Meiotic DNA Repair in the Nucleolus Employs a Nonhomologous End-Joining Mechanism

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Ribosomal RNA genes are arranged in large arrays with hundreds of rDNA units in tandem. These highly repetitive DNA elements pose a risk to genome stability since they can undergo nonallelic exchanges. During meiosis, DNA double-strand breaks (DSBs) are induced as part of the regular program to generate gametes. Meiotic DSBs initiate homologous recombination (HR), which subsequently ensures genetic exchange and chromosome disjunction. In Arabidopsis (Arabidopsis thaliana), we demonstrate that all 45S rDNA arrays become transcriptionally active and are recruited into the nucleolus early in meiosis. This shields the rDNA from acquiring canonical meiotic chromatin modifications and meiotic cohesin and allows only very limited meiosis-specific DSB formation. DNA lesions within the rDNA arrays are repaired in an RAD51-independent but LIG4-dependent manner, establishing that nonhomologous end-joining maintains rDNA integrity during meiosis. Utilizing ectopically integrated rDNA repeats, we validate our findings and demonstrate that the rDNA constitutes an HR-refractory genome environment.

INTRODUCTION

DNA damage, if not reliably repaired, may lead to loss of genetic information, genomic rearrangements, or cell cycle arrest. The most deleterious DNA insults are DNA double-strand breaks (DSBs), which may occur through exposure to genotoxic agents or as a result of errors during endogenous processes, such as DNA replication or meiotic recombination. DSBs can be repaired by either nonhomologous end-joining (NHEJ) or homologous recombination (HR) DNA repair pathways (Ceccaldi et al., 2016). NHEJ, differentiated in canonical NHEJ and microhomology-mediated end-joining pathways, is believed to work during all cell cycle stages, while HR appears to be the most prominent DSB repair pathway during S and G2 phases (Ceccaldi et al., 2016). The common denominator of all NHEJ pathways is direct ligation of the processed DNA ends (McVey and Lee, 2008; Chang et al., 2017). HR utilizes a repair template, either from the sister chromatid or the corresponding homolog, copying information that may have been lost during DNA damage or processing (Ceccaldi et al., 2016).

A special case of HR takes place during meiosis prior to gamete formation in sexually reproducing organisms. Meiosis is characterized by two consecutive cell divisions reducing the genome by half and by recombination of paternal and maternal chromosomes. Meiotic recombination is initiated by enzymatically mediated DNA DSBs, which are a prerequisite for subsequent crossover formation (Hunter, 2015; Mercier et al., 2015). Genomic loci that are composed of repetitive sequences, such as the rRNA gene clusters, present a challenge during meiotic recombination because of nonallelic alignments (Sasaki et al., 2010).

Ribosomal RNA genes (or rDNA) encode the RNA subunits of ribosomes and are highly conserved repetitive regions in the genomes of eukaryotes (Bell et al., 1989; Shaw and Jordan, 1995). The Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 (Col-0) 5S rDNA is located on chromosomes III, IV, and V, and the 45S rDNA is located in subtelomeric clusters on chromosomes II and IV (Copenhaver et al., 1995; Copenhaver and Pikaard, 1996b; Murata et al., 1997). Transcribed 45S rDNA units form the nucleolus organizer regions (NORs) and are part of the nucleolus (Preuss and Pikaard, 2007). In the ecotype Col-0, each of the two 45S rDNA clusters contains ~400 repeats, which are 4 Mb in length (Copenhaver and Pikaard, 1996b). Artificially induced DSBs in the rDNA of mammalian somatic cells lead to transcriptional shutdown, reorganization of the nucleolus, and unscheduled DNA synthesis (van Sluis and McStay, 2015; Larsen and Stucki, 2016; McStay, 2016; Warmerdam et al., 2016). It appears that a first wave of DNA repair operates via NHEJ, and that persisting damage is repaired via HR (Harding et al., 2015; van Sluis and McStay, 2015; Larsen and Stucki, 2016; McStay, 2016). fas1 Arabidopsis mutants, deficient for histone replacement, are characterized by enrichment of γH2Ax at the rDNA, mitotic anaphase bridges between the rDNA loci, and loss of rDNA copy number. rDNA loss in fas1−/−is alleviated in a rad51−/−background, indicating that it is a consequence of HR (Muchová et al., 2015; Varas et al., 2017). In Saccharomyces cerevisiae, meiotic DSBs are suppressed within the rDNA array by the histone deacetylase Sir2 and the AAA+ ATPases Pch2 and Orc1 (Gottlieb and Esposito, 1989; Ozenberger and Roeder, 1991; Mieczkowski et al., 2007; Vader et al., 2011). Sequencing of SPO11-associated oligonucleotides in yeast (Saccharomyces cerevisiae) suggests that very few, if any, meiotic DSBs occur in the rDNA (Pan et al., 2011). A study in maize identified a significant portion of meiotic DSBs within the rDNA (He...
et al., 2017), but the authors state that the number of rDNA copies used in their calculations was likely an underestimate, and therefore, meiotic DSBs in the rDNA may be quite infrequent.

Given the importance of rRNA genes and their central function for any living organisms, we set out to identify the molecular mechanisms that maintain rDNA clusters over generations in Arabidopsis.

RESULTS

Both 45S rDNA Loci Are Transcribed at the Onset of Meiosis and Localize within the Nucleolus

To understand how rDNA repeat units are maintained during meiosis, we first determined their spatial and temporal distribution and their transcriptional status. Previously, transcription has been correlated with recruitment into the nucleolus (Preuss and Pikaard, 2007) and with genome instability due to collisions of replication and transcription machineries (Larsen and Stucki, 2016). Utilizing a fluorescence in situ hybridization (FISH) probe against the 45S rDNA region and an antibody that binds DNA:RNA hybrids (S9.6, R-loops; García-Rubio et al., 2015), we established that the rDNA arrays on chromosomes 2 and 4 are transcribed early in meiosis (n = 12 cells; Figure 1A; Supplemental Figure 1A). By contrast, only NOR4 in Arabidopsis Col-0 adult somatic cells is actively transcribed, whereas NOR2 is silenced (Mohannath et al., 2016; n = 10 cells; Figure 1B). We validated these findings by extracting meiocytes of different stages from young buds and performing rRNA expression analysis of specific-length polymorphisms (short repetitive sequences) present in the 3' ETS (Pontvianne et al., 2007; Durut et al., 2014; Mohannath et al., 2016). In adult leaves, predominantly rRNA variants 2 and 3 from NOR4 are expressed, whereas variants 1, 3, and 4, residing on both NOR2 and NOR4, are detected in early and late meiocytes. In siliques, containing fertilized embryos, all rRNA variants are strongly expressed (Figure 1C; Supplemental Figure 1B).

We performed whole-mount FISH on anthers to generate three-dimensional reconstructions of meiotic cells. After the premeiotic S-phase, the NORs are localized in a canonical somatic configuration around the nucleolus (Pontvianne et al., 2013; n = 30 cells from different anthers), while during the meiotic prophase, from leptotene onward, they localize within the nucleolus (n = 30 cells from different anthers; Figure 1D; Supplemental Movies 1 and 2). Moreover, from zygotene onward, both NORs form a unified structure and only disengage at the end of meiotic prophase I, during diakinesis, when the nuclear envelope breaks down and paired chromosomes condense in preparation for segregation. In agreement with the rRNA expression data, DNA:RNA hybrids, which mark actively transcribed genes, colocalized with the rDNA throughout prophase I of meiosis (Supplemental Figure 1B).

Meiotic rDNA Is Embedded in a Unique Chromatin Environment

With the rDNA loci residing in the nucleolus from leptotene onward, they are partitioned from the rest of the chromatin during meiosis. To probe the functional relevance of this sequestration, we first analyzed potential differences in chromatin architecture and modification. During leptotene and zygotene, the axis protein ASY1 or the synaptonemal complex (SC) protein ZYP1 fails to colocalize with the 45S signal (rDNA; n = 17; Figure 2A). In pachytene, the formation of the SC corresponds to extended stretches of ZYP1 along the paired chromosomes and the
depletion of ASY1 (Higgins et al., 2005). Remarkably, at this stage, the rDNA loci acquire a prominent ASY1 signal, while the rest of the chromatin is largely devoid of it ($n = 25$; Figure 2A). Whole-mount immuno-FISH, which preserves the spatial relation of the nucleolus and chromatin within the nucleus, revealed that the nucleolus itself is free of ASY1 ($n = 32$ cells; Figure 2B; Supplemental Movies 3 and 4). To understand the three-dimensional (3D) relationship of rDNA, axis, and SC, we simultaneously stained for ASY1 and ZYP1 on spread chromatin of Pollen Mother Cells (PMCs) at pachytene and imaged the meiocytes using super-resolution confocal microscopy. At a 160-nm resolution, it is apparent that the strong ASY1 signals represent four spatially separated chromatin stretches devoid of any ZYP1 signal. The previous experiments established that these stretches represent the NORs of chromosomes 2 and 4. This demonstrates that, in contrast with the rest of the genome, the homologous chromosomes at the NORs do not undergo synapsis ($n = 5$ cells; Figure 2C; Supplemental Movie 5).

We furthermore used super-resolution microscopy and found that both REC8 (Cai et al., 2003) and SCC3 (Chelysheva et al., 2005) localize to chromosome axes during zygotene ($n = 18$ cells, REC8; $n = 11$ cells, SCC3). During pachytene, with ASY1 predominantly localizing to rDNA, only SCC3 but not REC8 colocalized with ASY1 ($n = 7$ cells, REC8; $n = 8$ cells, SCC3; Figure 2D; Supplemental Figure 2). This demonstrates that rDNA acquires a cohesin complex that excludes the

**Figure 1.** Both NORs Are Highly Dynamic Regions and Transcribed During Meiosis.

(A) and (B) Nuclei stained with S9.6 antibody directed against DNA:RNA hybrids (red), 45S rDNA visualized with a specific FISH probe (green), and DNA stained with DAPI; white). (A) Spread nucleus of a pollen mother cell at leptotene stage. All NORs have a strong S9.6 signal. (B) Spread somatic cell nucleus. Arrows indicate the two active NORs (green) colocalizing with the S9.6 signal (red). Scale bars = 10 μm. (C) Top: Illustration of Arabidopsis chromosomes 2 and 4 (Chr2 and Chr4); the localization of NORs, the corresponding 45S variants, and their transcriptional status in somatic cells are indicated. Bottom: Expression analysis of rDNA variants by RT-PCR during prophase I (early meiosis [Early]), post-prophase I and meiosis II (late meiosis [Late]), siliques containing fertilized embryos (Embryos), and in somatic tissue (adult leaves [Leaves]). The agarose gel separates the four DNA bands representing rDNA 3′ external transcribed sequence (3′ETS) variants 1 (VAR1), VAR2, VAR3, and VAR4. (D) Single optical layer of meiotic nuclei after a whole-mount FISH preparation. 45S rDNA has been visualized via FISH using a specific probe (red), and DNA has been stained with DAPI (cyan). Dashed circles highlight the nucleolus. Scale bars = 2 μm.
Monomethylation of H3K27 is strongly enriched at the somatically silenced NOR2 and is introduced by the two methyltransferases ATXR5 and ATXR6 (Mohannath et al., 2016). By contrast, H4Ac4, a known euchromatin mark, is mainly associated with the active NOR4 in adult somatic cells. Histone Deacetylase 6 (HDA6) has been shown to remove H4 acetylation from NOR2, contributing to its silenced state (Probst et al., 2004; Earley et al., 2006; To et al., 2011). We performed immuno-FISH on spread...
chromatin from PMCs and found that in zygotene, when all 45S rDNA resides within the nucleolus and is assembled into a unified structure, both NORs are enriched in H3K27me1 but depleted in H4Ac4 (n = 12 cells, H3K27me1; n = 16 cells, H4Ac; Figure 3; Supplemental Figure 3), indicating that their somatically distinct chromatin environments have been matched, and that at this stage both NORs carry marks of a repressed chromatin state. To validate these results, we performed similar analyses in the corresponding mutant backgrounds. The atrx5 atrx6 double mutants do not show H3K27me1 marks on meiotic NORs (Supplemental Figure 3A), and meiocytes of the hda6-6 mutant display NORs that are enriched in H4Ac4 (Supplemental Figure 3B). Moreover, the NORs in hda6-6 mutants do not form a compact, unified structure, with most cells displaying two separate rDNA clusters (90% of all observed cells at zygotene/pachytene stage; n = 31), indicating that H4 deacetylation is needed to maintain rDNA clustering within the nucleolus during meiosis. Similarly, in atrx5 atrx6 double mutants, 30% of all observed cells (n = 26) have separated rDNA clusters (Figure 4A; Supplemental Figure 4A).

The Nucleolus Shields rDNA from SPO11-Mediated DSB Formation and Homologous Recombination

To determine whether the specific meiotic chromatin environment of the 45S rDNA has an impact on DSB formation and repair, we performed immuno-FISH with an RAD51 antibody (Kurzbauer et al., 2012) and a 45S DNA probe on chromatin spreads (Figure 4A; Supplemental Figure 4A). On average, only 3.09 ± 2.62 (2.7%) of all RAD51 foci were localized at the rDNA (166 ± 65 RAD51 foci at leptotene/zygotene in total; n = 29). This is significantly lower (P = 0.0001) than the expected 11.8 RAD51 foci on rDNA, assuming a random distribution across the genome (on average, 7.6% of the area of a spread meiotic nucleus is rDNA; n = 15; in agreement, 7% of the Arabidopsis Col-0 genome has been estimated to be rDNA [Copenhaver and Pikaard, 1996a]). This indicates a mechanism that shields the rDNA from acquiring RAD51 foci. We therefore investigated RAD51 foci numbers in spreads of PMCs of hda6-6 plants. We observed a significant increase in RAD51 foci colocalizing with the rDNA compared with wild type (17.9 ± 10.6 on rDNA; n = 18; P < 0.0001; Figure 4A and 4B; Supplemental Figure 4A). We also analyzed plants deficient in

![Figure 3](image-url)
the conserved plant nucleolin gene NUC2 that have altered rDNA transcription and NOR morphology compared with wild-type plants (Pontvianne et al., 2007; Durut et al., 2014) and observed elevated numbers of RAD51 foci colocalizing with the rDNA compared with the wild type (8.18 ± 4.86 on rDNA; n = 27; P < 0.0001; Figure 4B; Supplemental Figure 4C). We also investigated pch2 mutant lines (Lambing et al., 2015) motivated by the findings in yeast that the PCH2 protein is instrumental in protecting the repetitive rDNA from recombination events (Vader et al., 2011) and detected a significant increase of RAD51 foci numbers on rDNA compared with wild type (5.4 ± 2.71 on rDNA; n = 26; P < 0.0001). Also, in atrx5 atrx6 double mutants, a significant increase of RAD51 foci numbers colocalizing with the rDNA was observed (6.69 ± 3.25 on rDNA; n = 23; P < 0.001; Figure 4B; Supplemental Figure 4C). It is important to note that the total (genome-wide) number of RAD51 foci in all tested mutant lines did not significantly differ from wild type (P > 0.39), apart from plants carrying the nuc2-2 mutant allele with slightly reduced overall RAD51 foci numbers (P < 0.05; Supplemental Figure 4B). The increase of 45S rDNA–associated RAD51 foci in hda6-6 mutants is mostly dependent on SPO11. In spo11-2-3 hda6-6 double mutants, we observe a significant decrease of RAD51 foci colocalizing with the rDNA (10.72 ± 5.4; n = 18) when compared with hda6-6 single mutants (17.94 ± 10.6; n = 18; P < 0.03). A similar trend is apparent in spo11-2-3 nuc2-2 double mutants (6.21 ± 3; n = 14) compared with nuc2-2 single mutants (8.18 ± 4.86 on rDNA; n = 27; P = 0.2; Figure 4B). Other breaks (RAD51 foci) may be explained by collisions between replication and transcription machineries (Aguilera and Gaillard, 2014), due to elevated rDNA transcription in the hda6-6 mutants.

RAD51 at 45S rDNA in hda6-6 and nuc2-2 meiocytes may support homologous recombination DNA repair events, with the potential of nonallelic interactions among rDNA repeats. To test this, we performed FISH using probes for 45S and 5S rDNA loci, allowing us to distinguish the five Arabidopsis chromosomes. In wild-type plants, only 24% (n = 17) of meiocytes at diakinesis

**Figure 4.** The nucleolus shields rDNA from meiotic DSB formation and deleterious HR.

(A) Immuno-FISH spreads of wild-type (Col-0) and mutant (hda6-6) PMCs at zygotene. The axis has been stained with an anti-ASY1 antibody (green) and the recombinase RAD51 with a specific antibody (red). The 45S rDNA has been visualized with a specific FISH probe (white). Arrows indicate separated NORs in the hda6-6 mutant.

(B) Total RAD51 foci colocalizing with the 45S rDNA probe. Statistical analysis was performed using a Mann-Whitney test.

(C) Graph depicting the percentage of nuclei with associated or clearly separated NORs counted from PMC spreads at the diakinesis stage. The DNA has been hybridized with a 45S rDNA probe (green) and a 5S rDNA probe (red). Statistical analysis was performed using a binary logistic regression. Arrow indicates two fused NORs. Scale bars = 10 μm.
show interconnected 45S rDNA signals from chromosomes 2 and 4, but in hda6-6 and nuc2–2, 82% (n = 17; P = 0.0004) and 59% (n = 15; P = 0.015) of meiocytes, respectively, had these connections (Figure 4C).

Repair of rDNA Depends on NHEJ Mechanisms

To directly investigate break formation in the 45S rDNA and identify the corresponding DNA repair mechanism, we quantified rDNA integrity at the end of meiosis in wild-type and mutant lines. We performed FISH using 45S rDNA probes on meiocytes at anaphase II to the tetrad stage and counted the number of individual 45S rDNA signals. Eight nonfragmented rDNA signals are expected in each wild-type tetrad (two NORs per haploid microspore cell). 45S rDNA fragmentation is expected to result in tetrads with more than eight signals. We observed no more than eight 45S rDNA signals in wild-type meiocytes (n = 25) but saw significantly more tetrads with greater than eight fragments in lig4-4 (21%; n = 32; P = 0.0029 compared to Col-0) and mre11-1 (16%; n = 31; P = 0.017) mutants (Figure 5A to 5C; Supplemental Figure 5). LIGASE4 is a well-conserved hallmark factor in the canonical NHEJ DNA repair pathway (Friessner and Britt, 2003). MRE11 is required for both HR and microhomology-mediated end-joining. To test if HR has a role in DNA repair of 45S rDNA during meiosis, we quantified 45S rDNA FISH signals in com1-1 and rad51-1 mutants. Canonical meiotic breaks are not repaired in these two mutants, leading to severe chromosome fragmentation during meiosis (Li et al., 2004; Uanschou et al., 2007), but the 45S rDNA is not fragmented. The fragmentation in lig4-4 and mre11-1 mutants is not alleviated in the corresponding spo11-2-3 lig4-4 (22% of cells have >8 rDNA signals; n = 22; P = 0.004) and spo11-2-3 mre11-1-4 (14% of cells have >8 rDNA signals; n = 27; P = 0.0185) double mutants. Consistently, MRE11 colocalizes with the 45S rDNA during pachytene (Figure 5D). Furthermore, fragmentation observed in lig4-4 rad51-1 double mutants is not different compared with lig4-4 single mutants (P = 0.86; Figure 5C; Supplemental Figure 5).

Essential for maintaining 45S rDNA in somatic cells, we determined the relative rDNA copy number by qPCR in wild-type and lig4-4 mutant lines grown side by side. We picked rosette leaves of different ages from plants grown for 67 d under short-day conditions, which strongly delays the transition from a vegetative meristem, producing leaves, to a generative meristem, producing flowers. This allowed us to assay cumulative 45S rDNA copy numbers within meristematic tissues. Newer leaves (from older meristems) had a significant reduction in 45S rDNA copy number in lig4-4 plants compared with older leaves that had been produced earlier during meristematic growth (Figure 5E), but wild-type plants showed no differences across the time course (P > 0.03). The effect is most pronounced in young leaves from relatively old meristems. We also performed this experiment with plants grown under long-day conditions and found no difference between wild-type and lig4-4 mutants (Figure 5F).

The rDNA Creates an HR-Refractory Chromatin Environment

To test the idea that the 45S rDNA is sequestered in an HR-refractory chromatin environment during meiosis, we generated plant lines carrying single ectopic rDNA insertions (ErDNA), each containing only a few rDNA units, to test the effect of rDNA on meiotic recombination (Figure 6; Supplemental Figure 6). The transformation (T-DNA) vector contained one complete rDNA unit (variant 1) and includes a unique sequence of 20 nucleotides within a variable portion of the 25S rDNA region, enabling its specific detection (Wanzenböck et al., 1997). Only transformants with a 3:1 segregation pattern, indicating a single locus insertion, were used. The insertion sites were mapped, ectopic rDNA copy numbers were evaluated, and active transcription was confirmed (Supplemental Figure 6A and 6B). To control for T-DNA-related effects not functionally linked to the rDNA, we obtained T-DNA insertion lines from T-DNA insertion collections near the genomic positions of ErDNA3 and ErDNA5 (TDNA3: SALK_137758; TDNA5: SAIL_713_A12). We generated a negative control using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (CAS9; Fauser et al., 2014; Steinert et al., 2015) to delete the rDNA portion, but left the T-DNA backbone intact, within the ErDNA5 transgene (ErDNA5-del; Supplemental Figure 6C). For all analyses described below, lines homozygous for the ectopic rDNA insertion sites have been used.

We designed FISH probes that hybridize to a 500-kb window surrounding the ectopic rDNA integration sites on chromosomes 3 and 5. We performed whole-mount immuno–FISH experiments to determine the relative position of these regions in relation to the nucleolus, in wild-type plants compared with ErDNA3, ErDNA5, and ErDNA5-del (Figure 6A; Supplemental Movie 6). The presence of ectopic rDNA caused the corresponding 500-kb regions on chromosome 3 or 5 (ErDNA3, ErDNA5) to localize or associate with the nucleolus significantly more often than the same region without the ectopic repeat (wild type, ErDNA5-del). To quantify the localization patterns, we sorted the FISH signals into three classes (Figure 6A). Furthermore, the genomic regions on chromosomes 3 and 5 were significantly more often paired with their corresponding homologous partner sites when the ectopic rDNA was present (Figure 6B).

These observations demonstrate that ectopic rDNA units mirror the characteristics of endogenous NORs. We therefore asked whether the presence of an ectopic rDNA unit also decreases the number of RAD51 foci in its vicinity. We performed immuno–FISH experiments, simultaneously detecting the ErDNA integration site (a 500-kb window around the target sites), ASY1 to mark the chromosomal axis (for staging), and RAD51 (Figure 6C). We found that the number of RAD51 foci within the 500-kb window is significantly reduced in the presence of the ectopic rDNA in ErDNA5 (0.9% of all RAD51 foci colocalize with the FISH signal; n = 19; P = 0.0004) compared with wild type (2.3% colocalization; n = 26). The TDNA5 (1.9% colocalization; n = 28; P = 0.7) and ErDNA5-del (1.6% colocalization; n = 29; P = 0.8) control lines were not significantly different from wild type. We obtained similar results for ErDNA3 with only 0.6% of RAD51 foci colocalizing with the FISH signal (n = 25; P = 0.0001), while in wild-type and TDNA3 control cells, 1.3% (n = 24) and 1.7% (n = 24; P = 0.07) of RAD51 foci colocalized with the FISH signal, respectively (Figure 6D). The total numbers of RAD51 foci were not significantly different in any of the lines (P ≥ 0.7 for all cases; Supplemental Figure 6D). These results demonstrate that ectopic 45S rDNA units inhibit
Figure 5. The rDNA is Repaired by NHEJ.

(A) Left: illustration representing a meiotic tetrad stage of a wild-type (Col-0) meiocyte. Middle: spread nuclei of a meiocyte at the tetrad stage followed by FISH to visualize the 45S rDNA (red) and the centromeres (green). Right: panel depicts only the FISH signals that visualize the 45S rDNA (red), showing eight distinct signals.

(B) Left: illustration representing a meiotic tetrad-like stage of an mre11-4 mutant meiocyte. Middle: spread nuclei of a meiocyte at a tetrad-like stage followed by FISH to visualize the 45S rDNA (red) and the centromeres (green). Right: panel depicts only the FISH signals that visualize the 45S rDNA (red), showing 10 distinct signals.
recombination intermediates in their vicinity, in line with our observations with the endogenous NORs.

To measure the effect of 45S rDNA on crossover frequency directly, we measured genetic distances in the presence or absence of ectopic rDNA units. We crossed the ErDNA lines to tester lines carrying flanking transgenic markers in a qrt1 background. The transgene markers, called FTLs (Fluorescent-Tagged Lines), encode fluorescent proteins expressed by the LAT52 postmeiotic, pollen-specific promoter (Francis et al., 2007). The qrt1 background causes the pollen products of each meiosis to be released as a tetrad, thus enabling tetrad analysis. The genetic distance between pairs of FTL markers can be calculated by scoring their segregation in the pollen tetrads (Berchowitz and Copenhaver, 2008).

We flanked the ectopic 45S rDNA integration site on chromosome 2 with two FTL markers (6.7 Mb interval) that show a genetic distance of 22.2 cM (n = 1773 tetrad, four plants) in wild-type plants but only 19.4 cM (n = 1688 tetrad, 5 plants; P = 0.003) in the presence of ectopic 45S rDNA (line ErDNA2-FTL). We observed a similar result with line ErDNA3. Flanking the ectopic 45S rDNA integration site on chromosome 3 with two FTL markers (7.9 Mb interval) that show a genetic distance of 41.6 cM (n = 1088 tetrad, six plants) in wild-type plants, we obtained a significantly reduced genetic distance in the presence of ectopic rDNA (line ErDNA3-FTL; 31.1 cM; n = 730 tetrad, six plants; P = 0.0009; Figure 6E). The ectopic 45S rDNA integration sites on chromosomes 2 and 3 reside within recombination-proficient genomic regions (Choi et al., 2013), facilitating detection of changes in recombination frequencies. By contrast, the integration site of ErDNA5 on chromosome 5 resides within a genomic region that also contains a recombination “cold spot.” We flanked the ErDNA5 integration site with two FTL markers (1.7 Mb interval), which have a genetic distance of 6.1 cM (n = 4682 tetrad, six plants) in wild-type plants. In the presence of ectopic 45S rDNA (line ErDNA5-FTL), the genetic distance is not significantly different (6.8 cM; n = 2691 tetrad, four plants; P = 0.07). These results suggest that crossover (CO) suppression by 45S rDNA acts locally and in recombination-competent contexts (Choi et al., 2013). The results with ErDNA5 suggest that rDNA-mediated CO suppression is not additive with the influence of existing cold spots.

**DISCUSSION**

Over the last decades, many factors and mechanisms that influence meiotic recombination have been identified (Hunter, 2015; Mercier et al., 2015). However, the question of how repetitive genetic elements are stably inherited remains open. Sequence repeats create a liability during recombination because they can undergo nonallelic exchange and are a potential source of deletions, duplications, inversions, or translocations.

Our study, utilizing the model plant Arabidopsis, supports a model in which the highly repetitive 45S rDNA arrays are protected from SPO11-induced meiotic DSBs as well as the meiotic recombination machinery by their recruitment into the nucleolus, and that DNA lesions within the 45S rDNA are repaired via non-HR pathways.

**The rDNA Creates a Unique Chromatin Environment during Meiosis**

We show that in Arabidopsis Col-0, both 45S rDNA arrays, located on chromosomes 2 and 4, become transcriptionally active at the onset of meiosis in contrast with adult somatic tissues, in which only the chromosome 4 array is active (Tucker et al., 2010). Earlier studies demonstrated that in embryos and seedlings, both rDNA arrays are transcriptionally active, until one array is silenced (Pontvianne et al., 2007). Our data reveal that the initial reactivation occurs before fertilization during meiosis. It is not clear if transcription is maintained over the entire course of meiosis since, in somatic cells, rDNA transcription is silenced upon mitotic entry (Klein and Grummt, 1999b). We propose that transcriptional activity of both 45S rDNA arrays is crucial for their association with the nucleolus in the prophase of meiosis I. Grob et al. (2014) showed that in human HT1080 cells, a synthetic NOR sequence would only form a new nucleolus if actively transcribed. Consistently, the actively transcribed, ectopic rDNA units are preferentially associated with the nucleolus.

During meiosis I, both NOR2 and NOR4 obtain identical patterns of chromatin modifications and fuse into a unified structure, indicating that the somatic distinction of the two is alleviated. 45S rDNA is also set apart from the rest of the genome during leptotene and zygotene by the exclusion of the axis protein ASY1, which is also involved in establishing interhomolog bias during meiotic HR (Sanchez-Moran et al., 2007). Based on those observations, we hypothesize that its exclusion from the 45S rDNA (and the nucleolus in general) relaxes interhomolog bias. Only after genome-wide DSB formation and repair has been accomplished does ASY1 localize to the 45S rDNA in pachytene while being removed from other genomic sites. During pachytene, the SC is established between homologous chromosomes but it is omitted in the region of 45S rDNA. The SC not only stabilizes interhomolog pairing events but also promotes the formation of crossovers (Mercier et al., 2015). Further evidence that the 45S rDNA does not acquire a canonical meiotic chromosome morphology is the absence of the meiosis-specific cohesin subunit REC8, which is part of the meiotic axis and

**Figure 5.** (continued).

(C) Quantification of 45S signals per individual meiocyte. Only stages from anaphase II to tetrad were analyzed. lig4-4, mre11-4, spo11-2-3 lig4-4, and spo11-2-3 mre11-4 show an increase in 45S foci numbers compared with Col-0. Statistical analysis was performed by using a binary logistic regression.

(D) Immuno-FISH spreads of wild-type (Col-0) PMCs at leptotene, zygotene, and pachytene. The axis has been stained with an anti-ASY1 antibody (green) and MRE11 with a specific antibody (red). The 45S rDNA has been visualized with a specific FISH probe (white).

(E) Graph depicting the loss of 45S copy numbers in an lig4-4 mutant plant, compared with wild type (Col-0), grown under short-day conditions. t0 to t3 refers to the age (young to old) of the meristem when leaves were generated. Statistical analysis was performed using a t test. Scale bars = 10 μm.
Figure 6. Ectopically Integrated rDNA Units Associate with the Nucleolus, Promote Homolog Pairing, and Suppress Meiotic Recombination.

(A) Graph depicts incidence of different classes of nucleolus association of genomic regions with (ectopic rDNA 3 [ErDNA3], ErDNA5) or without (Col-0) the ectopically integrated rDNA and in a control line (ectopic rDNA 5 deleted [ErDNA5-del]). Data have been obtained from whole-mount FISH preparations of anthers. DNA was hybridized with BAC probes for the 500-kb regions of interest and with a 45S rDNA probe. Statistical analysis was performed using a binary logistic regression.

(B) Graph depicts incidence of homologous alignments of genomic regions with (ErDNA3, ErDNA5) or without (Col-0) the ectopically integrated rDNA and in control lines (T-DNA, ErDNA5-del). Data have been obtained from Immuno-FISH preparations. DNA was hybridized with BAC probes for the 500-kb regions of interest. Statistical analysis was performed using a binary logistic regression.

(C) Immuno-FISH spreads of wild-type (Col-0) PMC at zygotene (single 160-nm optical layer shown). The axis has been stained with an anti-ASY1 antibody (green) and the recombinase RAD51 with a specific antibody (red). The 500-kb region of interest on chromosome 5 has been visualized with a specific FISH probe (white). Small panels on the right demonstrate RAD51–FISH probe colocalization analysis. Only RAD51 foci that had an overlap of at least 50% with the FISH signal were counted as “colocalizing.”

(D) Graph showing the percentage of RAD51 foci colocalizing with a 500-kb region on chromosome 3 or chromosome 5 in relation to the total RAD51 foci counts per meiocyte. Colocalizing events were counted in wild type (Col-0), ErDNA lines, and control lines (T-DNA, ErDNA5-del). Statistical analysis was performed using a Mann-Whitney test.

(E) Crossover frequencies measured by fluorescent pollen markers (FTLs) in three different intervals. Genetic distances were analyzed in the absence (wild type, Col-0) or presence (ErDNA2, ErDNA3, and ErDNA5) of ectopic rDNA repeats. Statistical analysis was performed using a binary logistic regression. Scale bar = 2 μm.
helps promote interhomolog interactions (Schwacha and Kleckner, 1997; Klein et al., 1999a; Cai et al., 2003). Despite its absence, sister chromatids in the 45S rDNA regions are held together, and the hawk protein SCC3 (Chelysheva et al., 2005) can be detected there, suggesting that a non-meiosis-specific cohesin variant provides sister chromatid cohesion for 45S rDNA.

**DNA Lesions in rDNA Are Repaired by NHEJ during Meiosis**

Our experiments revealed that the number of DNA lesions, as measured by RAD51 foci, is significantly lower in 45S rDNA compared with the rest of the genome. This suggests that the 45S rDNA is shielded from the meiotic recombination machinery. Indeed, mutants with compromised nuclear integrity (hda6-6, nuc2-2; Earley et al., 2006; Durut et al., 2014) have significantly more RAD51 foci, which are then SPO11 dependent. As a direct readout for DNA lesions, we quantified the number of 45S rDNA loci at the end of meiosis II. We found that 45S rDNA integrity is only affected in mutants acting in NHEJ DNA repair pathways (lig4, mre11-4) but not in mutants acting in HR repair (com1, rad51). In an spo11-2-3 mutant background, 45S rDNA fragmentation observed in lig4-4 and mre11-4 is not alleviated. This demonstrates that 45S rDNA is protected from SPO11-mediated DSB formation, and that SPO11-independent breaks that occur in the rDNA are shielded from HR and repaired via NHEJ pathways. Interestingly, LIG4 is also needed to maintain 45S rDNA during extended somatic growth. In this context, it is important to mention two recent studies that highlight the involvement of Fanconi Anemia Complementation group J (FANCJ) and Regulator of Telomere Helicase (RTHEL) helicases in 45S rDNA maintenance, most likely by resolving complex replication intermediates (Röhrig et al., 2016; Dorn et al., 2019). Future studies are needed to clarify the epistatic relation of these factors with LIG4.

**The 45S rDNA Is Sequestered in an HR-Refractory Chromatin Environment**

Our results suggested that 45S rDNA constitutes a local, HR-refractory chromatin environment. To test this prediction, we examined ectopically integrated 45S rDNA units. Indeed, the ectopic repeats are actively transcribed, associate predominantly with the nucleolus, and promote pairing of the corresponding genomic regions. Importantly, the DNA around the ectopic rDNA sites acquired fewer RAD51 foci, and meiotic recombination was significantly reduced at two of the sites that reside in recombination-competent regions. Our results also suggest that the sequence of 45S rDNA is not itself CO suppressive, but rather that it becomes suppressed when it is sequestered in the nucleolus and acquires a unique chromatin signature.

Our study thereby provides a comprehensive answer to the question of how the repetitive rDNA arrays are faithfully inherited.

**METHODS**

**Arabidopsis Growth Conditions**

Arabidopsis (Arabidopsis thaliana) seeds were stratified in water in the dark at 4°C for 2 d before sowing on soil/perlite 3:1 mixture (ED 63, Premium Perlite). Pots were covered with a transparent lid until cotyledons were fully developed and first primary leaves visible. Plants were grown under long-day conditions in controlled-environment rooms (16 h of light, 8 h of darkness, 60–80% humidity, 21°C, 15,550 lux, T5 Tube illumination) or under short day conditions (8 h of light, 16 h of darkness, 60–80% humidity, 21°C, 15,550 lux light intensity).

**Arabidopsis lines used in this study were as follows:** com1-1 (Janschou et al., 2007), mre11-4 (Šamanič et al., 2016), hda6-6 (Murfett et al., 2001; Probst et al., 2004; Earley et al., 2006), atrx5-1 atrx6-1 (Jacob et al., 2009), phz2-1 (Lambing et al., 2015), nuc2-2 (Durut et al., 2014), spo11-2-3 (Hartung et al., 2007), lig4-4 (Heacock et al., 2007), and rad51-1 (Li et al., 2004).

**Selection of Transgenic Lines**

Arabidopsis plants were transformed by the floral dip method (Clough and Bent, 1998). Transgenic plants carrying a BASTA resistance gene were selected from a population by spraying the respective herbicide (BASTA, 1:750 in distilled water, Bayer CropScience) when plants had at least two true leaves. Spraying was repeated every other day until survivors were clearly distinguishable from nonresistant plants. Transgenic lines carrying the Venus-yellow fluorescent protein gene under the At253 seed storage promoter were selected under a fluorescence stereomicroscope by collecting seeds positive for yellow fluorescent protein fluorescence.

**Generation of an Antibody Directed against Arabidopsis ASY1**

The full-length coding sequence of ASY1 was amplified using primers ASY1-Xhol-fwd and ASY1-PacI-rev (primer sequences in the Supplemental Table) and cloned into pFastBac HT A vector for expression in Hi5 insect cells. Both insert and vector were digested with Xhol and PacI restriction enzymes. Transformation and expression of ASY1 was performed at the Vienna BioCenter Facilities (VBCF) protein technologies facility.

The cell pellet was frozen at −80°C and thawed on ice. Subsequent resuspension of the pellet in ice-cold buffer A (1-g pellet per 5 mL of 50 mM phosphate buffer, pH 8, 500 mM NaCl, 2 mM β-mercaptoethanol, 0.05% Nonidet P-40, complete Mini EDTA-free; Roche) was followed by sonication on ice, and an additional round of centrifugation (20,000g, 30′, 4°C) yielded a precleared lysate that was incubated with Ni-beads (Profinity IMAC-Ni-charged resin; Bio-Rad) for 1 h at 4°C. The Ni-beads were washed three times with buffer A, and the recombinant 6×His-tagged proteins were eluted with 1 M imidazole, pH 8, in buffer A. The purity and concentration of the recombinant proteins in the eluates were analyzed by SDS-PAGE using Coomassie Brilliant Blue R 250 staining and Bradford assay according to standard techniques. After removal of imidazole, the protein solution was concentrated with the help of Vivaspin 20 10,000 MWCO centrifugal concentrators (Sartorius Stedim Biotech), and gel filtration was performed using an AKTA fast protein liquid chromatography system and a Superdex 200 16/60 column (GE Healthcare Life Sciences). The fractions that contained the His-tagged ASY1 protein were pooled, and ~0.5 mg of the affinity-purified recombinant protein was used for antibody production performed by Eurogentec (Belgium).

**Generation and Characterization of Arabidopsis ErDNA Lines**

Arabidopsis plants were transformed via the floral dip method with Agrobacterium tumefaciens cells containing the R4 binary vector. Transformed plants were selected on Murashige and Skoog plates (7 g/L bacto agar, 500 mg/L carbenicillin, 12 mg/L glucose and ammonium) supplemented with 30 μg/mL hygromycin B. Resistant plants were transferred from the plates to soil pots and placed in growth chambers. T3 lines that segregated 3:1 were selected as single insertion lines. To map the
Expression Analysis of Endogenous rDNA Variants and Ectopically Integrated rDNA Repeats

Expression analysis of rDNA variants (length polymorphism in the 3′-ETS; Pontvianne et al., 2010) was performed by RT-PCR utilizing staged PMCs. Inflorescences from primary and secondary shoots were collected, and each bud was dissected individually. Anthers were modestly squashed to force the release of columns (meiotic cells in syncytia). These were collected in artificial pond water (0.5 mM NaCl, 0.2 mM NaHCO3, 0.05 mM KCl, 0.4 mM CaCl2 [Miller and Gow, 1989]). All meiocytes in prophase I were pooled. Anthers that released single entities (dyads and tetrads) were grouped as meiosis I and II. Twenty to 30 anthers per category were collected and frozen immediately in liquid nitrogen. To extract RNA, the SV Total RNA Extraction Kit (Promega) was used following the product specifications. For reverse transcription of the RNA to cDNA, the iSCRIPT cDNA synthesis Kit (Bio-Rad) was used following the product specifications. Finally, to amplify the different rRNA/rDNA variants, a specific primer pair (3SrRNAVAR and 5SrRNAVAR) was used, as described by Pontvianne et al. (2010).

The specific expression of ErDNA was determined via RT-PCR using primers (BsrEII-RTag-Fw and rDNA-in-rv) directed against a short, integrated stretch of sequence (also including a BsrEII site) in the 25S rDNA. The BsrEII site served as an ErDNA-specific primer landing platform to exclusively amplify RNA transcribed from the ectopically integrated rDNA repeat(s). In all cases, expression of the ACTIN-7 (AT5G09810) gene served as reference (actin_ampl3_dn and actin_ampl3_up; see the Supplemental Table for primer sequences).

Determination of Endogenous and Ectopic rDNA Repeat Numbers

DNA was extracted by crushing leaves in UREA buffer (0.3 M NaCl, 30 mM TRIS-Cl, pH 8, 20 mM EDTA, pH 8, 1% [w/v] N-lauroylsarcosine, 7 M urea) and subsequently purified with phenol:chloroform:isoamylalcohol (25:24:1). The qPCR reaction was performed using the KAPA SYBR FAST kit following the product specifications. To quantify 45S rDNA copy numbers, 20–30 ng of genomic DNA was used together with one primer pair (18sRealDn and 18sRealup) as previously described for Arabidopsis (Muchová et al., 2015). The amplification of the rDNA and of the reference gene ACTIN-7 was performed in separate wells and in three technical replicates. The analysis was conducted with four separate biological replicates. The conditions used for the qPCR were 95°C 1 min initial denaturation, 95°C 30 s, 55°C 30 s, 72°C 30 s for 40 cycles with fluorescence detection after every elongation step. The PCR products were not longer than 250 bp and contained a GC content of ~50%. The experiment was performed on an Eppendorf Realplex 2 Mastercycler. Rosette leaves of different sizes, representing different ages, were collected from bottom to top as follows: t0 = 3.5-cm leaves, t1 = 2.5 cm, t2 = 1.5 cm, t3 = 1 cm for short-day conditions, and t0 = 2.5-cm leaves, t1 = 1.5 cm, t2 = 1 cm for long-day conditions.

For the plant lines containing the ectopic rDNA repeats, those with single insertion sites in the genome were selected according to their segregation patterns. The copy numbers of integrated rDNA repeats at these single insertion sites were determined by qPCR (again taking advantage of the short, integrated stretch of sequence including a BsrEII site in the 25S rDNA). To normalize the qPCR results and have an absolute quantification of gene copy number, we used primers (HPT_up_reverse and HYG_down) hybridizing to the hygromycin resistance gene and the previously characterized C2R line (Mittelsten Scheid et al., 2003), which harbors only a single T-DNA at a single integration site. See Supplemental Table for primer sequences.

Preparation of Pollen Mother Cells DAPI Spreads

Inflorescences were harvested into fresh fixative (3:1 96% [v/v] ethanol [Merck] and glacial acetic acid and kept overnight (O/N) for fixation. Once the fixative decolorized the inflorescences, they were placed in fresh fixative (can be stored for over a month at -20°C), and subsequently, one inflorescence was transferred to a watch glass. The yellow buds were removed to collect only white and transparent buds. The white buds were separated from the inflorescence and grouped according to size. This step is necessary to obtain preparations with separated meiotic stages.

Afterward, the buds were washed three times with citrate buffer (0.455 mL of 0.1 M citric acid, 0.555 mL of 0.1 M trisodium citrate in 10 mL of distilled water) and submerged in an enzyme mix (0.3% w/v cellulase, 0.3% w/v pectolyase in citrate buffer). Each bud has to be submerged for the digestion to work efficiently. The buds were incubated for 90 min in a moisture chamber at 37°C. Digestion was inhibited by adding cold citrate buffer (buds can be kept O/N at 4°C). At this point, the buds were transferred (maximum three to four buds of the same size) to a glass slide. Excess liquid was removed and 15 µL of 60% acetic acid added. The buds were suspended using a metal rod and an additional 10 µL of 60% acetic acid was added to the suspension. The droplet area was labeled using a diamond needle and fixed with fixative 3:1. Slides were dried for at least 2 h. To stage the meiocytes, 15 µL of 2 µg/ml 4′,6-diamidino-2-phenylindol (DAPI) diluted inVectashield (Vector Laboratories) was added to the slide and sealed with a glass cover slip. Images were taken on a Zeiss Axioplan microscope (Carl Zeiss) equipped with a mono cool-view charge-coupled device camera (Vignard et al., 2007).

Fluorescence in situ Hybridization and Detection of DNA–RNA Hybrids

The DAPI slides selected for FISH were washed in 100% ethanol until the cover slips could be easily removed (5–10 min) and subsequently washed in 4T (4× SCC and 0.05% w/v Tween20) for at least 1 h to remove the mounting medium.

After washing the slides in 2× SCC for 10 min, they were placed in prewarmed 0.01 M HCl with 250 µL of 10 mg/ml pepsin for 90 s at 37°C. The slides were then washed in 2× SCC for 10 min at room temperature. Fifteen microliters of 4% paraformaldehyde was added onto the slides, covered with a strip of autoclave bag, and placed for 10 min in the dark at room temperature. The slides were then washed with deionized water for 1 min and dehydrated by passing through an alcohol series of 70, 90, and 100%, for 2 min each. Slides were left to air dry for 30 min.

Meanwhile, the probe mix was prepared by diluting 1 µL of probe (2–3 µg of DNA) in a total of 20 µL of hybridization mix (10% dextran sulfate Molecular Weight [MW] 50,000, 50% formamide in 2× Saline Sodium-Citrate [SSC]).

In case the rDNA Locked Nucleic Acids (LNA) probe was applied, only 50 pmol (final concentration) was used per slide. The probe mix was denatured at 95°C for 10 min and then placed on ice for 5 min. Afterward, the probe mix was added to the slide, covered with a glass cover slip, sealed, and placed on a hot plate for 4 min in the dark at 75°C. Finally, the slides were placed in a humidity chamber overnight at 37°C. After hybridization, the cover slips were carefully removed and the slides were treated with 50% formamide in
2 × SCC for 5 min in the dark at 42°C. The slides were then washed twice with 2 × SCC for 5 min in the dark at room temperature.

To detect DNA-RNA hybrids, the Kerafast antibody [S9.6] (Kerafast #ENH001; 1:50 dilution in blocking solution: 1% BSA, 0.01% NaN₃ [w/v] in 1 × PBS) was added to the slides at this step and incubated for 30 min at 37°C. The slides were then washed once for 5 min in 2 × SCC, and an anti-mouse Alexa 568 antibody (Abcam #ab175473, 1:400 dilution) was added for 30 min at 37°C. At this point, the slides were washed one additional time in 2 × SCC.

Finally, 15 μL of DAPI-Vectashield solution was added to the slide and sealed with a cover slip. Images were taken on a Zeiss Axioplan microscope (Carl Zeiss) equipped with a mono-cool view charge-coupled device camera.

**Immun-FISH (Targeted Analysis of Chromatin Events)**

Inflorescences with at least two open flowers from the primary shoots of the plant were collected and placed in a Petri dish with wet filter paper. The buds were dissected with needles under a dissection microscope, and all anthers with transparent lobes were transferred to a droplet of artificial pond water (0.5 mM NaCl, 0.2 mM NaHCO₃, 0.05 mM KCl, 0.4 mM CaCl₂ in deionized water) on a glass slide. Afterward, the anthers were squashed between the tips of dissection forceps to release all meiocytes grouped in syncytia (columns).

The slide was transferred to a light microscope, and columns were collected into a 1.5-mL tube on ice with a glass capillary (Chen et al., 2010). Fifteen microliters of digestion solution (1% cytohelicase, 1.5% Suc, and 1% polyvinylpyrrolidone) was added to the meiocyte solution and carefully mixed. The tube was incubated for 10 min in the dark at room temperature.

Digestion was stopped by putting the tube on ice, and 8 μL of digested meiocytes was transferred to a clean and charged microscope slide. Twenty microliters of 2% (v/v) Liposol (Bibby Sterilin, no. 40023) was added to the droplet and mixed by tilting the slide. After 4 min at room temperature, 24 μL of 4% formaldehyde was added to the slide and left to air dry completely (Kurzbauer et al., 2012).

Dried slides were washed in 2 × SSC for 5 min, and the primary antibody mix was added after removing excess liquid. The slides were covered with a piece of autoclave bag and incubated in a humidity chamber at 4°C overnight.

The autoclave bag cover was removed, the slides were washed in 2 × SSC for 5 min, and the appropriate secondary antibody mix was added. The slides were again covered with an autoclave bag and incubated in a humidity chamber for 1 h at 37°C.

The slides were washed for 5 min in 2 × SSC, and 15 μL of 4% formaldehyde fixing solution was added to the slide, covered with a strip of autoclave bag, and incubated for 10 min in the dark.

Afterward, slides were rinsed by dipping into deionized water and dehydrated by passing through an alcohol series of 70, 90, 100% ethanol, for 2 min each. Finally, the slides were left to dry completely for 30 min in the dark.

To prepare the probe mix, 14 μL of hybridization solution was added to 2-3 μL of Bacterial Artificial Chromosome (BAC) probes (2-3 μg) or 1 μL of 1 μmol LNA probe and filled up to a final volume of 20 μL with deionized water.

The probe mix was denatured for 10 min at 95°C and then placed on ice for 5 min. After cooling, the probe mix was added to the slide and covered with a glass cover slip. The borders of the cover slip were sealed with rubber cement and placed on a hot plate for 4 min in the dark at 75–80°C (75°C for repetitive DNA and 80°C for multiple BACs).

The slides were incubated in a humidity chamber O/N at 37°C. If an LNA probe was used, 3–4 h were sufficient for a successful hybridization.

Thereafter, the slides were washed in 50% formamide–2 × SSC for 5 min in the dark at 42°C and twice in 2 × SSC for 5 min.

Finally, 15 μL of DAPI-Vectashield was added to the slide and covered with a glass cover slip. The borders of the glass slide were sealed with transparent nail polish. Slides were imaged with a conventional fluorescence microscope (Zeiss Axioplan). 2-stacks with 100-nm intervals were acquired, deconvolved using AutoQuant software (Media Cybernetics), and are presented as projections done with the HeliconFocus software (HeliconSoft). Super-resolution images were acquired using the STEDYCON system (Abberior).

All BACs were labeled using the Nick Translation mix from Roche following the manufacturer’s instructions. Each BAC was labeled individually and concentrated in a tube. In this study, fluorescently labeled nucleotides Chromatide Alexa Fluor 488-5-dUTP (Thermo Fisher Scientific), Chromatide Texas Red-12-dUTP (Thermo Fisher Scientific), and Cy5-dUTP (GE Healthcare) were used.

The dilutions used for the different primary and secondary antibodies are as follows: anti-H3K27me1: 1:200, anti-H4:ac1: 1:50, anti-AS1: 1:10,000, anti-RAD51: 1:300 (Kurzbauer et al., 2012), anti-ZYP1: 1:500 (Higgins et al., 2005), anti-SCCC1: 1:500 (Chelysheva et al., 2005), anti-REC8: 1:250 (Cai et al., 2003), anti-MRE11:1:200 (Lohmiller et al., 2008), anti-guinea pig Alexa 488: 1:400 (Abcam #ab150185), anti-rabbit Alexa 568: 1:400 (Abcam #ab175471), anti-rat Alexa 568: 1:300 (Abcam #ab175476), anti-mouse Alexa 568: 1:400 (Abcam #ab175473), anti-guinea pig STAR 580 (for STEDYCON only) 1:500 (Abberior #201120057), and anti-rabbit STAR RED (for STEDYCON only) 1:250 (Abberior #200120119).

**Whole Mount Immun-FISH**

This method is an adapted version of the “whole-mount FISH in a tube” from (Bey et al., 2018). Inflorescences were collected from the primary shoots of an Arabidopsis plant, and all open flowers and pollen-containing buds were removed with forceps and a needle under a dissection microscope. All remaining buds were opened with the help of two needles to increase accessibility of anthers to the enzyme solution and the FISH probe. The inflorescences were placed in 500 μL of fixation solution (1% formaldehyde, 10% DMSO, 1 × PBS, 60 mM EGTA), and all buds were submerged.

The solution with the buds was placed under vacuum for 10 min at room temperature and incubated for a further 30 min at room temperature.

After removing the fixation solution, the samples were incubated 2 × 10 min in 500 μL of methanol.

Methanol was removed, and samples were incubated for 2 × 5 min in 500 μL of ethanol.

Finally, after the ethanol was removed, the samples were incubated in 500 μL of xylene/ethanol (1:1) for 15 min. The samples were transferred to a new tube with 500 μL of xylene and incubated for 30 min at 50°C. Samples were washed twice in 500 μL of ethanol and 500 μL of methanol and washed three times in 500 μL of PBT (1 × PBS with 0.01% v/v Tween 20).

The samples were digested by incubating the buds in the enzyme solution (0.6% cytohelicase, 0.6% pectolyase, 0.6% cellulase in citrate buffer, pH 4.5) for 1 h at 37°C.

The samples were gently washed twice in 500 μL of 2 × SSC and then incubated in 500 μL of 0.1 mg/ml RNaseA in 2 × SSC for 1 h at 37°C. Afterward, the samples were washed twice in 500 μL of 1 × PBT and fixed in 1% formaldehyde (in 1 × PBT) for 30 min.

Samples were then washed twice in 500 μL of 1 × PBT and once in 500 μL of 2 × SSC, followed by incubation in 500 μL of a 1:1 mix of HB50 (50% formamide, 2 × SSC, 50 mM NaH₂PO₄) and 2 × SSC for 30 min, incubated in 500 μL of HB50 for 30 min, and finally incubated in 30 μL of hybridization solution (labeled DNA probe 0.5–1.5 μg for DNA repeats and 2–5 μg for unique sequences, 50% formamide in 2 × SSC) in the dark for 1 h.

To denature the probe and the target sequence, the tube was placed in a heating block for 4 min at 85°C and, afterward, on ice for 3 min. Finally, to
hybridize the labeled probe to the sample, the tube was left overnight in the dark at 37°C.

The samples were washed in 500 μL of HB50, incubated in 500 μL of fresh HB50 at 42°C for 1 h, and washed in 500 μL of PBT for two times, 10 min each.

To successfully immune label proteins in a whole-mount preparation, once the samples were washed in PBT, they were incubated in 50 μL of primary antibody mix under vacuum for 30 min and then transferred for 3 h to 37°C or overnight at 16°C. The primary antibody mix was 10 times more concentrated than what is usually used for regular spreads. Afterward, the samples were washed four times in 1 x PBT and incubated with 50 μL of the secondary antibody solution for 30 min under vacuum and placed for 3 h at 37°C.

Finally, the sample was washed three times for 15 min in 1 x PBT, incubated in 100 μL of 1 x PBS with 8 μL of DAPI 5 μg/ml for 30 min, and placed on a glass slide with 20 μL of DAPI-Vectorshield. Imaging of whole-mount samples was performed with a Zeiss LSM710 equipped with an Airyscan Unit with 160 nm resolution in x, y, and z. The images were deconvolved and 3D rendered with the Huygens Software (SVI software).

Scoring Recombination Rates
Recombination analysis was performed according to the protocol published by Berchowitz and Copenhaver (2008). In brief, individual flowers were collected from the main shoot and tapped on a glass slide with pollen sorting buffer (10 mM CaCl₂, 1 mM KCl, 2 mM MES and 5% [v/v] Suc, pH 6.5) (Yelina et al., 2013) for 2–3 min until all pollen was released. Tetrads were scored with an inverted microscope equipped with three filter sets for green, red, and cyan fluorescent proteins. All plant lines used for tetrad analysis were in the qrt1-2 mutant background.

We flanked the ectopic 45S rDNA integration (ErDNA2) at nucleotide position 1,704,488 on chromosome 2 with FTL 1431 (at nucleotide position 1,521,041) and FTL 2269 (at nucleotide position 8,276,753). ErDNA3 at nucleotide position 10,863,135 on chromosome 3 with FTL 1019 (at nucleotide position 1,517,290) and FTL 1046 (at nucleotide position 10,863,135) and ErDNA5 at nucleotide position 4,999,535 on chromosome 5 with FTL 1143 at nucleotide position 3,760,756 and FTL 2450 at nucleotide position 4,999,535. For all analyses, lines homozygous for the ectopic rDNA insertion sites have been used.

CRISPR/Cas9-Mediated ErDNA Deletion
To specifically delete the rDNA portion of the R4 transgene (Wanzenböck et al., 1997) but retain the resistance marker and the left border and the right border of the vector after integration in the plant genome, a CRISPR/Cas9 vector was constructed as described before (Richter et al., 2018). Two guide RNAs (gRNAs) were designed that bind between the left border of the R4 binary vector and the start of the rDNA sequence and between the 3’ETS and the promoter of the hygromycin-resistant gene, respectively. All gRNAs were designed to contain at least 50% GCs and to be next to Protoscaler Adjacent Motif (PAM) sequence. The plants transformed with the Cas9 construct were selected with BASTA and the survivors evaluated for Cas9 activity on both target sites. This was achieved by amplifying, by PCR, the gRNA target regions within the T-DNA, followed by subsequent Sanger sequencing.

Only T1 plants that showed Cas9 activities on both target sites were propagated to the T2 generation and genotyped for loss of the ectopic rDNA unit within the transgene. It was necessary to go through at least two plant generations to select for a homozygous deletion and for loss of the Cas9 transgene.

Definition and Quantification of RAD51 Foci
RAD51 foci were counted manually on deconvolved, slice-aligned, and 16-bit projected images. Only RAD51 foci colocalizing with the chromatin (DAPI) were accepted. Only RAD51 foci overlapping with 50% or more with the genomic region specifically labeled with a FISH probe were scored as a colocalizing event.

Quantification of Fluorescence Intensity
Fluorescence intensity was calculated with Fiji using the profile Plot analysis tool. Fluorescence intensity for each channel was normalized to the highest fluorescent value.

Quantification of rDNA Fragmentation via FISH
rDNA fragments were counted manually on 16-bit images of meiotic cells from metaphase II to the tetrad stage. Only rDNA signals (FISH) that overlapped with a chromatin signal (DAPI) were accepted.

Nucleolus Association of the ErDNA
Whole-mount immuno-FISH images were 3D reconstructed to locate the nucleolus as an empty pocket within the nucleus. The specific genomic regions (FISH signals) were accepted as “nucleolus associated” in case they were located at the borders or within the nucleolus itself. All experiments were performed by also detecting the endogenous 45S rDNA as a reference for the nucleolus location.

Statistical Analysis
All statistical analyses, t Tests, Mann-Whitney test, and binary logistic regression tests (as indicated in the figure legends) were performed using GraphPad Prism software (GraphPad Software) and http://statpages.org/logistic.html.

Unpaired, two-tailed Mann-Whitney tests were performed, since D’Agostino Pearson omnibus K2 normality testing revealed that most data were not sampled from a Gaussian population, and nonparametric tests were therefore required. Error bars indicate standard deviations.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers COM1 AT3G52115, MRE11 AT5G54280, HDA6 AT5G63110, ATXR5 ATXR6 AT5G09790-AT5G24330, PCH2 AT4G24710, NUC2 AT3G18610, SPO11-2 AT1G63990, LIG4 AT5G57160, RAD51 AT5G20850.

Supplemental Data

Supplemental Figure 1. The NORs are highly dynamic regions that are both transcribed during meiosis.

Supplemental Figure 2. The rDNA acquires distinct chromatin characteristics during meiosis.

Supplemental Figure 3. The rDNA acquires a specific chromatin environment during meiosis.

Supplemental Figure 4. The nucleolus shields rDNA from meiotic DSB formation and deleterious HR.

Supplemental Figure 5. The rDNA is repaired by NHEJ.

Supplemental Figure 6. Ectopically integrated rDNA units associate with the nucleolus, promote homolog pairing and suppress meiotic recombination.
Supplemental Table. Primers and BACs used in this study.

Supplemental Movie 1. In G2 the rDNA is not localized within the nucleolus.

Supplemental Movie 2. During leptotene the rDNA is localized within the nucleolus.

Supplemental Movie 3. ASY1 does not localize within the nucleolus and to the rDNA during leptotene.

Supplemental Movie 4. ASY1 does not localize within the nucleolus and to the rDNA during zygotene.

Supplemental Movie 5. ASY1 localizes to the NORs of chromosome 2 and chromosome 4 during pachytene.

Supplemental Movie 6. Nucleolus association of an ectopic rDNA unit (ErDNA).

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