtSN1, and soloLTR overexpression in cmt3 CTD-OX plants strongly implicated 21-nt siRNA-mediated RNAi as the basis for CTD-OX action. This led us to examine whether 21-nt siRNAs matching the NRPE1 CTD are produced in cmt3 CTD-OX plants but lost in the mutants. Indeed, this is the case, as shown in Figure 2C.

A missense allele of the condensin subunit gene SMC4 suppresses the SDC phenotype

Unlike the rdr6 mutant (m17), mutant m73 showed no loss of 21-nt tasiRNAs [Fig. 2A] yet had lost 21-nt siRNAs matching the NRPE1 CTD [Fig. 2C], suggesting a defect in transgene-induced RNAi but not tasiRNA biogenesis. The recessive nature of the m73 mutation and the dominant nature of the CTD-OX transgene for 21-nt siRNA production were evident upon crossing m73 with wild-type Col-0, yielding F1 progeny expressing high levels of 21-nt siRNAs matching the NRPE1 CTD [Fig. 2C].

Using bulked-segregant analysis coupled with deep genome sequencing, the causative mutation in m73 was identified as a C-to-T transition in the SMC4 gene (AT5G48600), defining the allele smc4-1. The missense mutation in smc4-1 results in a proline-to-serine substitution at amino acid 22 [P22S] at the edge of the conserved
ATPase domain [Fig. 2D]. Because a T-DNA insertion allele of smc4 is embryonic-lethal when homozygous (Siddiqui et al. 2006; Smith et al. 2014), smc4-1 is likely a hypomorphic allele.

To confirm that the smc4-1 mutation is causative, we transformed the m73 mutant with a transgene expressing a full-length SMC4 gene [gSMC4], which restored SDC expression [Fig. 2E]. Likewise, AtSN1 and soloLTR elements that had been silenced in the smc4-1 [m73] mutant were again derepressed as in the starting cmt3 CTD-OX line [Fig. 2E] or in nrpd1 [pol IV] or nrpe1 [pol V] mutants.

Collectively, the results of the mutant screen are best explained by the interpretation that aberrant RNAs generated by the single-copy CTD-OX transgene give rise to RDR6- and SGS3-dependent 21-nt siRNAs that disrupt Pol V activity via RNAi. This impairs RdDM, resulting in SDC, AtSN1, and soloLTR expression. In the RNAi mutants, Pol V activity is no longer disrupted. We deduce that SMC4, as a key subunit of condensin, is somehow required for the CTD-OX transgene to produce aberrant RNAs.

To circumvent the uncertainties associated with the cmt3 CTD-OX genetic background, we outcrossed m73 to wild-type Col-0 and identified F2 progeny that were homozygous for smc4-1, homozygous wild-type for CMT3, and devoid of the CTD-OX transgene. These smc4-1 homozygotes were further backcrossed twice to wild-type Col-0, yielding a homozygous line referred to here as smc4-1 [Col-0] or simply smc4-1. This line was used for all subsequent assays.

SMC4 is required for pericentromeric transposon silencing and chromocenter condensation

Genome-wide effects of SMC4 on gene expression were investigated by conducting mRNA deep sequencing comparing smc4-1 with wild-type plants. The most striking finding was that hundreds of transposable elements [TEs] are derepressed to high levels in smc4-1, as categorized by Zemach et al. (2013) (Fig. 3C). Strikingly, TE expression in the indicated genotypes, including the RdDM mutants but not in RdDM or cmt3 mutants, was also represented.

Using RT–PCR, we confirmed the derepression in smc4-1 of several TEs identified by RNA sequencing [RNA-seq], comparing these with soloLTR, which is silenced by RdDM, while also testing a variety of mutants, including the RdDM mutants nrpd1 [pol IV], nrpe1 [pol V], and drm1 drm2 or the maintenance methylation mutants met1, cmt2, and cmt3 [Fig. 3D]. Strikingly, TEs derepressed in smc4-1 are also derepressed in met1 or cmt3 mutants but not in RdDM or cmt2 mutants [Fig. 3D]. Collectively, these results suggest that condensin is needed in addition to CG and CHG methylation for silencing of TEs located in pericentromeric heterochromatin. Consistent with this interpretation,
heterochromatic chromocenters become decondensed in smc4-1 mutants as in met1, cmt3, or ddm1 mutants (Fig. 3E; Supplemental Fig. S3; see also Soppe et al. 2002).

MET1, DDM1, H3K9 dimethylation (H3K9me2), H3K27 monomethylation (H3K27me1), and SMC4 silence overlapping subsets of TEs

To examine how transposons silenced by SMC4 overlap with transposons silenced by CG or CHG methylation, we compared smc4-1 mRNA-seq data with published (Stroud et al. 2013) met1, ddm1, cmt3, or suvh4/5/6 mRNA-seq data sets (Fig. 4). Two-thirds of all TEs derepressed fourfold or more in met1 are also derepressed in ddm1 mutants (636 of 956) [Fig. 4A], as reported previously [Stroud et al. 2013]. Fewer TEs are derepressed in smc4-1 [286] than in met1 [956], but 63% [181 of 286] of these SMCE-dependent TEs overlap with TEs derepressed in met1, which is highly significant [P = 3.60 × 10^{-20}] given an expectation of only 3.1% overlap by chance. It is important to note that smc4-1 mutants are viable, whereas a smc4 T-DNA insertion is lethal. Thus, the number of TEs up-regulated in smc4-1 as a loss-of-function allele but not a null allele may underestimate the full effects of SMC4. Fifty-nine percent [169 of 286] of the TEs derepressed in smc4-1 are derepressed in both met1 and ddm1 [Fig. 4A], which is also highly significant [P value of essentially 0] given an expected overlap [by chance] of only 0.08%.

In cmt3-11 mutants, only 32 TEs are derepressed fourfold or more, however, 47% [15 of 32] of these overlap with TEs derepressed in smc4-1 [Fig. 4B]—significantly more than the 0.92% expected by chance [P = 9.27 × 10^{-23}]. Moreover, 31% [10 of 32] of the TEs derepressed in cmt3 overlap with TEs derepressed in smc4-1, met1, and ddm1 [Fig. 4B,C]—significantly more than the 0.0007% expected by chance [P = 1.99 × 10^{-44}].

Comparing smc4-1 data with published nrpe1 (pol V) mRNA-seq data [Blevins et al. 2014], only one derepressed TE was common to both data sets [Supplemental Fig. S4A], which is not statistically significant [P = 0.25]. TEs derepressed in smc4-1 also showed no further increase in expression in smc4-1 nrpe1 or smc4-1 nrpd1 double mutants [Supplemental Fig. S4B]. Collectively, the results implicate condensin in MET1-, CMT3-, and DDM1-dependent transposon silencing but not silencing by RdDM.

Turning from DNA to histone methylation, 139 TEs are derepressed in a triple mutant for the H3K9 dimethylases SULVH4, SULVH5, and SULVH6. Of these TEs, 26.6% [37 of 139] are also derepressed in smc4-1 [Fig. 4D], a
significant fraction ($P = 1.2 \times 10^{-43}$), indicating that a subset of TEs requires both H3K9me2 and SMC4 for silencing. Using the H3K9me2 ChIP-seq (chromatin immunoprecipitation [ChIP] combined with high-throughput sequencing) data of Stroud et al. (2014), we plotted the density of H3K9me2 (relative to total H3) at the 286 TEs derepressed in $smc4-1$, comparing these elements with a training set of 313 TEs randomly selected from the Arabidopsis thaliana genome (see Supplemental Tables S5, S6 for the lists of transposons examined).

SMC4-dependent TEs show H3K9me2 enrichment throughout the elements in wild-type plants (Supplemental Fig. S5A) and lose this enrichment in $suvh4$ $suvh5$ $suvh6$ triple mutants (Supplemental Fig. S5B). In contrast, the 313 randomly selected TEs show a lesser degree of H3K9me2 enrichment that is independent of $SUVH4/5/6$. Interestingly, genes whose repression involves SMC4 show no enrichment for H3K9me2 [Supplemental Fig. S5A,B], indicating that not all SMC4 targets are enriched for H3K9me2.

The H3K27 monomethylases ATXR5 and ATXR6 are functionally redundant paralogs important for the stability and silencing of pericentromeric heterochromatin [Jacob et al. 2009, 2010]. TEs derepressed in atxr5/6 double mutants show substantial overlap with TEs derepressed in $ddm1$ or $met1$ (Stroud et al. 2012). The H3K27me1 mark is enriched within the bodies of SMC4-dependent TEs as well as randomly selected TEs but not genes [Supplemental Fig. S5C]. Therefore, we compared TEs derepressed fourfold or more in atxr5/6 with the 169 TEs whose repression involves SMC4, $MET1$, and $DDM1$-dependent, revealing a 37% (62 of 169) overlap [Fig. 4E], which is significantly higher than the 0.32% expected by chance ($P = 4.34 \times 10^{-119}$).

Collectively, the overlap between H3K9me2, H3K27me1, and TEs repressed by SMC4 correlates with the enrichment of all three features in pericentromeric regions, as shown for chromosome 1 in Figure 4F.
DNA methylation and siRNA accumulation are unaltered in smc4-1 mutants

Because SMC4/condensin partners with cytosine methylation proteins in TE repression, we conducted genome-wide bisulfite sequencing to test whether SMC4 is required for DNA methylation. Transposons that are derepressed in smc4-1 mutants are heavily methylated in all sequence contexts, as shown in the heat maps of Figure 5A. Their CG methylation is almost completely lost in met1 mutants, their CHG methylation is greatly diminished in cmt3 mutants, and their CHH methylation is depleted in cmt2 mutants (Fig. 5A). Methylation in all three of these sequence contexts is also substantially reduced in ddm1 mutants but not in drm1 drm2 or nrpe1 (pol V) mutants, consistent with maintenance methylation rather than RdDM. Importantly, DNA methylation is not appreciably affected in any sequence context in smc4-1 mutants either at TEs regulated by SMC4 (Fig. 5A) or genome-wide (Supplemental Fig. S6A). This is also apparent upon examining methylation patterns at individual loci, as shown for three TE loci in Figure 5B.

Recall that transgene-induced 21-nt siRNAs matching the NRPE1 CTD region in the cmt3 CTD-OX line were absent in the m73 mutant. This led us to conduct small RNA deep sequencing [small RNA-seq] to see whether small RNA levels are affected by smc4-1 genome-wide. We detected no change in siRNA levels relative to wild-type Col-0 in the smc4-1 mutant for either 21- or 24-nt siRNAs (Fig. 5C, Supplemental Fig. S6B,C). This suggests that condensin somehow affects the CTD-OX transgene in a locus-specific manner without having genome-wide effects on 21-nt siRNA biogenesis.

MORC ATPases are thought to function downstream from DNA methylation to affect heterochromatin on MET1, DDM1, SMC4, and ATXR5/6 using RT–PCR assays [Fig. 4G]. This gel-based assay confirmed the conclusions from the RNA-seq data, showing that the TEs are silenced in wild-type plants [Col-0] but derepressed in each of the mutants.

Displaying RNA-seq data as a heat map, we compared the relative expression levels of the 169 SMC4-, MET1-, and DDM1-dependent TEs in cmt3, atxr5/6, smc4, met1, or ddm1 mutants [Fig. 4H]. DDM1 and MET1 exert the strongest repression of the largest number of TEs, with SMC4 also needed for moderate to strong repression in most cases. ATXR5 and ATXR6 exert a less pronounced effect on TE expression, but a subset of TEs requires SMC4, MET1, DDM1, and ATXR5/6 for strong repression.

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condensation (Jacob and Martienssen 2012, Lorkovic et al. 2012, Moissiard et al. 2012), possibly in ways similar to SMC ATPases (Iyer et al. 2008). Analyzing mRNA-seq data for *atmorc1* and *atmorc6* double mutants (Moissiard et al. 2012, Stroud et al. 2013), we found that 59% [26 of 44] of the TEs derepressed in *atmorc1*/*atmorc6* overlap with TEs derepressed in *met1* and *ddm1* (Supplemental Fig. S7A), which is significantly higher than the 0.08% expected by chance (*P* = 1.07 × 10^-66*) and comparable with the 59% overlap between TEs derepressed in *smc4-1*, *met1*, and *ddm1*. Of the TEs derepressed in *atmorc1*/*atmorc6*, 41% [18 of 44] overlap with TEs derepressed in *smc4-1* (Supplemental Fig. S7B).

Collectively, our results indicate that TEs silenced in an *SMC4*-dependent manner are subject to multiple levels of control, including cytosine hypermethylation, histone H3K9me and H3K27me, and assembly into higher-order complexes that also involve MORC ATPases.

Both condensins I and II are involved in *SMC4*-dependent transposon silencing

*SMC4* is a core subunit of condensin I and condensin II, making it unclear whether defective TE silencing in *smc4-1* stems from impairment of one or both forms of condensin. To address this question, we identified and assayed for expression of seven *SMC4*-dependent TEs derepressed in *smc4-1* mutants defective for two subunits specific for condensin I [Sakamoto et al. 2011]. Moreover, *smc4-1* mutants display prominent RAD51 repair foci (Fig. 6E), as in atrx5/6 nuclei in which DNA damage is known to occur (Feng et al. 2017).

A flowering gene found by RNA-seq to be dramatically up-regulated in *smc4-1* mutants is FLOWERING LOCUS T (FT), which we verified using both gel-based RT–PCR assays (Fig. 6D) and qPCR (Fig. 7A). This likely explains the speedier transition to flowering observed under long-day conditions [16 h light, 8 h dark] for the original *m73* mutant [genotype: *smc4-1 CTD-OX cnt3*] as well as the *smc4-1* line resulting from repeated backcrossing to wild-type Col-0 (Fig. 7B,C).

Another important gene regulated by *SMC4* is *ROS1*, which encodes a DNA glycosylase that facilitates the removal and replacement of methylated cytosines by DNA repair (Gong et al. 2002). *ROS1* is up-regulated approximately fivefold in *smc4-1* relative to the *smc4-1* single mutant (Fig. 7D), indicating that condensin limits the basal expression level of *ROS1*. Consistent with previous studies (He et al. 2009, Lei et al. 2015), *ROS1* expression is dependent on RdDM such that ROS1 expression is reduced in *nrdp1* (*pol IV*) *smc4-1* or *nrpe1* (*pol V*) *smc4-1* double mutants relative to the *smc4-1* single mutant (Fig. 7D) yet still remains higher than in a *pol V* single mutant. *ROS1* transcription levels positively correlate with RdDM-dependent methylation levels within a TE near the gene promoter (Lei et al. 2015, Williams et al. 2015). We observed a modest gain of methylation in all sequence contexts at this TE in the *smc4-1* mutant [Supplemental Fig. S8]. Condensin may thus limit the extent of RdDM at this site in wild-type plants.

Discussion

Previous case studies have documented roles for condensin in repressing specific loci. For instance, in budding yeast, condensin helps repress silent mating type loci and also represses Pol II transcription within the intergenic spacers of Pol I transcribed ribosomal RNA genes (Bhal et al. 2002, Machin et al. 2004, He et al. 2016). Consistent with the latter studies, the non-SMC condensin subunit AtCAP-H2 of *Arabidopsis* localizes within the nucleolus (Fujimoto et al. 2005), and rRNA genes become decondensed in *Arabidopsis* RNAi lines with reduced *SMC4* levels (Smith et al. 2014). In flies, condensin is involved in position effect variegation (Lupo et al. 2001, Dej et al. 2004). In *C. elegans*, a specialized condensin is involved in X-chromosome dosage control (Meyer 2010), and, in mice, condensin helps maintain T-cell quiescence (Rawlings et al. 2011). Our results extend these case...
studies to a whole-genome level, made possible by the identification of smc4-1 as a viable, yet deleterious, mutation of the essential SMC4 gene. Our findings indicate that condensin acts as a corepressor of both genes and transposons, affecting hundreds of loci.

Our results indicate that condensin partners with symmetrical cytosine methylation and repressive histone modifications, particularly ATXR5/6-dependent H3K27me1, in the repression of pericentromeric transposons. MET1 and ATXR5/6 are thought to be recruited to DNA replication forks by interacting with PCNA (Chuang et al. 1997; Raynaud et al. 2006; Hale et al. 2016). Interestingly, condensin has been detected at stalled DNA replication forks (D’Ambrosio et al. 2008) and shown to interact with DNA methyltransferases in mammalian cells (Geiman et al. 2004). These observations suggest that compact repressive chromatin structures might assemble quickly following DNA replication.

Condensin is needed for Drosophila Gypsy family retrotransposons to cluster within distinct chromatin bodies (Gerasimova et al. 2000) and for LTR retroelements in Schizosaccharomyces pombe to cluster in the vicinity of centromeres (Cam et al. 2008; Tanaka et al. 2012; Murton et al. 2016). How are these dispersed elements recognized and brought together? The fact that Arabidopsis loci that are heavily cytosine methylated or sparsely methylated can be regulated by condensin argues against DNA methylation as a primary determinant of condensin recruitment, as does the fact that flies and yeast do not appreciably methylate their DNA. Conserved histone modifications or histone variants present in all eukaryotes seem more likely as marks that enable condensin recruitment. Architectural chromatin proteins such as heterochromatin protein 1 (HP1) family members, Polycomb-repressive complex 1 (PRC1) family proteins, or yeast silent information regulator (SIR) proteins might then serve as intermediaries for condensin recruitment (McBryant et al. 2006; Woodcock and Ghosh 2010; Grossniklaus and Paro 2014).

The mutation in the smc4-1 allele P22S is intriguing in that the substituted proline is highly conserved except in budding yeast, which has a serine at this position, as in smc4-1 (see Fig. 2D). It is plausible that the P-to-S mutation might be tolerated as a hypomorphic mutant in other model organisms, including mammals, in which null mutants are lethal. Given that somatic mutations in condensin subunits occur in multiple types of cancer (Leiserson et al. 2015; Uhlmann 2016), generating this mutation by gene editing might prove useful for genome-wide identification of loci that become derepressed in human cells when condensin function is compromised.
Materials and methods

Plant materials

A. thaliana ecotype Col-0 was used in all experiments. met1-3 and ddm1-2 were described in Saze et al. (2003), and ndr1-1 (pol IV) and ndr1-11 (pol V) mutants were described previously (Onodera et al. 2005; Pontes et al. 2006). atr5 atr6 was provided by Scott Michaels, and met1-1 was provided by Eric Richards. drm2-2 and cmt3-11t were obtained from the Arabidopsis Biological Resource Center (ABRC). The cmt3-11t met1-1 double mutant was created by crossing cmt3-11t to met1-1 (Pontvianne et al. 2013). The SMC4 T-DNA insertion mutant line SAIL-86-D02 (Siddiqui et al. 2006) was obtained from the ABRC. Mutant lines of the condensin subunits cap-e1 (CS84719), cap-d2 [SALK_077796C], cap-d3 [SALK_094776], cap-g2 [SALK_049790C], and cap-h2 [SALK_059304] were obtained from the ABRC.

The CTD-OX transgene corresponding to NRPE1 sequences encoding amino acids 1249–1976 was cloned into pEarleyGate 202 (Earley et al. 2006) and then transformed into cmt3-11t by the floral dip method (Clough and Bent 1998). Transgenic plants were selected by Basta herbicide resistance, and 431 positive transformants were further screened to identify 242 lines with Basta resistance, segregating 3:1. These 242 lines were subjected to Southern blot analysis to identify plants with single T-DNA left and right border fragments. Line #389 [cmt3 OX-CTD] was chosen for EMS mutagenesis. An OX-CTD line was obtained by backcrossing #389 [cmt3 OX-CTD] to Col-0 and identifying F2 individuals lacking cmt3 mutant alleles.

EMS mutagenesis and genetic mapping

Approximately 22,000 cmt3 CTD-OX line seeds were mutagenized with EMS as described (Kim et al. 2006), except that the EMS concentration was 0.3%. Seeds of resulting M1 plants were harvested, and resulting M2 plants with a wild-type (as opposed to SDC) phenotype were identified. Bulked-segregant analysis and sequence-based mapping were conducted as described by Hartwig et al. (2012). Briefly, putative suppressor mutants were first backcrossed to the cmt3 CTD-OX parental line, and resulting F1 plants were grown and allowed to self-pollinate to produce F2 seeds. F2 plants were then grown, and 50 plants displaying SDC or wild-type phenotypes were pooled, and their DNA was subjected to library construction and deep sequencing, with an estimated genome coverage of 30×. The cmt3 CTD-OX line was also sequenced. Candidate EMS-induced mutations absent in the parental line and displaying high allele frequencies in suppressor mutants were confirmed by sequencing of PCR-amplified genomic DNA.

Complementation of smc4-1 by an SMC4 transgene

Plant transformation vector pHPT carrying a full-length wild-type SMC4 genomic clone insert (Siddiqui et al. 2006) was provided by Daniel Riggs. The plasmid was transformed into Agrobacterium tumefaciens strain GV3101 and subsequently provided by Daniel Riggs. The plasmid was transformed into A. tumefaciens strain GV3101 and subsequently provided by Daniel Riggs. The plasmid was transformed into A. tumefaciens strain GV3101.
DNA methylation analyses

Genomic DNA was isolated from 2-wk-old plants using the Nucleon PhytoPure DNA extraction kit [Amersham]. Chop-PCR assays were performed using 100 ng of restriction endonuclease-digested (“chopped”) genomic DNA as in Blevins et al. (2017). Bisulfite sequencing analysis of the ROS1 promoter regions was according to Blevins et al. (2014). In brief, PCR fragments amplified from bisulfite-treated DNA were cloned into pGEM-T-Easy and sequenced using a T7 primer. Forty-eight sequences per amplicon were analyzed in CyMATE (Hetz et al. 2007). Chop-PCR and bisulfite sequencing primers are listed in Supplemental Table S4.

Semiquantitative RT–PCR

Total RNA was extracted from three to four leaves of 2-wk-old plants using the Spectrum plant total RNA kit (Sigma-Aldrich). RNA (1.5 µg) was then treated using a Turbo DNA-free kit (Thermo Fisher Scientific) and used for random-primed cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen). Resulting cDNA was used for PCR amplification using GoTaq Green polymerase (Promega) and primers listed in Supplemental Table S4.

RNA-seq and data analysis

Total RNA was extracted from 2-wk-old Arabidopsis leaves using TRI reagent [MRC, Inc.]. Libraries for three biological replicates of each genotype were constructed and sequenced using the Illumina NextSeq 500 platform. TrueSeq adaptor sequences were trimmed using Trimmomatic version 0.33. Reads post-trimming were filtered with a quality score cutoff of 20 and length cutoff of 30. Filtered reads were mapped to the TAIR10 genome using TopHat 2.0.10. The number of uniquely mapped reads corresponding to filtered reads were mapped to the TAIR10 genome using TopHat 2.0.10. The number of uniquely mapped reads corresponding to annotated protein-coding genes or TEs were computed using a quality score cutoff of 20 over a window of 3 bases. Reads >15 bases post-trimming were excluded. Trimmed and filtered reads were then mapped to the TAIR10 genome reference genome sequence using Bowtie version 1.1.2 (Langmead et al. 2009), and only perfectly matched 21- to 25-nt RNAs were analyzed further. Any reads aligning to 45S rRNA, chloroplast, or mitochondria were excluded. Read alignments were further filtered for size classes 21 and 24 nt.

For small RNA Northern blot analyses, ~100 µg of total RNA was extracted from 2-wk-old seedlings using TRIzol [ThermoFisher Scientific] and then size-fractionated on RNAeasy minicolumns (Qiagen) [Blevins et al. 2006]. The low-molecular-weight RNA fraction was then used as described previously [Blevins et al. 2015].

Accession numbers

Sequence data generated in this study have been deposited in the NCBI Sequence Read Archive [http://www.ncbi.nlm.nih.gov/sra] under accession number SRP105760.

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