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Journal

Proceedings of the National Academy of Sciences of the United States of America, 111(45)

ISSN

0027-8424

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Publication Date

2014-11-11

DOI

10.1073/pnas.1418564111

Peer reviewed

The AAA-ATPase molecular chaperone Cdc48/p97 disassembles sumoylated centromeres, decondenses heterochromatin, and activates ribosomal RNA genes

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Contributed by Robert L. Fischer, September 26, 2014 (sent for review August 29, 2014)

Centromeres mediate chromosome segregation and are defined by the centromere-specific histone H3 variant (CenH3)/centromere protein A (CENP-A). Removal of CenH3 from centromeres is a general property of terminally differentiated cells, and the persistence of CenH3 increases the risk of diseases such as cancer. However, active mechanisms of centromere disassembly are unknown. Nondividing *Arabidopsis* pollen vegetative cells, which transport engulfed sperm by extended tip growth, undergo loss of CenH3; centromeric heterochromatin decondensation; and bulk activation of silent rRNA genes, accompanied by their translocation into the nucleolus. Here, we show that these processes are blocked by mutations in the evolutionarily conserved AAA-ATPase molecular chaperone, *CDC48A*, homologous to yeast Cdc48 and human p97 proteins, both of which are implicated in ubiquitin/small ubiquitin-like modifier (SUMO)-targeted protein degradation. We demonstrate that *CDC48A* physically associates with its heterodimeric cofactor UFD1-NPL4, known to bind ubiquitin and SUMO, as well as with SUMO1-modified CenH3 and mutations in *NPL4* phenocopy *cdc48a* mutations. In WT vegetative cell nuclei, genetically unlinked ribosomal DNA (rDNA) loci are uniquely clustered together within the nucleolus and all major rRNA gene variants, including those rDNA variants silenced in leaves, are transcribed. In *cdc48a* mutant vegetative cell nuclei, however, these rDNA loci frequently colocalized with condensed centromeric heterochromatin at the external periphery of the nucleolus. Our results indicate that the *CDC48A*^{NPL4} complex actively removes sumoylated CenH3 from centromeres and disrupts centromeric heterochromatin to release bulk rRNA genes into the nucleolus for ribosome production, which fuels single nucleus-driven pollen tube growth and is essential for plant reproduction.

centromere disassembly | heterochromatin decondensation | chromosome dynamics | rDNA activation | pollen tip growth

Centromeres are required for chromosomes to attach to spindle fibers and segregate during mitosis and meiosis. Of central importance to centromeres are nucleosomes with the centromere-specific histone H3 variant (CenH3)/centromere protein A (CENP-A) (1). Centromeres are intimately associated with heterochromatin, which is frequently formed adjacent to an array of CenH3 nucleosomes, upon which the kinetochore is assembled, and heterochromatin is required to promote CenH3 assembly at centromeres (2). In both mammals and plants, constitutively heterochromatic megabase-sized centromeric regions are rich in highly repetitive tandem arrays of satellite DNA and moderately repetitive sequences, such as transposons, and show a low level of transcription (3). Centromeric heterochromatin remains condensed after mitosis and is associated with repressive chromatin marks, including DNA cytosine methylation (4) and histone H3 Lys9 methylation (5). Chromosomes from terminally differentiated cells that have exited the cell cycle often have little CenH3 (6), presumably to prevent them from undergoing defective mitoses

and oncogenesis (7). In contrast to the relatively well-known process of de novo CenH3 assembly at centromeres, little is known about how CenH3 is actively removed from centromeres, leading to centromere disassembly.

Unlike animals, whose gametes represent the direct products of meiosis, flowering plants form multicellular haploid gametophytes by means of postmeiotic mitoses (8). In stamens, each haploid pollen microspore undergoes an asymmetrical mitosis to produce a large, terminally differentiated vegetative cell and a smaller generative cell, which have different fates. The generative cell, engulfed in the cytoplasm of the vegetative cell, undergoes a second mitosis to form two sperm cells. The primary role of the vegetative cell in plant reproduction is to germinate and form a rapid tip-growing pollen tube, sometimes centimeters in length, which transports two sperm cells to the female gametophyte for double fertilization. We reported previously that the *Arabidopsis thaliana* pollen vegetative cell invariably undergoes CenH3 removal from centromeres, coincident with loss of the demethylated histone H3 Lys9 (H3K9me2) heterochromatin mark and centromeric heterochromatin decondensation (9), although the mechanism and biological significance of these processes remain unclear.

Significance

Centromeres are the fundamental unit required for segregation of chromosomes during mitosis and meiosis, and they are defined by the centromere-specific histone H3 variant (CenH3)/centromere protein A (CENP-A). In contrast to the relatively well-known process of de novo assembly of CenH3 at centromeres, little is known of how CenH3 is actively removed, leading to centromere disassembly, an essential biological process during the life of a cell. This study describes the process of centromere disassembly, demonstrating that it occurs via an active, proteolytic mechanism, which is also linked to major changes in chromosome dynamics: chromatin decondensation and bulk rRNA gene activation.

Author contributions: Z.M., N.C., M.G.-A., T.-F.H., T.N., V.K.S., K.M., D.Z., R.L.F., and H.T. designed research; Z.M., N.C., M.G.-A., T.-F.H., T.N., V.K.S., J.B., L.S., S.A., and S.O. performed research; Z.M., N.C., M.G.-A., T.-F.H., T.N., V.K.S., K.M., D.Z., R.L.F., and H.T. analyzed data; and D.Z., R.L.F., and H.T. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1418564111/-DCSupplemental.

Here, we describe the active mechanism of centromere disassembly, which, directed by the CDC48A AAA-ATPase molecular chaperone, makes novel mechanistic links to chromosome dynamics: decondensation of constitutive heterochromatin and translocation of hitherto silent ribosomal DNA (rDNA) repeats into the nucleolus, coincident with activation of bulk rRNA genes.

Results

CDC48A Is Required for Active Removal of CenH3 from Centromeres in the Pollen Vegetative Cell. To understand the mechanism for CenH3 removal, we carried out a forward genetic screen to search for *Arabidopsis* mutants that retain condensed CenH3 centromeres in the pollen vegetative cell nucleus. Mature pollen from chemically mutagenized transgenic plants expressing GFP-fused CenH3 under the control of its native promoter, and RFP-fused histone H2B driven by a vegetative cell-specific *LAT52* promoter (9), were examined using fluorescence microscopy. We identified one mutant, *izanagi* (*igi*), which we named after a Nipponese god of fertility, that showed five CenH3-GFP centromeric foci within the RFP-positive vegetative cell nucleus (Fig. 1A). We mapped the *igi* mutation to *CDC48A* (AT3G09840) (10) using a combination of high-throughput whole-genome sequencing and conventional Sanger DNA sequencing. *CDC48A* encodes a functional AAA-ATPase (11) homologous to yeast Cdc48 and human p97, which is highly conserved from Archaeobacteria to mammals (SI Appendix, Figs. S1 and S2) and is associated with diverse cellular activities involving ubiquitin-proteasome-mediated protein degradation (12, 13). We found a G-to-A substitution mutation in a conserved Ser codon in the amino acid motif called D1, implicated in ATP binding and hexamerization (14) (Fig. 1B and SI Appendix, Fig. S2). Two independently isolated loss-of-function transfer (T)-DNA insertion alleles, *cdc48a-1* and *cdc48a-3* (15), phenocopied *igi*, showing CenH3 centromeric foci in the vegetative cell nucleus (Fig. 1C), and the WT *CDC48A* transgene complemented the *igi* mutation in two independent transformants (SI Appendix, Fig. S3). The *Arabidopsis* genome encodes two additional Cdc48/p97 homologs, CDC48B (At3g53230) and CDC48C (At5g03340) (15) (SI Appendix, Fig. S1), although mutations in these genes did not phenocopy *cdc48a* mutations. We therefore concluded that *CDC48A* is *IGI*.

The *igi* mutant plants show phenotypes previously described for *cdc48a* mutant plants (15). That is, the *igi* mutation does not cause obvious alterations in pollen mitoses, viability, or morphology (SI Appendix, Fig. S4 and Table S1), but the mutant *igi* allele is rarely transmitted paternally and is transmitted maternally at a reduced level (SI Appendix, Table S2). Consequently, mutant *igi* lines are maintained only as heterozygotes. The inability to transmit the paternal *igi* allele primarily reflects defective pollen tube formation by the vegetative cell (15) (Fig. 1D and SI Appendix, Fig. S5). We inspected the expression pattern of *CDC48A* protein during pollen development using transgenic plants carrying BFP-tagged *CDC48A* under the control of its native promoter. We found that *CDC48A* accumulates in the microspore nucleus before the first asymmetrical mitosis that gives rise to the vegetative and generative cells (Fig. 1E). Consistent with its role in pollen tube growth, *CDC48A* is preferentially expressed in the vegetative cell in the binucleate and trinucleate stages of pollen (Fig. 1E).

CDC48A Associates with Sumoylated CenH3 in Early Stages of Pollen.

We next explored the possibility that *CDC48A* might have a direct role in removing CenH3 from centromeres. To identify *CDC48A*-associated proteins, we generated two transgenic lines expressing either Myc-tagged *CDC48A* (*CDC48A*-Myc) alone or *CDC48A*-Myc and GFP-fused CenH3 (CenH3-GFP). Centromeric CenH3-GFP foci disappear in the vegetative cell nucleus when it is formed following completion of the first asymmetrical pollen mitosis (SI Appendix, Fig. S6), suggesting that the most likely timing of *CDC48A* action is at the binucleate stage of pollen development.

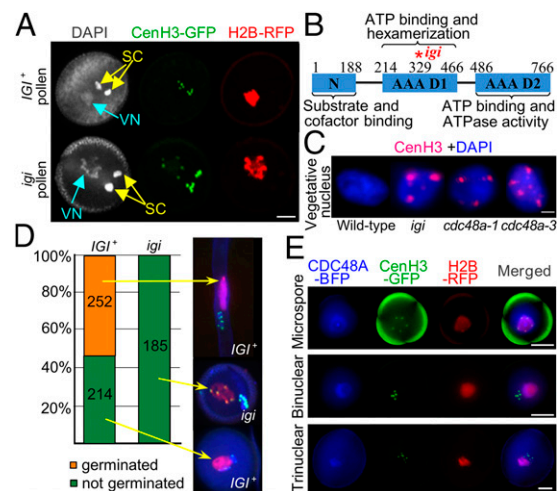


Fig. 1. *IGI* is *CDC48A* and is required for active CenH3 removal from centromeres in the pollen vegetative cell and pollen tube formation. (A) Fluorescence microscopy of an *A. thaliana* *IGI+* and an *igi* mutant mature pollen grain carrying *pCenH3::CenH3::GFP* and *pLAT52::H2B::RFP*. Approximately 1000 pollen vegetative cell nuclei from a *+igi* heterozygous plant homozygous for *pCenH3::CenH3::GFP* and *pLAT52::H2B::RFP* were inspected, and ~35% of them showed CenH3-GFP centromeric foci. SC, sperm cell; VN, vegetative cell nucleus. (Scale bar: 10 μ m.) (B) Protein domain organization of *A. thaliana* *CDC48A*. Amino acid coordinates are indicated. The position of a substitution mutation in allele *cdc48a-igi* is marked with an asterisk. (C) Immunolocalization of CenH3 in WT, *igi*, *cdc48a-1*, and *cdc48a-3* vegetative cell nuclei from mature pollen, using anti-CenH3/HTR12 antibodies. Nuclei were counterstained with DAPI (blue). (Scale bar: 1 μ m.) (D) Pollen germination assays on solid media. Percentages of germinated (orange bar) and nongerminated (green bars) pollen grains from *IGI+/igi* heterozygous plants in the *pCenH3::CenH3::GFP pLAT52::H2B::RFP* background were examined for the *igi* phenotype showing centromeric CenH3-GFP dots in the RFP-positive vegetative cell nucleus under the fluorescence microscope. The pollen phenotype (*IGI+* or *igi*) is indicated. Numbers on the bars indicate the number of pollen grains examined. (E) Fluorescence microscopy of a pollen grain at mononucleate microspore, dinucleate, and trinucleate mature stages from a transgenic plant homozygous for *pCDC48A::CDC48A::BFP*, *pCenH3::CenH3::GFP*, and *pLAT52::H2B::RFP*. (Scale bars: 10 μ m.) All of ~100 pollen grains at each stage showed an equivalent *CDC48A* expression pattern.

Therefore, we isolated transgenic pollen from young flower buds enriched in pollen at early stages (SI Appendix, Fig. S7), extracted proteins, immunoprecipitated *CDC48A*-Myc using the anti-Myc antibody, and carried out protein blot analyses. Strikingly, proteins immunoprecipitated from both lines produced robust band signals when immunoblotted with the antibody against *A. thaliana* CenH3/HTR12 (16), whose sizes were larger than scarcely detectable unmodified CenH3 or CenH3-GFP, indicating covalent modification of CenH3 (Fig. 2A and B). MS analysis of trypsin-digested peptides extracted from acrylamide gel slices containing the larger CenH3 bands (Fig. 2C) identified SMALL UBIQUITIN-LIKE MODIFIER1 (SUMO1) (At4g26840) (Fig. 2F and SI Appendix, Table S3) in addition to CenH3 itself. However, no ubiquitin peptide was found. To address the possibility that *CDC48A*-associated CenH3 is SUMO1-conjugated, similar protein blot analyses were performed using the antibody against *A. thaliana* SUMO1. We detected signals that correspond to the size predicted to be disumoylated CenH3 in the *CDC48A*-Myc line (Fig. 2A) or disumoylated CenH3 and disumoylated CenH3-GFP in the *CDC48A*-Myc CenH3-GFP line (Fig. 2B), respectively. The control line without the *CDC48A::Myc* transgene gave no signal (Fig. 2B). As a second test of whether CenH3 is sumoylated, we immunoprecipitated CenH3-GFP from the

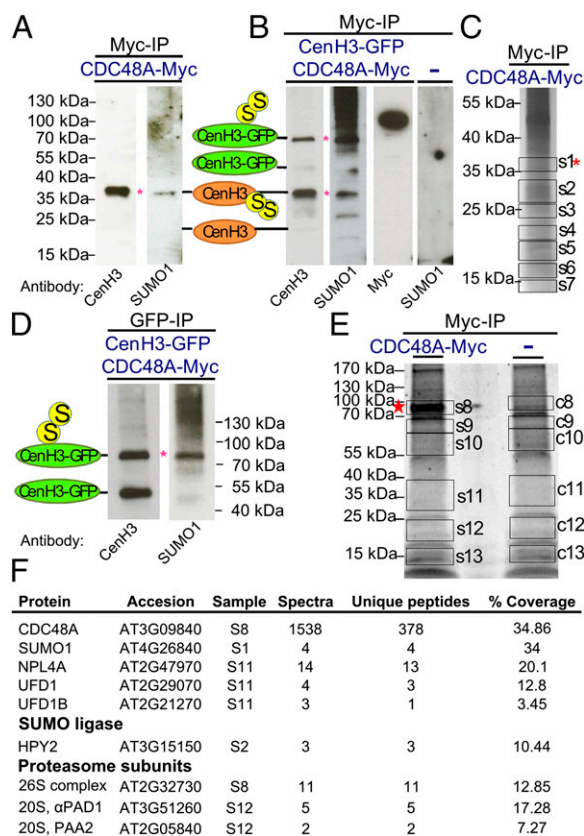


Fig. 2. CenH3 is subjected to sumoylation, and CDC48A associates with sumoylated CenH3 in early-stage pollen. Coimmunoprecipitation of CDC48A-interacting proteins extracted from early-stage pollen of transgenic lines expressing CDC48A-Myc (A) and CDC48A-Myc and CenH3-GFP or CenH3-GFP without (–) CDC48A-Myc (B) using anti-Myc antibodies, followed by protein blot analyses with antibodies against CenH3, SUMO1, or Myc. Modified CenH3 band signals are marked by asterisks. S, sumoylation. (C) Immunoprecipitated proteins were fractionated by 4–20% gradient SDS/PAGE and stained with silver. Acrylamide gel slices s1–s7 were cut out and in-gel trypsin-digested peptides were extracted from each gel sample for MS analysis. SUMO1 was identified only in s1 (indicated with an asterisk), whose position corresponded to the modified CenH3 band in A. (D) Immunoprecipitation of CenH3-GFP from early-stage pollen extracts of the CDC48A-Myc CenH3-GFP line using anti-GFP antibodies, followed by protein blot analyses with antibodies against CenH3 and SUMO1. (E) Early-stage pollen proteins were immunoprecipitated from lines with or without (–) the *pCDC48A::CDC48A-Myc* transgene using anti-Myc antibodies, fractionated and silver-stained. Trypsin-digested peptides were extracted from gel slices s8–s13, which showed protein bands unique to the CDC48A-Myc line (Left), and from the WT control (Right) counterparts c8–c13, and were then analyzed by MS. The position of CDC48A-Myc bait protein is indicated with an asterisk. Proteins that were detected in both lines were excluded as false-positive findings. (F) CDC48A-associated cofactors and SUMO-proteasome pathway components were detected by MS. Spectra represent the number of all peptides that map to the accession indicated in the second column. The percentage coverage (% coverage) takes into account unique peptides that map only to the accession.

CDC48A-Myc CenH3-GFP line using the anti-GFP antibody. The immunoprecipitated protein population abundantly consisted of both unmodified CenH3-GFP and sumoylated CenH3-GFP (Fig. 2D). These data suggest that CDC48A physically associates with SUMO1-modified CenH3 in pollen vegetative cells.

CDC48A and NPL4 Cooperate to Remove Sumoylated CenH3 from Centromeres in the Pollen Vegetative Cell. FISH and immunolocalization analyses of *cdc48a* mutant vegetative cell nuclei from mature pollen detected accumulation of SUMO1 that colocalizes with condensed centromeric repeat loci (Fig. 3A). In contrast, both the

centromeric repeats and SUMO1 signals are dispersed in WT vegetative cell nuclei (Fig. 3B), whereas in control sperm nuclei, SUMO1 signals remain excluded from condensed centromeric repeats (Fig. 3C). Taken together, these results indicate that CDC48A binds SUMO1-modified CenH3 and removes it from centromeres.

Cdc48/p97 interacts with a number of protein cofactors, and these interactions are important for its target specificity and functional diversity. Many of these cofactors have distinct ubiquitin-binding domains and act as ubiquitin adaptors (13). To elucidate how CDC48A is targeted to sumoylated CenH3, we searched for adaptor proteins required for CDC48A function in pollen. Ufd1-Npl4, a known heterodimeric cofactor of Cdc48/p97 (17), binds to both ubiquitin and SUMO (18). The *Arabidopsis* genome encodes three Ufd1 homologs, which we named UFD1A (AT2g29070), UFD1B (AT2g21270), and UFD1C (AT4g38930), and two Npl4 homologs, which we named NPL4A (AT2g47970) and NPL4B (AT3g63000). MS analysis of the CDC48A-associated proteins from the early stages of pollen (Fig. 2E) identified peptides that match these NPL4 and UFD1 proteins (Fig. 2F and *SI Appendix*, Table S3), supporting the notion that CDC48A forms a complex with NPL4 and UFD1.

Arabidopsis T-DNA insertion lines with mutations in *NPL4A* and the highly related *NPL4B* gene, which encode proteins with 83.5% amino acid identity, allowed us to carry out genetic analyses to understand the role of NPL4 in this pathway. Single-homozygous mutant plants, *npl4a/npl4a* or *npl4b/npl4b*, do not display pollen defects; however, double-mutant *npl4a npl4b* pollen, similar to *cdc48a* mutant pollen, undergoes mitoses and maturation normally (*SI Appendix*, Table S1) but is infertile (*SI Appendix*, Tables S2 and S4). Strikingly, *npl4a npl4b* pollen vegetative cell nuclei exhibited condensed centromeric repeat loci that colocalize with SUMO1, phenocopying *cdc48a* mutations (Fig. 3D). These data indicate that NPL4A and NPL4B redundantly form a complex with CDC48A, and that their interaction with CDC48A is required to bind SUMO1-modified CenH3 at centromeres for removal and proteolysis. Consistent with the inference, we identified a SUMO ligase and three proteasome subunits in the CDC48A-Myc pulled-down fractions (Fig. 2E and F and *SI Appendix*, Table S3).

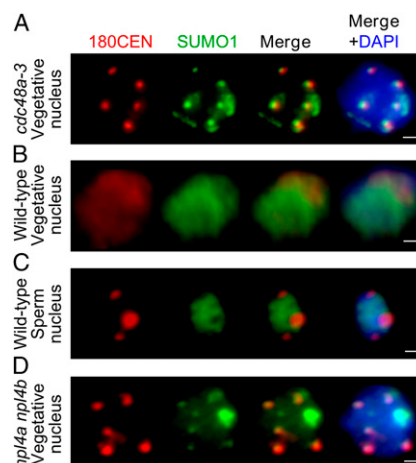


Fig. 3. CDC48A and NPL4 cooperate to remove SUMO1-modified CenH3 from centromeres in the pollen vegetative cell. Dual-FISH/immunolocalization of 180-bp centromeric DNA repeats (180CEN) and SUMO1 in *cdc48a-3* vegetative cell (A), WT vegetative cell (B), WT sperm (C), and *npl4a npl4b* vegetative cell (D) nuclei from mature pollen is shown. (Scale bars: 1 μ m.) Approximately 300 pollen vegetative cell nuclei from *+cdc48a-3* and *npl4a/npl4a +npl4b* plants were inspected, and ~15% of them showed SUMO1 focal signals that colocalized with condensed 180CEN foci, whereas all of >500 WT vegetative cell nuclei showed dispersed SUMO1 signals.

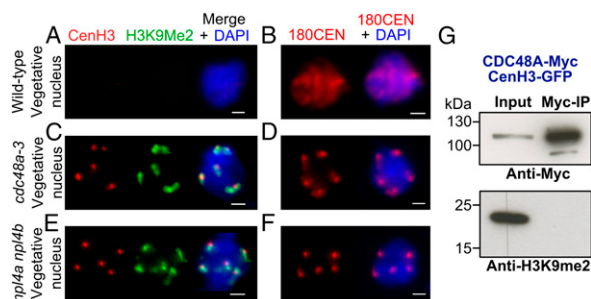


Fig. 4. CDC48A-directed CenH3 removal from centromeres is coupled with loss of H3K9me2 and centromeric heterochromatin decondensation. Dual immunolocalization of CenH3 and H3K9me2 (A, C, and E) and FISH analysis of 180CEN (B, D, and F) in WT, *cdc48a-3*, and *npl4a npl4b* vegetative cell nuclei from mature pollen is shown. (Scale bars: 1 μ m.) Approximately 300 pollen vegetative cell nuclei from *+cdc48a-3* and *npl4a/npl4a +/npl4b* plants were examined, and ~20% of them showed condensed CenH3, H3K9me2, and 180CEN foci, whereas in all of >1,000 WT vegetative cell nuclei, 180CEN repeats were dispersed and neither CenH3 nor H3K9me2 signal was detected. (G) Protein blot analyses of CDC48A-Myc-associated early-stage pollen proteins using antibodies to Myc and H3K9me2. See also Fig. 2B.

CDC48A-Directed CenH3 Removal from Centromeres Is Coupled with Loss of H3K9me2 and Centromeric Heterochromatin Decondensation.

In WT vegetative cells, the removal of CenH3 is concurrent with loss of H3K9me2 (Fig. 4A) and centromeric heterochromatin decondensation (Fig. 4B). In contrast, *cdc48a* vegetative cell nuclei showed robust heterochromatic H3K9me2 signals radiating from condensed CenH3-associated centromeres (Fig. 4C) and condensed 180-bp centromeric repeat loci (Fig. 4D). Similarly, *npl4a npl4b* mutant vegetative cell nuclei from *npl4a/npl4a +/npl4b* or *+npl4a npl4b/npl4b* plants had strong H3K9me2 signals that coincided with CenH3 and centromeric repeat foci (Fig. 4E and F). These results indicate that CDC48A^{NPL4} is required for the overall chromatin reconfiguration observed in the pollen vegetative cell. However, no H3K9me2 signal was detected in the CDC48A-Myc immunoprecipitated fraction under conditions that produced a strong signal in total proteins (Fig. 4G), suggesting that CDC48A is indirectly responsible for H3K9me2 removal from centromeric heterochromatin.

CDC48A-Mediated Centromeric Heterochromatin Decondensation Coincides with Bulk Activation of Silent rRNA Genes.

We next addressed the functional significance of centromeric heterochromatin decondensation. In mammals and plants, thousands of tandem 45S rDNA repeats, each encoding 18S, 5.8S, and 25S rRNA, the major structural and functional components of the ribosome, are located adjacent to the centromere or the telomere (19) and form facultative heterochromatin, whose epigenetic state reversibly switches from silent to active, thereby controlling the number of transcribed rRNA genes in response to cellular demands for ribosomes and protein synthesis (20). The *Arabidopsis* genome has two rDNA repeat loci located near telomeric regions of chromosomes 2 and 4 (21). In somatic leaf nuclei, condensed silent heterochromatic portions of rDNA loci coincide with chromocenters that contain centromeric heterochromatin at the external periphery of the nucleolus, a sub-nuclear compartment where rDNA transcription and ribosome assembly take place, and only relatively few rRNA genes are located within the nucleolus to be transcribed (22). Distinct megabase-sized clusters of rRNA gene variant repeats are selectively silenced during early embryonic development; are excluded from the nucleolus; and are not transcribed in somatic tissues, including leaves (23, 24).

We examined the distribution of centromeric repeat and 45S rDNA loci in WT leaf and pollen vegetative cell nuclei using FISH and protein immunolocalization. As described previously (22), in control leaf diploid nuclei, the two genetically unlinked rDNA loci coincided with

condensed centromeric repeats (Fig. 5A) located external to the nucleolus marker fibrillarin (Fig. 5B). In contrast, in the vegetative cell nuclei we examined, these two rDNA loci are invariably clustered together and are no longer colocalized with dispersed centromeric repeats (Fig. 5C), largely spatially overlapping the nucleolus instead (Fig. 5D). The topology of rDNA loci in vegetative cells is in sharp contrast to the topology in leaf cells, where large portions of rDNA loci are external to the nucleolus. These data suggest that vast portions of the rDNA repeats are within the nucleolus in the vegetative cell nucleus, and therefore have the potential to be transcribed. Consistent with this notion, we found that all major rDNA variants (Fig. 5E) are transcribed in pollen, including the longest variant repeats (VAR1), which represent >50% of the megabase-sized total rDNA pool (25) and are entirely silenced and not transcribed in leaves (26) (Fig. 5F).

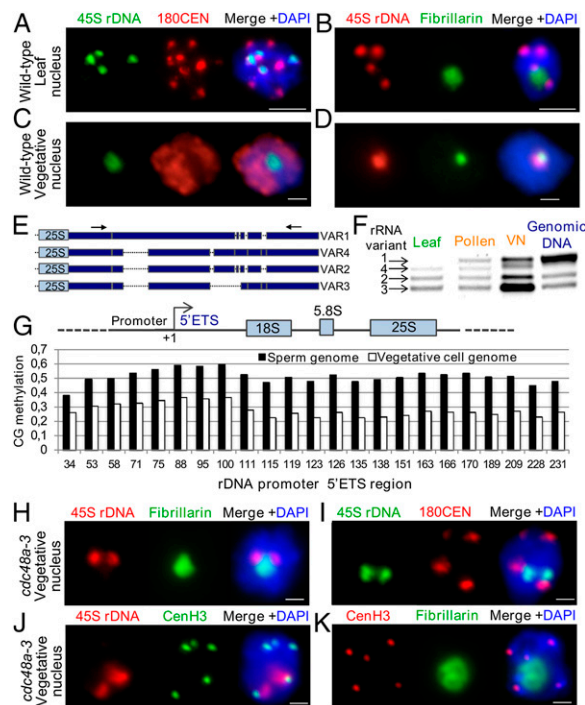


Fig. 5. CDC48A-mediated centromeric heterochromatin decondensation coincides with activation of silent rRNA genes within the nucleolus. Dual-FISH analysis of 45S rDNA and 180CEN (A and C) and FISH/immunolocalization of 45S rDNA and fibrillarin (B and D) in WT leaf and mature pollen vegetative cell nuclei are shown. Note that leaf diploid nuclei typically show ten 180CEN and four rDNA focal signals. (Scale bars: 1 μ m.) All of >1,000 pollen vegetative cell nuclei exhibited clustering of two rDNA loci that largely overlap the fibrillarin nucleolus marker. (E) Diagram illustrates the polymorphic regions of *A. thaliana* rDNA sequences in which insertions/deletions in the 3' external transcribed region define the major repeats from WT sperm and vegetative cell genomes. Positions of CG sites relative to the transcription start site (+1) are indicated beneath the graph. (H–K) FISH/immunolocalization of 45S rDNA, fibrillarin, 180CEN, and CenH3 in *cdc48a-3* mutant vegetative cell nuclei from mature pollen. (Scale bars: 1 μ m.) Approximately 300 pollen vegetative cell nuclei from *+cdc48a-3* plants were inspected in each experiment, and ~20% of them showed two separate rDNA loci colocalizing with centromeres external to the nucleolus. Equivalent results were obtained with *cdc48a-igi* and *cdc48a-1* alleles.

Active rRNA genes transcribed in the nucleolus are demethylated at promoter CG sites in the 5' external transcribed spacer (ETS) region, whereas silent nucleoplasmic copies are heavily methylated, and loss-of-function *met1* DNA methyltransferase mutant alleles disrupt rRNA gene silencing (24). To test whether the expression of full-set rRNA variants in pollen vegetative cells correlates with rDNA demethylation, we analyzed previously published methylome data (27) derived from purified sperm and vegetative cell nuclei by fluorescence-activated cell sorting (FACS) (9, 28) (Fig. 5G). Consistent with rRNA gene activation, we detected substantially lower levels (average 1.83-fold reduction) of CG methylation in the vegetative cell genome relative to the control sperm genome, which are separated by one mitosis during pollen development.

We found that *cdc48a* mutations have a significant effect on the organization of centromeric heterochromatin and rDNA repeat loci. In contrast to WT vegetative cell nuclei, *cdc48a* vegetative cell nuclei displayed two separate portions of rDNA loci, which are localized at the external periphery of the nucleolus (Fig. 5H). One or both of the two rDNA loci were frequently found to colocalize with condensed centromeric repeats (Fig. 5I) and CenH3 centromeres (Fig. 5J) located external to the nucleolus (Fig. 5K). These results highlight the interrelationship between the centromeric constitutive heterochromatin and rDNA facultative heterochromatin, and reveal that CDC48A is required for the reactivation of silent rRNA genes within the nucleolus.

Discussion

The process of de novo CenH3 assembly at centromeres has been described previously and shown to depend on the histone chaperone HJURP/Scm3 (29, 30) and the Mis18 heterotetramer (31). Here, we describe the active mechanism for the removal of CenH3 from centromeres. Recent studies have shown that sumoylation, as well as ubiquitination, can be a signal for protein removal from complexes and transference into degradation pathways (32, 33). Our results demonstrate that CenH3 is subjected to sumoylation and provide evidence for the CDC48A^{NPL4}-directed centromere disassembly through active unloading of sumoylated CenH3 from native centromeres.

Numbers of SUMO-modified proteins contain the tetrapeptide consensus motif "ΨKXE," where Ψ is a large hydrophobic residue, K is the Lys to which SUMO is conjugated by E2 ligase, X is any amino acid, and E is Glu (34). The *A. thaliana* CenH3 (HTR12) does not possess this motif. Recent proteomics studies showed that a considerable part of sumoylation occurs on nonconsensus sites (35). Of note, HTR12 has XKDΨE and AKRSR sequences that were found to be sumoylated in SAE2 protein from HeLa cells (36, 37).

The molecular chaperone activity of CDC48A^{NPL4} complex members, or their homologs, may complement the proteolytic mechanisms used to reduce the number of inappropriate CenH3 neocentromeres at ectopic sites on chromosomes. If left unchecked, neocentromeres result in the loss of chromosome integrity, which leads to diseases, including cancer, in humans (1, 38). Below a certain threshold of cellular CenH3 levels, cells cease mitosis in *Arabidopsis* (39). Similarly, in human cells, the posttranslational reduction of CenH3 levels results in senescence, where cells lose the ability to divide, likely to prevent defective mitoses (7), which may be a process that requires p97. An intriguing question is how CDC48A^{NPL4} would distinguish between native and ectopically incorporated CenH3 for removal.

The precise relationship between CDC48A^{NPL4}-mediated disassembly of sumoylated CenH3 centromeres, loss of the H3K9me2 heterochromatin mark, and centromeric heterochromatin decondensation is not yet known. It remains unclear how the function of the CDC48A^{NPL4} complex, which does not directly associate with H3K9me2, is coupled to the loss of this mark in the pollen vegetative cell. Stable centromeres are characterized by long arrays of CenH3 nucleosomes, which are conducive to kinetochore formation (1). It is possible that the removal of long CenH3 nucleosome arrays

initiates a broader decondensation of centromeric heterochromatin. Alternatively, the CDC48A^{NPL4} complex may be targeted to other, yet to be defined targets that regulate the large-scale chromatin dynamics. Interestingly, mouse primordial germ cells undergo similar dynamic chromatin reconfigurations, including loss of H3K9me3 and decondensation of chromocenters, during the acquisition of totipotency (40).

Ribosome biogenesis and cell growth are coupled processes (41). Remarkably, RNA polymerase I mutant (*pol I*) pollen from *+pol I* heterozygous plants are fertile, and the *pol I* mutant allele is transmitted paternally to the next generation at a reduced level, whereas it is not transmitted maternally (42). This observation supports the view that the pollen vegetative cell employs an extremely efficient rRNA gene transcription system that allows pollen tube formation with a limited number of Pol I molecules. The requirement of CDC48A for vigorous pollen tube growth and reproductive success (15) suggests that extensive heterochromatin decondensation, rather than being a byproduct of pollen development, is a vital biological process tied to the unique function of the terminally differentiated vegetative cell that does not require centromeres anymore. We propose that CDC48A^{NPL4} decondenses centromeric heterochromatin to "euchromatinize" whole rDNA loci, facilitating rRNA gene transcription by Pol I in the nucleolus, which is essential to support the high metabolic needs of single-celled pollen tubes that elongate by tip growth and transport sperm cells to ovules over long distances. Our results imply that active CenH3 removal from centromeres functions not only to prevent defective cell division but also to promote cell growth.

Mutations in *p97* cause motor neuron degeneration and diseases of the nervous system, such as dementia (43). Future work will show whether the CDC48A-mediated heterochromatin decondensation and bulk rRNA gene activation represent a universal mechanism used by other terminally differentiated cell types, including neurons, in which a single nucleus drives extended tip growth.

Materials and Methods

Plant Lines. The *cdc48a-1* (SALK-064573), *cdc48a-3* (SALK-064893), *npl4a* (SALK-139523), and *npl4b* (SALK-064164) T-DNA insertion lines were obtained from the Nottingham *Arabidopsis* Stock Centre. Plants carrying the T-DNA insertion were selected by PCR using the T-DNA-specific primer and the gene-specific primers in *SI Appendix, Table S6*. The *npl4a/npl4a* and *npl4b/npl4b* single-homozygous mutant plants were selected and crossed, and *npl4a/npl4a +npl4b* and *+npl4a npl4b/npl4b* plants were generated. All of the lines used were in the Col-0 ecotype.

Ethyl Methanesulfonate Mutagenesis and *A. thaliana* Mutant Screening. Seeds harvested from the *pCenH3:CenH3:GFP pLAT:H2B:RFP* transgenic line in the Col-0 ecotype (9) were mutagenized with 0.2% ethyl methanesulfonate. Pollen from M1 plants was screened using a Zeiss M1 inverted microscope supplied with a spinning-disk unit (UltraView VoX; PerkinElmer). The recorded pollen images were processed with Definiens Image Analysis software to identify mutant trinucleate pollen grains showing five CenH3-GFP centromeric foci within the RFP-positive vegetative cell nucleus. A more detailed description is provided in *SI Appendix*.

Mapping the *igi* Mutation. After a back-cross to the parental *pCenH3:CenH3:GFP pLAT:H2B:RFP* line (9), mutant progeny was self-pollinated and each F1 plant was inspected for the *igi* phenotype showing CenH3-GFP foci in the pollen vegetative cell nucleus. Genomic DNA from five *igi* mutant and 115 *IGI* WT plants was pooled separately and subjected to whole-genome sequencing using the Illumina HiSeq2000m platform. Sequence reads were mapped to the reference genome (*A. thaliana* TAIR9, The Arabidopsis Information Resource). CLC Genomics Workbench 4.7.1 was used to identify SNPs, which caused amino acid substitutions and were detected in approximately half of the reads exclusively from the mutant pool due to heterozygosity, and 19 candidate genes were shortlisted. After four more back-crosses, WT and *IGI/igi* mutant progeny were genotyped for the individual candidate genes to determine the causative mutation using primers in *SI Appendix, Table S7*. A more detailed description is provided in *SI Appendix*.

Protein Immunolocalization and FISH. Preparation of pollen nuclei, slide preparation, immunostaining, and FISH were carried out as described (9). Antibodies

used are described in *SI Appendix*. The 45S ribosomal DNA probes were generated from plasmid R2 (44) using Nick Translation Mix (Roche Applied Science).

Analysis of rRNA Variants. Total RNA from 2-wk-old leaves or mature pollen of *A. thaliana* WT (Col-0) plants was isolated using an RNeasy Plant Mini Kit (Qiagen) as previously described (9). FACS-sorted vegetative cell nuclei (9, 28) were directly collected into TRIzol LS Reagent (Life Technologies), and total RNA was isolated according to the manufacturer's protocol. RNA samples were treated with DNase (Thermo Scientific) and precipitated. Analysis of rRNA variants was performed using a OneStep RT-PCR Kit (Qiagen). Primers used for detection of rRNA variants, VAR1–4, (26) are listed in *SI Appendix, Table S8*.

Analysis of rDNA Promoter Fractional Methylation. Bisulfite sequencing reads from previously published methylome data (27) derived from purified sperm and vegetative cell nuclei by FACS were aligned to a copy of the reference sequence of an rDNA 5' ETS region (+1–243), as shown in *SI Appendix, Table S9*, using Bowtie and custom Perl scripts as described (27, 45). For each cytosine residue, the number of reads in which it was sequenced as C or T was used to calculate its average fractional methylation $[\#C/(\#C + \#T)]$ in rRNA gene repeats.

Immunoprecipitation. To coimmunoprecipitate proteins associated with CDC48A-Myc, ~100 μ L of wet-volume, early-stage pollen was lysed in immunoprecipitation buffer [50 mM Hepes-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 1% Protease Inhibitor Mixture (Sigma), and a protease inhibitor tablet (Roche)] by vortexing with glass beads for 10 min. Homogenized pollen was filtered through two layers of 10- μ m nylon mesh to remove pollen debris. The flow-

through fraction was sonicated for 10 min to shear chromatin, and nuclear debris was then removed by centrifugation at 16,100 $\times$ g for 20 min. The supernatant was used for immunoprecipitation utilizing α -Myc or α -GFP magnetic beads (Miltenyi Biotech) according to the manufacturer's manual, except that washing was done with immunoprecipitation buffer.

Western Blot Analysis. Western blot analysis was done as described by Rossmann and Stillman (46) using the antibodies described in *SI Appendix*.

Nano-Liquid Chromatography/MS Analysis. Proteins coimmunoprecipitated with CDC48A-Myc were fractionated by SDS/PAGE using 4–20% (wt/vol) precast acrylamide gels (Bio-Rad). Protein bands were visualized with silver staining, cut out from gel, and processed for MS. The nano-HPLC system used was an UltiMate 3000 HPLC Rapid Separation liquid chromatography nanosystem coupled to a Q Exactive mass spectrometer, equipped with a Proxeon nanospray source (all from Thermo Fisher Scientific). For peptide identification, the RAW files were loaded into Proteome Discoverer (version 1.4.0.288; Thermo Scientific). All thereby created tandem MS spectra were searched using Mascot 2.2.07 (Matrix Science) against the *A. thaliana* protein sequence database.

ACKNOWLEDGMENTS. We thank the Vienna Biocenter BioOptics team for processing automated microscopy images and sorting pollen nuclei, the Protein Chemistry team for MS analyses, Dmitry Smetanin for SNP analyses, Jenny Frost for editing the manuscript, Ingo Schubert for anti-CenH3, Craig Pikaard for rDNA sequences and discussion, and Marjori Matzke and Paul Talbert for discussion. H.T. thanks Mary Coffin for inspiration. The research was supported by Austrian Science Fond Grants P21389-B03 and P24918-B21 (to H.T.), and NIH Grant R01GM069415 (to R.L.F. and D.Z.).

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