



Arabidopsis thaliana FANCD2 Promotes Meiotic Crossover Formation ^{OPEN}

Marie-Therese Kurzbauer,^a Mónica Pradillo,^b Claudia Kerzendorfer,^a Jason Sims,^a Rene Ladurner,^{a,c,1} Cecilia Oliver,^{b,2} Michael Peter Janisiw,^{a,3} Magdalena Mosiolek,^{a,c} Dieter Schweizer,^{a,c} Gregory P. Copenhagen,^d and Peter Schlögelhofer^{a,4}

^aDepartment of Chromosome Biology, Max F. Perutz Laboratories, University of Vienna, Vienna Biocenter, 1030 Vienna, Austria

^bDepartamento de Genética, Facultad de Biología, Universidad Complutense de Madrid, 28040 Madrid, Spain

^cGregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Vienna Biocenter, 1030 Vienna, Austria

^dDepartment of Biology and the Integrative Program for Biological and Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3280

ORCID IDs: 0000-0001-6625-6015 (M.P.); 0000-0001-9261-7304 (R.L.); 0000-0002-7412-0676 (M.P.J.); 0000-0002-7962-3862 (G.P.C.); 0000-0002-0909-3587 (P.S.)

Fanconi anemia (FA) is a human autosomal recessive disorder characterized by chromosomal instability, developmental pathologies, predisposition to cancer, and reduced fertility. So far, 19 genes have been implicated in FA, most of them involved in DNA repair. Some are conserved across higher eukaryotes, including plants. The *Arabidopsis thaliana* genome encodes a homolog of the *Fanconi anemia D2* gene (*FANCD2*) whose function in DNA repair is not yet fully understood. Here, we provide evidence that *AtFANCD2* is required for meiotic homologous recombination. Meiosis is a specialized cell division that ensures reduction of genomic content by half and DNA exchange between homologous chromosomes via crossovers (COs) prior to gamete formation. In plants, a mutation in *AtFANCD2* results in a 14% reduction of CO numbers. Genetic analysis demonstrated that *AtFANCD2* acts in parallel to both *MUTS HOMOLOG4 (AtMSH4)*, known for its role in promoting interfering COs and *MMS AND UV SENSITIVE81 (AtMUS81)*, known for its role in the formation of noninterfering COs. *AtFANCD2* promotes noninterfering COs in a *MUS81*-independent manner and is therefore part of an uncharted meiotic CO-promoting mechanism, in addition to those described previously.

INTRODUCTION

Fanconi anemia (FA) is a human autosomal recessive disorder characterized by chromosomal instability, bone marrow failure, congenital skeletal abnormalities, a predisposition for cancer, reduced fertility, hypogonadism, testicular failure, and cellular sensitivity to DNA cross-linking agents (Fanconi, 1967; Kee and D'Andrea, 2012). So far, mutations in 19 genes responsible for these clinical manifestations have been identified (Fanconi anemia complementation group D2, -I, -A, -B, -C, D1, -E, -F, -G, -J, -L, -M, -N, -O, -P, -Q, -R, -S, and -T; reviewed in Ceccaldi et al., 2016; Michl et al., 2016; Supplemental Data Set 1). Many interacting factors, not directly implicated in FA pathologies, have been identified, including Bloom syndrome RecQ helicase-like, DNA ligase 4, DNA-dependent protein kinase, both subunits of the KU

heterodimer (KU70 and KU80), and FANCD2/FANCD1-associated nuclease 1 (FAN1). All of these factors have a direct role in DNA repair and/or in DNA repair pathway choice (Nakanishi et al., 2005; Kottmann and Smogorzewska, 2013).

The mammalian FA DNA repair pathway is activated by the protein kinase ATR (ATM and Rad3-related; Andreassen et al., 2004). The FA core ubiquitin ligase complex, comprising FANCA, B, C, E, F, G, L, and M then modifies FANCD2 with a single ubiquitin moiety. Modified FANCD2 localizes to nuclear foci at DNA lesion sites together with its heterodimeric partner FANCD1 and other protein factors like RAD51 (FANCR), BRCA1 (FANCS), and BRCA2 (FANCD1) (reviewed in Jacquemont and Taniguchi, 2007). In addition, FANCD2 is phosphorylated by the kinase ATM (ataxia telangiectasia mutated) in response to DNA damage, eliciting a cell cycle checkpoint signal (Wang and D'Andrea, 2004).

FANCD2 is essential for DNA repair by homologous recombination (HR) in response to DNA interstrand cross-linking, but its molecular functions are still not fully understood. Despite the lack of a defined nuclease domain, it has been reported that the FANCD2 protein has a 3'-5' directed DNA exonuclease activity and modifies DNA ends so that they are less favorable substrates for error-prone repair pathways like nonhomologous end joining (NHEJ) and more likely repaired via the faithful HR pathway (Pace et al., 2010). Furthermore, in its ubiquitinated form, FANCD2 attracts the FAN1 nuclease to DNA interstrand crosslinks (ICLs) that block the progression of replication forks. FAN1 is endowed with endo- and 5'-3' exonuclease activities and is also essential for HR DNA repair and reestablishment of collapsed replication forks

¹ Current address: Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305.

² Current address: Institute of Human Genetics, Centre National de la Recherche Scientifique, 34396 Montpellier, France.

³ Current address: Center for Anatomy and Cell Biology, Division of Cell and Developmental Biology, Medical University of Vienna, 1090 Vienna, Austria.

⁴ Address correspondence to peter.schloegelhofer@univie.ac.at.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Peter Schlögelhofer (peter.schloegelhofer@univie.ac.at).

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(Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Klein Douwel et al., 2014; Pizzolato et al., 2015; Lachaud et al., 2016). Additionally, FANCD2 may serve as a scaffold for several other nucleases like XPF-ERCC1 and MUS81-EME1 via recruitment of SLX4 (Castor et al., 2013; Boisvert and Howlett, 2014; Klein Douwel et al., 2014). Yet, the precise orchestration of the different nucleases at sites of DNA lesions is still unknown. FANCD2 also attracts CtIP to DNA lesion sites and cooperates with it in the repair of ICLs (Murina et al., 2014). This latter finding is especially intriguing since CtIP, together with the MRE11-complex, is known to process DNA ends at DNA lesion sites promoting HR DNA repair (Liu and Huang, 2016).

Considering the importance of FANCD2 for human health, understanding the underlying molecular mechanisms is of crucial importance and motivated studies of FANCD2 homologs in different model organisms. The *fancd2*^{-/-} knockout mice display unpaired and mispaired chromosome regions in male meiotic cells but checkpoint activation followed by apoptosis impedes further analysis (Houghtaling et al., 2003). *Caenorhabditis elegans* encodes homologs of several FA factors (reviewed in Youds et al., 2009). It is the only animal model organism where FANCD2's (FCD-2) function in meiotic DNA repair has been extensively analyzed. The *C. elegans* FA pathway responds to double-strand breaks (DSBs) in replicating cells and *fcd-2* deletion mutants show hypersensitivity to ICL agents during somatic development (Collis et al., 2006; reviewed in Youds et al., 2009). During *C. elegans* meiosis, when crossover (CO) pathways are compromised, FCD-2 channels a subset of DNA repair intermediates into HR pathways, thereby preventing them from being processed by error-prone NHEJ pathways (Adamo et al., 2010).

Meiosis ensures the reduction of the parental genome prior to the formation of generative cells and allows the exchange of genetic information between maternal and paternal chromosomes, leading to novel combinations of genetic traits in the following generation. The molecular basis of this process is recombination between homologous chromosomes and depends on the formation of DSBs, introduced at nonrandom sites throughout the genome. DSBs are formed by an evolutionarily conserved protein complex, with the SPO11 transesterase being its catalytically active subunit (Keeney et al., 1997; Robert et al., 2016; Vrielynck et al., 2016). Following DNA cleavage, SPO11 is covalently bound to the 5' ends of DSBs and has to be removed prior to DNA end processing. Processing creates 3' single-stranded DNA overhangs, which are coated by the recombinases RAD51 and DMC1 to form nucleoprotein filaments that find and invade preferentially non-sister chromatids. Specialized meiotic DNA repair proteins, together with other DNA repair factors, mediate capture of the second DSB end, ligation, DNA synthesis, and resolution to generate COs (reviewed in Hunter, 2007; Muyt et al., 2009; de Massy, 2013).

Two classes of COs exist in most organisms. Class I COs are sensitive to interference (one CO inhibits formation of other COs nearby) and depend on the ZMM proteins: Zip1, Zip2, Zip3, Zip4/Spo22, Msh4/Msh5, and Mer3 in yeast (reviewed in Lynn et al., 2007). Class II COs are interference insensitive and depend on the resolvase Mus81 (Hollingsworth and Brill, 2004). Meiotic DSBs can also be repaired by mechanisms that result in non-crossovers, such as the synthesis-dependent strand annealing pathway

(McMahill et al., 2007). In most organisms, at least one CO per homologous chromosome pair is essential for correct chromosome segregation (obligatory CO), but the vast majority of DSBs are repaired by non-CO pathways or by sister chromatid interactions (reviewed in Muyt et al., 2009; Phadnis et al., 2011; Wang et al., 2015).

Meiotic processes and many protein factors are widely conserved among eukaryotes, including the model plant *Arabidopsis thaliana*. Homologs of several FA pathway members have been identified in *Arabidopsis* and implicated in DNA repair and, in the case of the helicase FANCM and its interaction partners MHF1 and 2, also in meiotic CO formation (Crismani et al., 2012; Knoll et al., 2012; Dangel et al. 2014; Girard et al., 2014). Here, we present the identification and characterization of the *Arabidopsis FANCD2* homolog, *AtFANCD2*, and demonstrate its importance for meiotic recombination. Fertility is reduced in *Atfancd2* mutants and 14% fewer meiotic COs are formed. Strikingly, *AtFANCD2* acts in parallel to both *AtMSH4*, known for its role in promoting interfering COs (class I), and *AtMUS81*, known for its role in promoting noninterfering COs (class II). Taken together, our findings suggest that *AtFANCD2* processes a subset of meiotic DNA repair intermediates into noninterfering COs. Previously, disrupting class I and II CO pathways, by mutating *MSH4* and *MUS81*, did not entirely eliminate COs, suggesting the existence of additional, as yet uncharacterized, CO-forming mechanisms. Our results suggest that *AtFANCD2* is part of such an additional mechanism contributing to class II CO formation.

RESULTS

Characterization of the *AtFANCD2* Gene and Mutant Alleles

We identified an *Arabidopsis* gene encoding a protein with 25% identity (and 44% similarity) to human FANCD2 by homology search and named it *AtFANCD2* (At4g14970). Cloning and sequencing of the cDNA revealed that the gene comprises 31 exons and its open reading frame is 4431 base pairs in length (GenBank accession number MF327383). RT-PCR detected transcripts in all tested plant organs but especially in proliferating tissue and rapidly dividing cell suspension culture cells (Figure 1A).

To study *AtFANCD2*'s function, two mutant lines carrying T-DNA insertions in *AtFANCD2* exons were identified in the Salk Institute Genomic Analysis Laboratory T-DNA (Alonso et al., 2003) and GABI-KAT (Kleinboelting et al., 2012) collections. The mutant lines were named *fancd2-1* (SALK_113293) and *fancd2-2* (GABI_209C01) and analyzed (Figures 1B to 1F). Sequencing the T-DNA insertion sites revealed that *fancd2-1* has two "left borders" and that a 9-bp fragment of the 6th exon of the *AtFANCD2* sequence is deleted in the mutants. The T-DNA insertion of *fancd2-2* plants has one "left" and one "right border" and 35 bp of exon 19 and 19 bp of the adjacent intron are deleted (Figure 1C). Both mutant alleles do not generate a continuous mRNA across the T-DNA insertion sites, indicating that no functional *AtFANCD2* protein can be produced (Figure 1D). Homozygous mutant plants develop normally and are mildly but significantly affected in fertility, with seed per silique counts of 50.5 in *fancd2-1* (± 5.4 ; $n = 150$ siliques) compared with 53.4 in the wild type (± 8.1 ; $n = 235$ siliques; $P < 0.0001$). Pollen viability, as assessed by

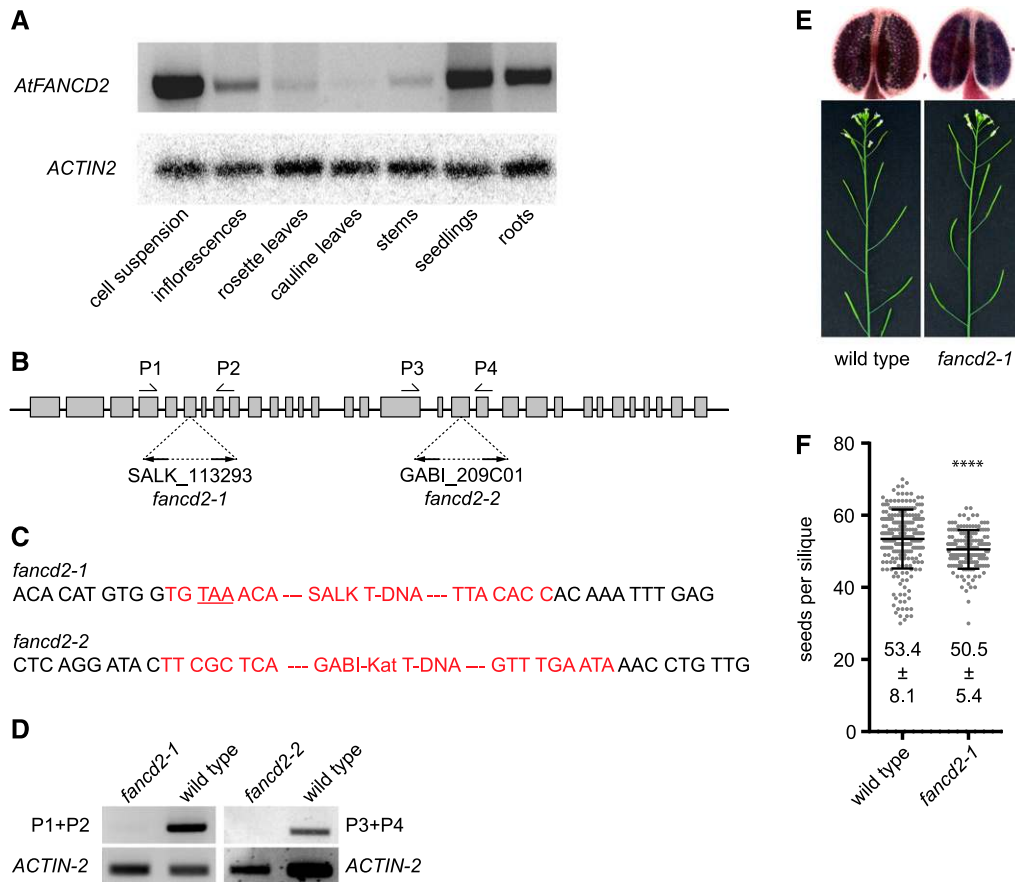


Figure 1. Characterization of the *AtFANCD2* Gene (At4g14970) and Two Corresponding T-DNA Insertion Mutants.

- (A)** The *AtFANCD2* gene is expressed in all tested plant organs.
- (B)** The exon and intron structure of the *AtFANCD2* gene has been defined by cloning and sequencing of the cDNA (GenBank accession number MF327383). The positions of the T-DNA insertion sites of the mutant lines SALK_113293 and GABI_209C01 are indicated.
- (C)** Sequences of regions flanking the T-DNA insertion sites are shown in black letters, with sequences corresponding to the T-DNA sequences in red letters. The first in-frame stop codon in mutant line *fancd2-1* is underlined, and the first in-frame stop codon in mutant line *fancd2-2* is found 72 bp further downstream within the T-DNA sequence.
- (D)** No *AtFANCD2* mRNA is produced across the T-DNA insertion sites in *fancd2-1* and *fancd2-2* mutant lines. Primer positions relative to the T-DNA insertions are indicated in **(B)**.
- (E)** Somatic growth, silique length, and pollen formation appear unaffected in *fancd2-1* mutants.
- (F)** *AtFANCD2* is required for full fertility. Average and SD of seeds per silique are indicated (**** $P \leq 0.0001$).

Alexander staining, appears similar to the wild type (Figures 1E and 1F) and *fancd2-2* mutants were phenotypically indistinguishable from *fancd2-1* mutant plants.

AtFANCD2 Is Involved in Meiotic DSB Repair

To analyze FANCD2's role in meiosis, spreads of pollen mother cells (PMCs) were prepared and chromosomes were visualized by light microscopy after 4',6-diamidino-2-phenylindole (DAPI) staining (Ross et al., 1996). Meiosis appeared to be normal in *fancd2-1* until the diplotene stage but in 12.6% of diakinesis and metaphase I cells unpaired chromosomes (four bivalents and one pair of univalents) were observed ($n = 190$ cells; Figure 2A).

Data from other organisms show that the FA pathway is activated by ATR and interacts with ATM, so we assessed the epistatic

relationship between *AtFANCD2*, *AtATM*, and *AtATR*. *atm-2* mutant plants have meiotic defects and only generate $6.8 (\pm 7.2; n = 364)$ seeds per silique (Figure 3; Garcia et al., 2003). However, in the *atm-2 fancd2-1* double mutant, fertility was further reduced to $0.9 (\pm 1.9; n = 317)$ seeds per silique. In *atm-2* mutant PMCs, pachytene nuclei appeared normal, whereas metaphase I chromosomes sometimes showed interconnections and chromosome fragmentation in later stages (Figure 3; Garcia et al., 2003). These phenotypes were aggravated in the *atm-2 fancd2-1* double mutant and could be recapitulated in *atm-2 mus81-2* mutants (Figure 3). Conversely, *fancd2-1 atr-2* double mutants had no aberrant meiotic chromosome segregation or structural integrity phenotypes (Supplemental Figure 1). Chromosome fragmentation was not observed in *atm-2 fancd2-1 spo11-2-3* triple mutants, demonstrating that the observed DSB

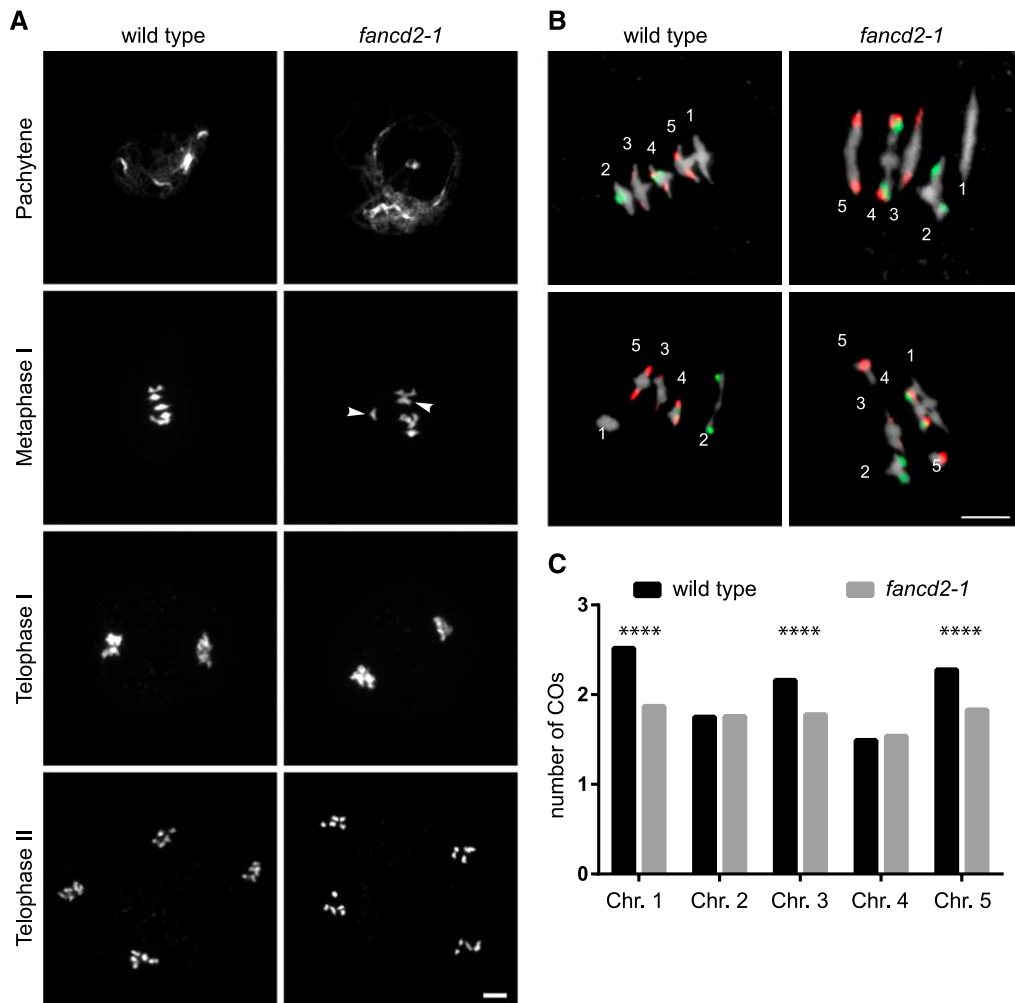


Figure 2. AtFANCD2 Promotes Meiotic Chiasma Formation.

(A) Chromosome spreads of wild-type and *fancd2-1* meiocytes with indicated stages. Arrowheads point to univalents. Bar = 5 μ m.

(B) Chromosome spreads of wild-type and *fancd2-1* metaphase I cells. FISH probes against 45S (green) and 5S (red) rDNA were used to identify chromosomes, allowing for chiasma counting depending on chromosome morphology. Chromosome identities are indicated. Bar = 5 μ m.

(C) Quantification of chiasmata per chromosome in wild-type (black bars) and *fancd2-1* mutant plants (gray bars) (**** $P \leq 0.0001$)

repair defects occur downstream of meiotic DSB formation (Supplemental Figure 2). Taken together, these results indicate that AtFANCD2 and AtATM function in separate meiotic pathways following DSB formation. Alternatively, and also discussed in more detailed below, meiocytes without functional AtATM may produce more DNA repair intermediates that depend on AtFANCD2 for correct processing, while these are not formed in cells devoid of AtATR.

AtFANCD2 Is Required for Normal Levels of Meiotic Crossovers

To analyze CO formation in *fancd2-1* mutants, we used DAPI staining and fluorescent in situ hybridization (FISH) of metaphase I chromosome spreads to count chiasmata on individual chromosome pairs (Sanchez-Moran et al., 2002; Armstrong, 2013). While 10.2 chiasmata (± 0.14) are formed on average per wild-type meiocyte ($n = 69$), only 8.78 chiasmata (± 0.13) were found in

fancd2-1 cells ($n = 111$), representing a statistically significant reduction of 14% of chiasma frequency ($P < 0.001$) (Figures 2B and 2C; Supplemental Table 1). Chiasmata were specifically reduced on the long chromosomes 1 ($P < 0.0001$), 3 ($P < 0.0001$), and 5 ($P < 0.0001$), while chiasma frequency was almost unaffected on the short, NOR-bearing, chromosomes 2 ($P = 0.974$) and 4 ($P = 0.644$).

To analyze meiotic recombination frequency more closely and in a defined chromosomal region, we used the fluorescent tagged lines (FTLs) system (Francis et al., 2007; Berchowitz and Copenhaver, 2008). Transgenes encoding fluorescent marker proteins, inserted at defined positions on the chromosomes, are driven by the pollen-specific, postmeiotic *LAT52* promoter. In addition, mutation of the *QUARTET1* gene enables tetrad analysis because the four pollen grains originating from each meiotic division remain physically attached and do not separate during development (Preuss et al., 1994; Francis et al., 2006). Assaying the marker

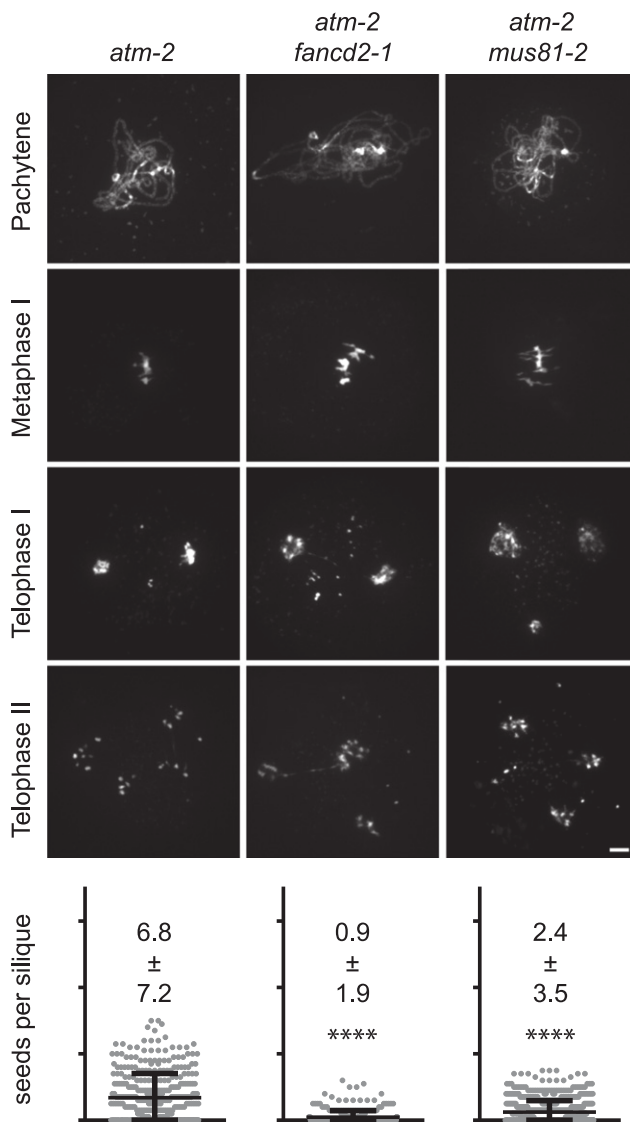


Figure 3. Meiotic Progression and Seed Formation in *atm-2* and Indicated Double Mutant Plants.

The stages of meiotic progression are indicated. *fancd2-1 atm-2* and *mus81-2 atm-2* double mutants are similarly affected with significantly reduced fertility compared with *atm-2* single mutants. Plots represent seed formation; average and SD are indicated (**** $P \leq 0.0001$). Bar = 5 μ m.

distribution in the four meiotically related pollen grains allows for the calculation of genetic distances within and interference between selected intervals. We determined CO frequency in two neighboring intervals, I5c and I5d, on chromosome 5 by counting fluorescent pollen tetrads from wild-type and *fancd2-1* mutant plants (Figure 4A). I5c (1.4 Mb) spans 5.9 cM and I5d (1.7 Mb) 6.2 cM in wild-type plants ($n = 4682$ pollen tetrads from five plants). No difference in recombination frequency was observed for interval I5c (5.9 cM) in *fancd2-1* mutants, but recombination was significantly reduced to 5.2 cM in I5d ($n = 4789$ pollen tetrads from six plants; $P = 0.0022$). We also analyzed two additional

intervals, I2a and I2b, on chromosome 2. No significant difference in recombination frequency was observed for I2b (1.5 Mb; 4.7 to 4.6 cM) between the wild type and *fancd2-1* mutants, but recombination was significantly reduced in the I2a interval (0.5 Mb), from 3 cM in the wild type to 2.5 cM in *fancd2-1* mutants ($P = 0.046$; wild type, $n = 4542$ pollen tetrads from five plants; *fancd2-1*, $n = 4544$ pollen tetrads from 10 plants). Interestingly, interference was in place in all analyzed regions and genotypes (interference ratios I2ab: wild type, 0.19; *fancd2-1*, 0.27; I5cd wild type, 0.36; *fancd2-1*, 0.28) (Figure 4A; Supplemental Table 2). Further evidence that the interference-sensitive CO I pathway remains unaffected in the *fancd2-1* mutant background is provided by the analysis of HEI10 foci numbers. HEI10 is an E3 ubiquitin ligase known to promote interference-sensitive COs (Ward et al., 2007; Chelysheva et al., 2012). The same numbers of HEI10 foci are observed in wild-type (10.4 ± 1.5 ; $n = 24$) and *fancd2-1* mutant (10.6 ± 1.5 ; $n = 21$) meocytes at pachytene stage ($P = 0.8581$; Figure 4B).

DMC1 but Not RAD51 Foci Numbers Are Altered in *fancd2-1*

Placing AtFANCD2 functions downstream of DSB formation but upstream of (or during) CO formation, we sought to define its role in meiotic recombination more precisely. To this end, we quantified the number of foci of the recombinase RAD51 in zygotene stages in PMCs using immunofluorescence. We observed an average of 210 RAD51 foci (± 40 ; $n = 22$ cells) in *fancd2-1* cells and 233 (± 45 ; $n = 14$ cells) foci in wild-type cells, representing a nonsignificant difference ($P = 0.0966$) (Figure 5A). We also stained for the meiosis-specific recombinase DMC1 and found a mild but significant increase in DMC1 foci ($P = 0.0001$) in the absence of AtFANCD2. In *fancd2-1* zygotene cells, 250 (± 32 ; $n = 10$ cells) DMC1 foci were observed, while only 188 (± 41 ; $n = 34$ cells) foci are formed in wild-type cells (Figure 5B). These numbers indicate that the absence of AtFANCD2 does not affect meiotic DSB formation, that DNA ends at meiotic DSB sites are processed, and that they acquire recombinase proteins. In case AtFANCD2 affected meiotic DSB formation (e.g., as part of a negative feedback regulatory loop), it would be expected that RAD51 and DMC1 foci numbers were equally altered. As only DMC1 foci are increased in the absence of AtFANCD2, this rather suggests that DNA repair events involving DMC1 are slowed (Duroc et al., 2014; Thacker et al., 2014) but not blocked, since complete synapsis is established in *fancd2-1* pachytene meocytes (Figure 6).

AtFANCD2 Promotes CO Formation in Parallel to Both AtMSH4 and AtMUS81

The indication that interhomolog DNA repair events may be affected in *Atfancd2-1* mutants together with the observed 14% reduction of chiasma numbers prompted us to analyze the epistasis of AtFANCD2 and genes encoding known CO-promoting factors. *fancd2-1* mutants were crossed to *msh4* mutant plants, defective in class I interference-sensitive COs, and *mus81-2* mutant plants, defective in class II, interference-insensitive COs (Higgins et al., 2004, 2008a; Hartung et al., 2006; Berchowitz et al., 2007). CO reduction and specifically loss of the so-called obligatory CO results in chromosome missegregation and the occurrence of univalent chromosomes instead of bivalent pairs. Using DAPI staining on

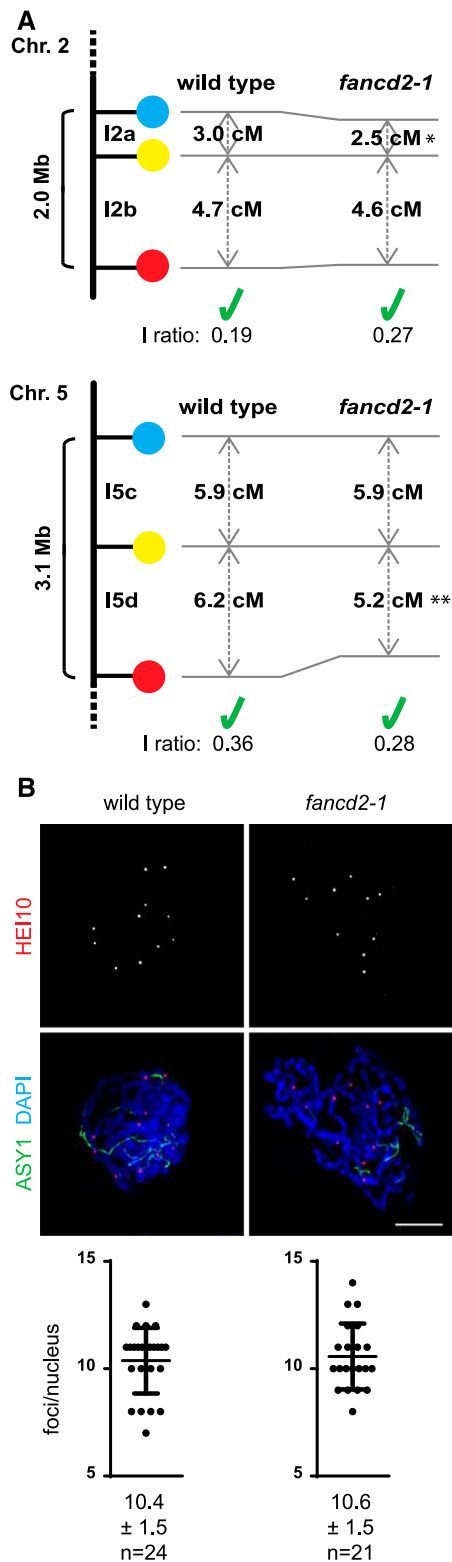


Figure 4. *AtFANCD2* Is Required for Normal Levels of Meiotic Interference-Insensitive Crossovers.

chromosomes of PMC spreads, we observed no univalents in wild-type cells ($n > 190$), whereas we found univalents in 12.6% of all analyzed *fancd2-1* cells ($n = 190$, $P < 0.0001$ compared with the wild type). We found univalents in 86.5% of *msh4* cells ($n = 37$), with many cells displaying only one, two, or three bivalents, consistent with published data (Higgins et al., 2004). In *msh4 fancd2-1* double mutants, all observed cells ($n = 36$) contained univalents and have a clear shift to less bivalents per meicyote, compared with *msh4* single mutants ($P = 0.0006$; Figure 7).

mus81-2 mutant plants have so far not been reported to display univalents, despite a $\sim 10\%$ reduction in meiotic CO frequency (Berchowitz et al., 2007; Higgins et al., 2008a). We observed two cells with univalents out of 46 *mus81-2* cells scored (Supplemental Figure 3). Crossing *fancd2-1* to *mus81-2*, the percentage of cells with univalents increased from 4.3% in *mus81-2* to 26.3% in the double mutant ($n = 38$, $P = 0.0038$ compared with *mus81-2*), indicating that *AtMUS81* and *AtFANCD2* are involved in formation of obligatory COs. We also observed cells with more than one chromosome pair affected in the double mutant (Figure 7).

Thus, *fancd2-1* exhibits an additive effect in combination with both *msh4* and *mus81-2*. It has been shown before that *msh4* and *mus81* mutant combinations have an additive effect on CO number reduction in various organisms (de los Santos et al., 2003; Argueso et al., 2004; Whitby, 2005; Berchowitz et al., 2007; Higgins et al., 2008a; Holloway et al., 2008) and the corresponding proteins have been placed in either the interfering CO (class I) or the noninterfering CO (class II) pathway (Copenhaver et al., 2002). Eliminating both pathways does not result in a complete absence of COs, which lead to the speculation that one or more additional CO-promoting mechanisms may exist (de los Santos et al., 2003; Argueso et al., 2004; Whitby, 2005; Berchowitz et al., 2007; Higgins et al., 2008a). *AtFANCD2* appears to be involved in a pathway that acts in parallel to both *AtMSH4* and *AtMUS81*.

To investigate this further, we generated a triple mutant line combining the *msh4*, *mus81-2*, and *fancd2-1* mutant alleles. The *msh4 mus81-2 fancd2-1* triple mutant ($n = 34$) has fewer bivalents than the *msh4 mus81-2* double mutant ($n = 41$) ($P < 0.0001$), presumably due to fewer COs; 67.6% of the *msh4 mus81-2 fancd2-1* triple mutant meicyotes contain no bivalents (2.4% in the double mutant), 26.5% of the cells display one, and only 5.9% have two bivalents (compared with 31.7% and 46.3%, respectively, in the *msh4 mus81-2* double mutants). Nonetheless, CO formation does not appear to be completely abrogated, since

(A) CO frequencies in two neighboring intervals on chromosomes 2 and 5 were analyzed. Recombination is significantly reduced in two of the four intervals in *fancd2-1* mutants, while interference is still in place (see text for details). Interval I2a (0.5 Mb): FTL1506 (eCFP) and FTL1524 (eYFP). Interval I2b (1.5 Mb): FTL1524 and FTL965 (DsRed2). Interval I5c (1.4 Mb): FTL 1963 (eCFP) and FTL 1143 (eYFP). Interval I5d (1.7 Mb): FTL1143 and FTL2450 (DsRed2). * $P \leq 0.05$ and ** $P \leq 0.01$.

(B) Spreads of pachytene cells stained for the axial element protein ASY1 (green) and the interference-sensitive CO marker HEI10 (red). Dot plots show foci numbers per nucleus. Average and SD are indicated. Foci numbers are not significantly different between wild-type and *fancd2-1* nuclei ($P = 0.8607$). Bar = 5 μm .

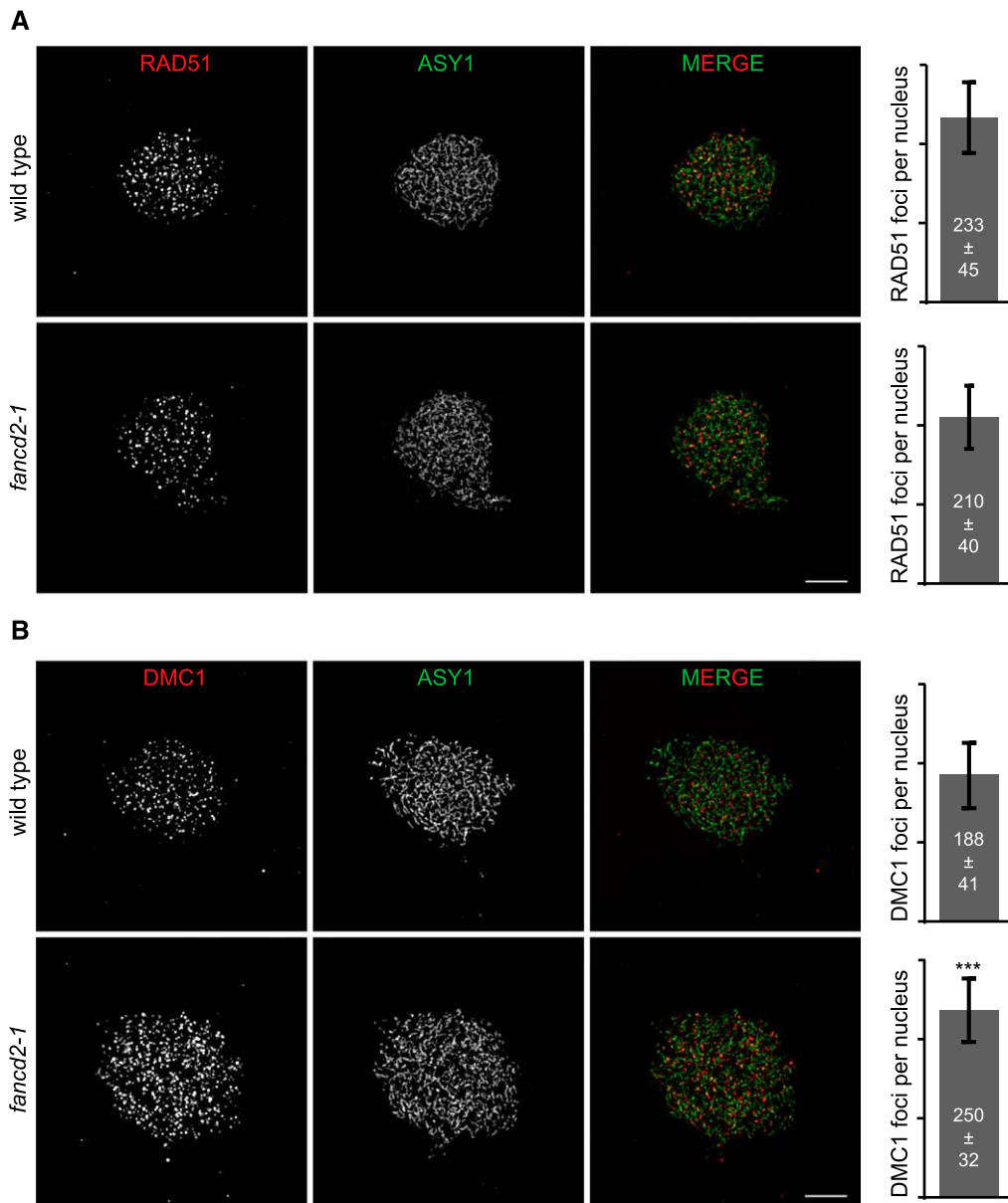


Figure 5. Immunolocalization of Recombinases RAD51 and DMC1 in Wild-Type and *fancd2-1* Meocytes.

(A) Spreads of zygotene cells stained for the axial element protein ASY1 (green) and the recombinase RAD51 (red). RAD51 foci numbers are not significantly different ($P = 0.0966$) between wild-type ($n = 14$) and *fancd2-1* mutant plants ($n = 22$).

(B) Spreads of zygotene cells stained for ASY1 (green) and DMC1 (red). DMC1 foci numbers are significantly different ($P = 0.0001$) between wild-type ($n = 34$) and *fancd2-1* mutant plants ($n = 10$). Bar graphs show average foci numbers per nucleus; sd is indicated. Bars = 5 μ m.

some bivalents are still observed. The data also demonstrate that in the *msh4 msh81-2* background *AtFANCD2* is needed for ~80% of the remaining COs, while in wild-type meocytes, it is required for only 14% of COs (Figure 7).

Mutation of *AtFAN1* Compromises Meiotic Progression

Reports from other organisms show that FANCD2 interacts with the FAN1 nuclease that is also essential for HR DNA repair and

reestablishment of replication forks (Liu et al., 2010; Smogorzewska et al., 2010). Inspired by these findings, we used homology searching to identify the Arabidopsis gene At1g48360 as a nonambiguous *FAN1* homolog, encoding a protein with 23% identity and 36% similarity to its human counterpart. An insertion mutant was identified in the Saskatoon Arabidopsis T-DNA collection (Robinson et al., 2009) and named *fan1-2* (Figure 8). Puchta and colleagues also independently identified *AtFAN1* and analyzed *fan1-1* mutants (Hermann et al., 2015). They found that *AtFAN1* is involved in

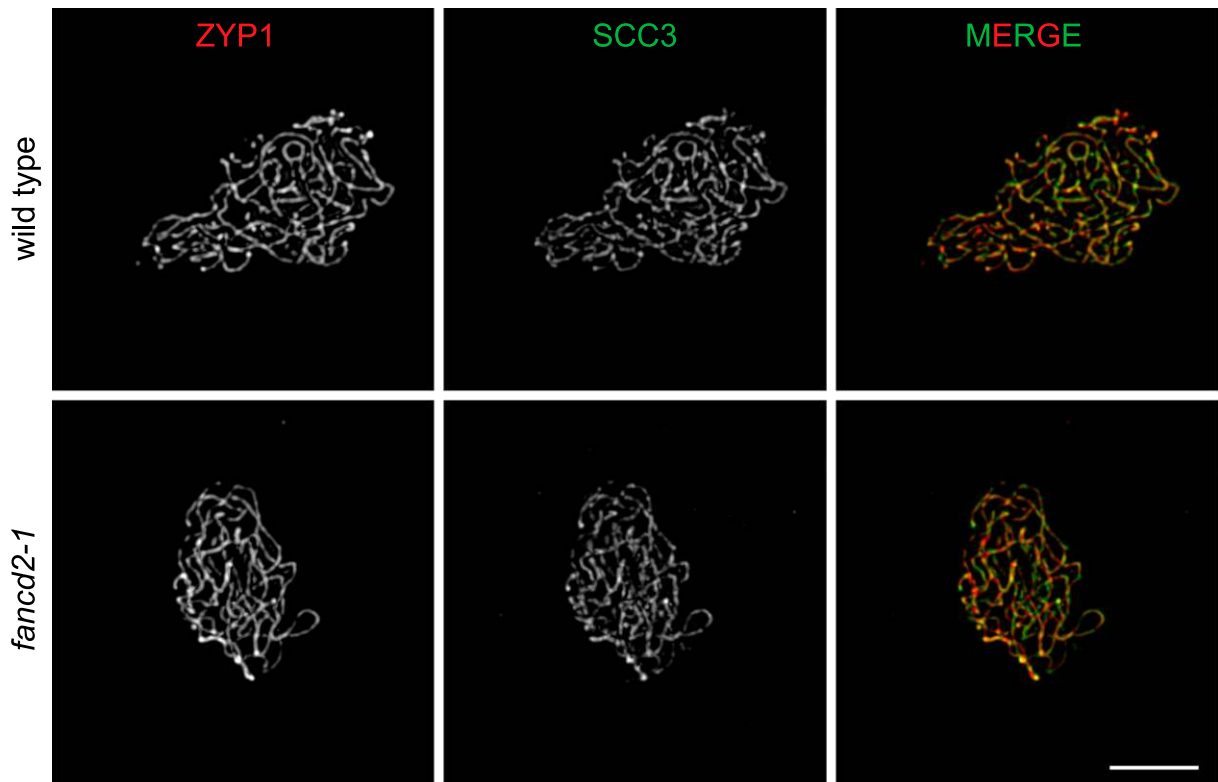


Figure 6. Regular Pachytene and SC Formation in the Absence of AtFANCD2.

Immunolocalization of the synaptonemal complex protein ZYP1 (red) and the cohesin protein SCC3 (green) in wild-type and *fancd2-1* meiotic cells. Continuous ZYP1 staining along all chromosomes in spread pachytene cells confirms that synapsis is complete in the absence of AtFANCD2. Bar = 5 μ m

somatic DNA crosslink repair acting independently of AtMUS81. Our analysis showed that *fan1-2* mutant plants displayed normal growth and vegetative development and that seed set was similar to the wild type (55.9 ± 4.4 , $n = 49$ siliques, $P = 0.0926$). Analysis of DAPI-stained *fan1-2* meiotic chromosomes revealed no obvious meiotic phenotype and meiotic progression seemed unaffected (Figure 8). In contrast to *fancd2-1*, none of the *fan1-2* meiotic cells showed univalents ($n = 58$), but importantly meiotic cells from *fan1-2 atm-2* double mutants displayed severe chromosome fragmentation and a drastic reduction of fertility (1.9 ± 2.8 ; $n = 123$ siliques; Figure 8), compared with *atm-2* single mutants (6.8 ± 7.2 ; $n = 364$ siliques; $P < 0.0001$; Figure 3). In this sense, the *fancd2-1* and *fan1-2* mutants behave very similarly. Based on these observations, we propose that in *atm-2* meiotic cells more DNA repair intermediates are generated, and these extra intermediates depend on AtFANCD2 and AtFAN1 for correct processing. Assuming that AtFAN1 is recruited by AtFANCD2, it should work in parallel to AtMUS81. This is consistent with published data on somatic DNA repair in plants (Herrmann et al., 2015).

DISCUSSION

Here, we report that the Arabidopsis homolog of FANCD2 is required for meiotic recombination. Fertility is mildly reduced in *fancd2* mutants and we observed occasional univalents in male meiotic cells, with $\sim 13\%$ of all cells displaying at least one chromosome pair without the obligatory CO. Even though these will

lead to a small number of chromosomally unbalanced male gametes, the majority of developing pollen grains in anthers will have the correct number of chromosomes and therefore not substantially compromise fertility.

We also observed an overall reduction of COs using both cytological and genetic assays. The latter experiment had further implications: First, we found that recombination is not homogeneously affected, with “hotter” regions showing larger changes and second, that CO interference is still intact in *fancd2-1* plants, implying that AtFANCD2 promotes noninterfering COs. Chiasma formation on chromosomes 2 and 4 was not affected in *fancd2-1*. We attribute this to the fact that interference-insensitive crossovers are already generally reduced on these short, NOR-bearing chromosomes (Lam et al., 2005).

AtFANCD2 has also been previously tested in a large survey for class I CO formation defect suppressors but was shown to not be involved. A decrease of bivalent formation was observed, corroborating our results (Girard et al., 2014).

The reduced recombination frequency phenotype motivated us to further investigate AtFANCD2's involvement in CO formation. We crossed *fancd2-1* mutants to *msh4* and *mus81-2* mutant lines. MSH4, together with its heterodimeric partner MSH5, is thought to form a protein ring around DNA repair intermediates that will mature into interfering COs (Higgins et al., 2004, 2008b; reviewed in Manhart and Alani, 2016). MUS81, together with its partner EME1, has been shown to be a structure specific nuclease, to

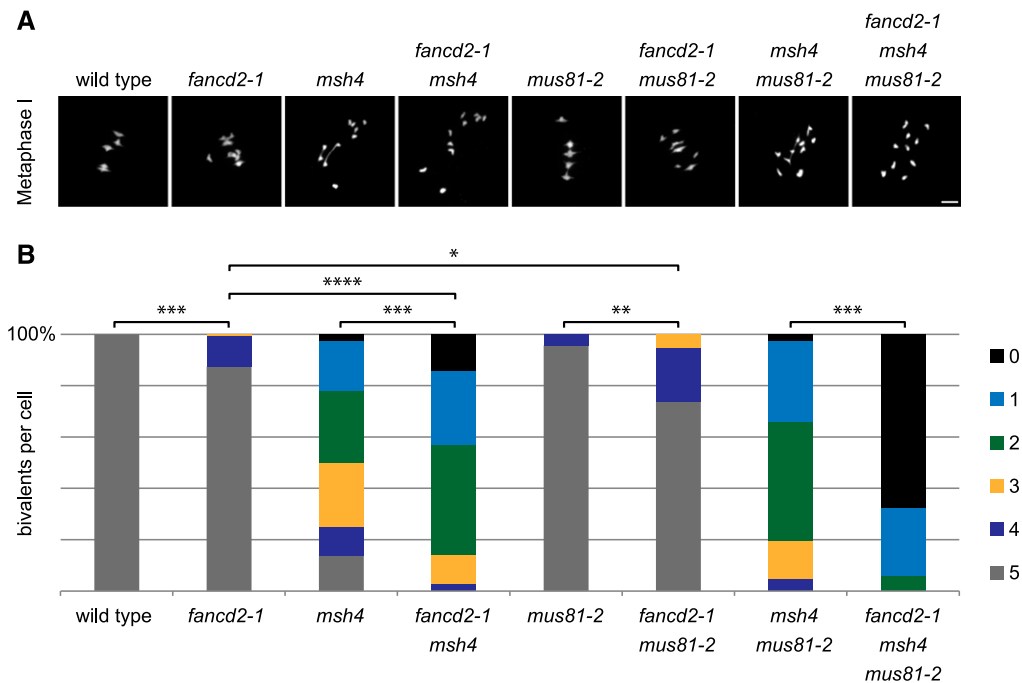


Figure 7. AtFANCD2 Promotes CO Formation Independently of AtMSH4 and AtMUS81.

(A) Representative images of metaphase I cells are shown.

(B) Bivalents, as a readout for CO formation, were assessed in chromosome spreads of the indicated mutant lines. The colors indicate bivalents per cell (gray indicates that all chromosomes found their respective partners, and black indicates that no bivalent has been formed) in percent. Mutation of *AtFANCD2* leads to a significant decrease ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $****P \leq 0.0001$) of bivalent formation in all tested mutant backgrounds. Bar = 5 μm .

process nicked and mature Holliday junctions (Hartung et al., 2006; Berchowitz et al., 2007; Amangyeld et al., 2014; reviewed in Ciccina et al., 2008; Wyatt and West, 2014; Blanco and Matos, 2015), yielding noninterfering COs or non-crossovers (Oke et al., 2014). In plants and other organisms, the MSH4-dependent and MUS81-dependent COs are generated in parallel. Interestingly, *Arabidopsis msh4 mus81* double mutants (and further double mutants that affect either one or the other mechanism) still form residual COs (Berchowitz et al., 2007; Higgins et al., 2008a), which has led to the suggestion that additional proteins and pathways may exist that contribute to CO formation. This CO pathway or mechanism was thought to exclusively generate noninterfering COs, since all mutations that affect the interfering COs are not additive and are believed to act in a single pathway.

AtFANCD2 is essential for 14% of all chiasmata and the remaining COs in the corresponding mutant plants show proficient interference. Furthermore, *AtFANCD2* acts in parallel to both, *AtMSH4* and *AtMUS81*, as the corresponding double mutants are more severely affected in terms of CO formation than the respective single mutants. Importantly, the remaining COs in the *msh4 mus81-2* double mutant plants depend to a large extent on *AtFANCD2*. Therefore, we suggest that *AtFANCD2* is part of a previously uncharacterized mechanism, yielding class II COs in plants, with at least equal importance as *AtMUS81*. *AtFANCD2* serves in parallel to the well-characterized interference-sensitive (class I) CO pathway and represents an additional player for the production of interference-insensitive (class II) COs, independent

of *AtMUS81*. It appears that meiotic DNA repair intermediates are processed by a range of different proteins that have affinity for them, rather than depending on a series of precisely orchestrated events for repair. *AtFANCD2* appears to be used for a subset of these intermediates in wild-type meiocytes. When the “class I” pathway is compromised or the structure specific resolvase *AtMUS81* is missing, more intermediates are channeled into CO repair via *AtFANCD2*. This is consistent with our observations that in the wild type, only 14% of the COs depend on *AtFANCD2*, but in *msh4 mus81-2* double mutants, most of the remaining COs depend on *AtFANCD2* (bivalent formation is reduced by 80% in the triple compared with the double mutant). Since CO formation is not fully abrogated in the triple mutants, further factors implicated in (most likely) class II CO formation remain to be identified.

Further support for the interpretations above comes from the analysis of the *atm-2 fancd2-1* double mutant. ATM has been established as a negative regulator of meiotic DSB formation in various organisms (Joyce et al., 2011; Lange et al., 2011; Zhang et al., 2011; Carballo et al., 2013; Garcia et al., 2015; Mohibullah and Keeney, 2017). We propose that the aggravation of the fertility and chromosome fragmentation defects observed in the double mutant compared with the *atm-2* single mutant is a result of supernumerary DSBs formed in the absence of ATM and that the additional DSBs may result in more DNA repair intermediates that depend on *AtFANCD2* and further class II CO factors. This is in line with our findings that the reduction of fertility is similar in *atm-2 mus81* and *atm-2 fan1-1* double mutants (Figures 3 and 8).

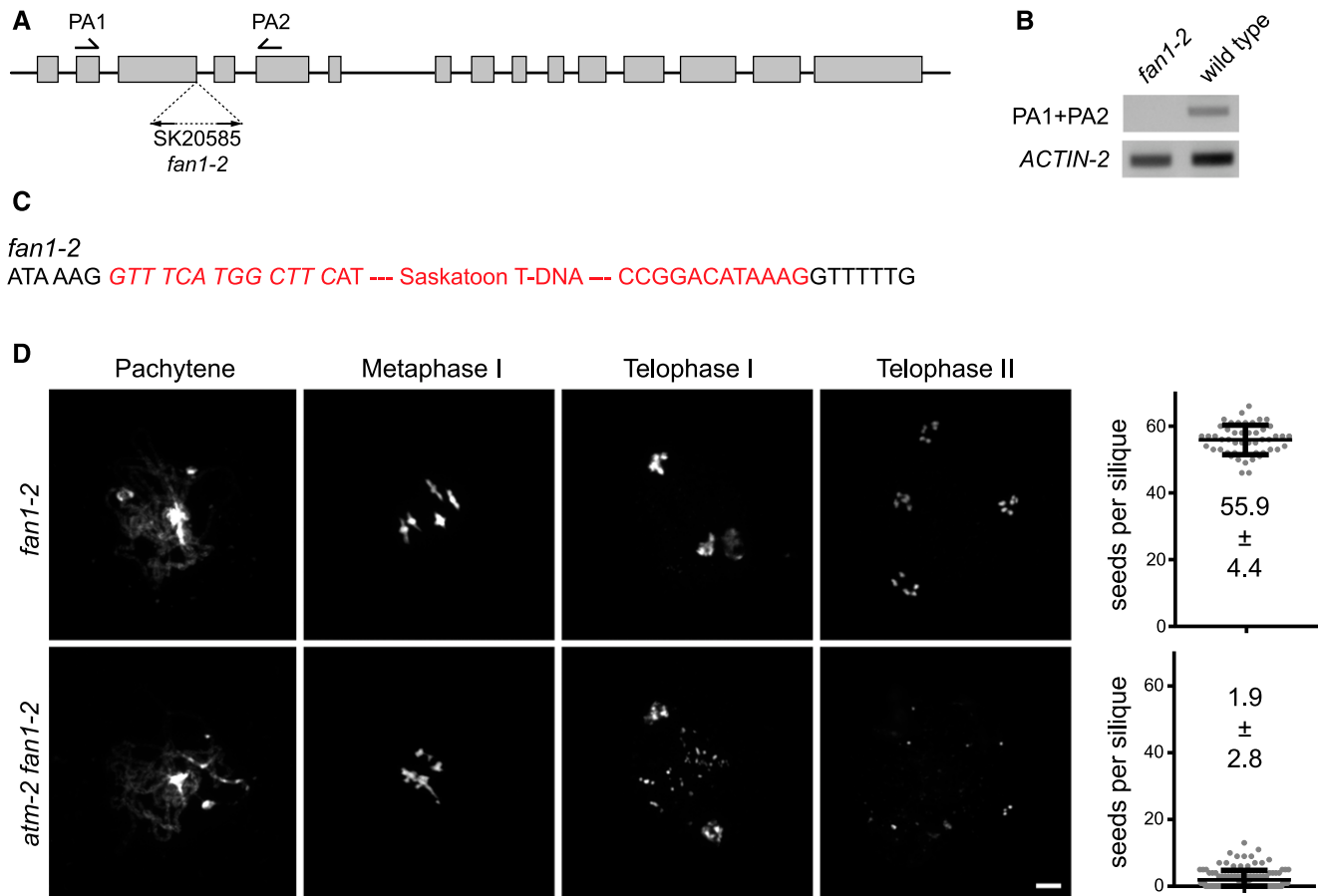


Figure 8. Characterization of the *Atfan1-2* T-DNA Insertion Mutant.

(A) Exons and introns of the Arabidopsis *FAN1* gene have been defined before (Herrmann et al. 2015). The positions of the T-DNA insertion site in the mutant line are indicated. Primer binding positions for expression analysis are given.

(B) Using primers spanning the insertion site, no expression of *AtFAN1* could be detected in *fan1-2* mutants.

(C) Sequences of genomic regions flanking the T-DNA insertion site are shown in black letters, and sequences corresponding to the T-DNA are in red letters. Inserted bases are given in italics. The first in-frame stop codon in the mutant line is found 66 bp downstream of the insertion site within the T-DNA sequence.

(D) Meiotic progression and seed formation in *fan1-2* and *atm-2 fan1-2* mutant plants. Meiotic stages are indicated.

Unexpectedly, our data also shed new light on CO assurance, a mechanism that ensures that each pair of homologs is connected by at least one CO (Jones and Franklin, 2006; Martini et al., 2006). It appears that both class I and II COs contribute to CO assurance.

In summary, we present data that establish *AtFANCD2* as a CO-promoting factor in plants. Considering the evolutionary conservation of *FANCD2* and its related proteins, we speculate that this function will also be conserved. A previous study implicated the *C. elegans* *FANCD2* protein (*FCD-2*) in meiotic DNA repair, yet the predominant *fcd-2* phenotype was aberrant DNA repair via NHEJ in the *msh-4* mutant background. Univalents in the single *fcd-2* mutant have not been observed. It should be noted that *C. elegans* lacks *DMC1* (Rinaldo et al., 2002) and has very rigorous CO interference control, with typically only one CO per bivalent (Hillers and Villeneuve, 2003). In Arabidopsis, it appears that especially the interhomolog, *DMC1*-dependent HR events are affected in the absence of *AtFANCD2*. This may be interpreted that DNA repair machineries in *C. elegans* are less redundant and upon

loss of *MSH-4* and *FANCD-2*, NHEJ proteins can acquire access to unrepaired DNA breaks or DNA repair intermediates. Future studies are needed to understand the precise molecular role of *FANCD2* during meiosis in different organisms. We advocate the idea that *FANCD2*, likely together with its conserved partner *FANCI*, serves as an adaptor for nucleases and other DNA repair factors, as it has been shown for somatic DNA repair (Garner and Smogorzewska, 2011; Yamamoto et al., 2011; Chaudhury et al., 2013; Boisvert and Howlett, 2014): First, it serves to recognize specific DNA repair intermediates (Park et al., 2005; Sobek et al., 2007; Sato et al., 2012; Liang et al., 2016) that, at least in the case of Arabidopsis, are formed in ~14% of all interhomolog meiotic DNA repair events, and, second, to recruit (meiotic) DNA repair proteins that then cleave and process the DNA (Kratz et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Boisvert and Howlett, 2014; Murina et al., 2014; Unno et al., 2014; Yeo et al., 2014). We furthermore present a plant candidate for such a recruited protein, the Arabidopsis relative of the nuclease *FAN1*, which recapitulates

the aggravation of DNA damage and loss of fertility in the *atm-2* mutant background. Future experiments will show which factors, possibly including AtFAN1, are recruited to meiotic chromatin by AtFANCD2.

METHODS

Plant Growth Conditions

Plants were grown under long-day conditions (16 h light, 8 h dark, 21°C; 60% humidity, 15,550 lux, T5 Tube illumination). All lines were *Arabidopsis thaliana*, ecotype Columbia.

Mutant Plant Lines

The following mutant plant lines were used: *fancd2-1* (SALK_113293), *fancd2-2* (GABI_209C01), *fan1-1* (GABI_815C08; Herrmann et al., 2015), *fan1-2* (SK20585), *atm-2* (SALK_006953; Garcia et al., 2003), *atr-2* (SALK_032841; Culligan et al., 2004), *msh4* (SALK_136296; Higgins et al., 2004), and *mus81-2* (SALK_107515; Hartung et al., 2006). Confirmation of genotyping was performed by PCR using the following primers: *fancd2-1*: FancUbp, P1, and SALK_LBa1; *fancd2-2*: FD2-2L, FD2-2R, and GABltest1; *fan1-1*: F1-1_LP, F1-1_RP, and GABltest1; *fan1-2*: PA1, FAN1_RP, and GABltest1; *atm-2*: ATM-F1, ATM-R1, and SALK_LBc1; *atr-2*: atr2_1, atr2_3, and SALK_LBa1; *msh4*: MSH4_F1, MSH4EXP_R1, and SALK_LBa1; *mus81-2*: Mus81_LP, Mus81_RP, and SALK_LBa1 (Supplemental Table 3).

Cloning of the AtFANCD2 cDNA

The cDNA has been cloned using primers based on the gene predictions of TAIR 9. Various primer combinations have been used to obtain exonic parts that were not correctly predicted. Ultimately, the full-length cDNA of AtFANCD2 was obtained ligating six PCR generated fragments by conventional cloning techniques. Fragments were constructed using the following primer pairs: F1CK and Mfe_up, Mfe_dn and F3CK, 3-no and Sac_up, Sac_dn and Aha_up, Aha_dn and Eco_up, and Eco_dn and Sma_up (Supplemental Table 3). The cDNA was submitted to GenBank (accession number MF327383).

Expression Analysis of AtFANCD2

RNA was isolated from various tissues using the TRI reagent solution (Sigma-Aldrich) according to the manufacturer's instructions and remaining DNA removed by a subsequent DNase treatment. Two micrograms of RNA was reverse-transcribed using RevertAid H minus M-MuLV reverse transcriptase (Fermentas) according to the manufacturer's instructions. A 20-cycle PCR was performed using primers Actin2.1 and Actin2.2 and Taq polymerase (Fermentas). PCR products were separated on an agarose gel and blotted on a nylon membrane. The ACTIN7 sequence (At5g09810) was detected using a probe constructed by PCR using primers Actin2.1 and Actin2.2 and cDNA obtained from buds as template. The intensity of the 1631-bp signal was measured using Quantity One (Bio-Rad) and normalized to the band with lowest expression. The cDNA was subsequently diluted according to the normalized expression and the same amount of cDNA was used in a subsequent PCR amplifying the AtFANCD2 cDNA using primers Aha_dn and Sma_up. Amplification products were analyzed on an agarose gel.

Sequencing of T-DNA Junctions

The upstream T-DNA junction of *fancd2-1* was amplified using primers LBc1 and FancUbp. The downstream border was amplified using primers

LBc1 and P2. The resulting fragments were cloned into pCR2.1 and sequenced using standard primers.

The upstream T-DNA junction of *fancd2-2* was amplified using primers GABltest1 and P3. The downstream border was amplified using primers LBc1 and P2. The resulting fragments were cloned into pCR2.1 and sequenced using standard primers.

The upstream T-DNA junction of *fan1-2* was amplified using primers PA1 and o8409. The downstream border was amplified using primers pAC106RB and FAN1_RP. The resulting fragments were cloned into pCR2.1 and sequenced using standard primers.

Seed per Silique Counts

Mature but still green siliques originating from the fifth to the thirtieth flower per stem were harvested into 96% ethanol and left at room temperature for 1 to 3 days for destaining. Ethanol was exchanged several times until the tissue was destained and seeds inside siliques were counted manually under a dissection microscope.

Cytology

Spreads of PMCs for DAPI staining were prepared as described (Vignard et al., 2007). Prophase spreads for cytological detection of proteins were prepared as described before (Kurzbaue et al., 2012). The following primary antibodies were used as described previously: anti-HEI10 raised in rabbit (Chelysheva et al., 2012), anti-ASY1 raised in rabbit (Armstrong et al., 2002) or rat (Higgins et al., 2004), anti-RAD51 raised in rat (Kurzbaue et al., 2012), anti-DMC1 raised in rabbit (Chelysheva et al., 2007), anti-SCC3 raised in rabbit (Chelysheva et al., 2005), and anti-ZYP1 raised in rat (Higgins et al., 2005). The secondary antibodies used were as follows: Goat-anti-rabbit conjugated to FITC (1:300; Sigma-Aldrich; F9887), goat-anti-rat conjugated to Cy3 (1:300; Chemicon; AP183C), goat-anti rabbit conjugated to Alexa Fluor 568 (1:500; Invitrogen; A11036), and donkey-anti-rat conjugated to Alexa Fluor 488 (1:500; Invitrogen; A21208).

Genome-Wide Chiasma Counting

Fixation, spread preparation of PMCs, and FISH were performed as previously described (Sanchez Moran et al., 2001; López et al., 2012). FISH using 45S and 5S rDNA probes, combined with chromosome morphology, allowed the unambiguous identification of each chromosome and chromosome arm (Sanchez-Moran et al., 2002).

Determination of Genetic Distances and Interference Ratios

Genetic distances and interference were determined as described before (Berchowitz and Copenhaver, 2008). The following lines were used: FTL1506, FTL1524, and FTL965 for analysis of chromosome II and FTL1963, FTL1143, and FTL2450 for analysis of chromosome V. Analyzed plants were all grown at the same time and tetrads from at least five individual plants per genotype were evaluated.

Statistical Analyses

Chiasma data were analyzed using the SPSS statistical package. Due to the binary nature of the results (recombination or no recombination), differences in genetic distances were analyzed by binary logistic regression. All other statistical analyses were performed using the GraphPad Prism software version 6.07. 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 database under accession number MF327383.

Supplemental Data

Supplemental Figure 1. Meiotic DNA repair is unaffected in *fancd2-1 atr-2* double mutants.

Supplemental Figure 2. Meiotic DNA repair defects in *atm-2 fancd2-1* double mutants depend on presence of *AtSPO11-2*.

Supplemental Figure 3. Univalents are formed in *mus81-2* meiocytes.

Supplemental Table 1. Mean chiasma frequencies per cell, per bivalent, and per bivalent arm.

Supplemental Table 2. Tetrad analysis

Supplemental Table 3. Oligonucleotides used in this study.

Supplemental Data Set 1. Fanconi anemia genes and their functions in mammals and plants.

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AUTHOR CONTRIBUTIONS

M.-T.K., M.P., C.K., J.S., R.L., C.O., M.P.J., and M.M. performed the experiments shown. M.-T.K., D.S., G.P.C., and P.S. conceived the experiments. M.-T.K., M.P., and P.S. analyzed the data. M.-T.K. and P.S. wrote the manuscript with substantial contribution from G.P.C.

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REFERENCES

- Adamo, A., Collis, S.J., Adelman, C.A., Silva, N., Horejsi, Z., Ward, J.D., Martinez-Perez, E., Boulton, S.J., and La Volpe, A.** (2010). Preventing nonhomologous end joining suppresses DNA repair defects of Fanconi anemia. *Mol. Cell* **39**: 25–35.
- Alonso, J.M., et al.** (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653–657.
- Amangyeld, T., Shin, Y.K., Lee, M., Kwon, B., and Seo, Y.S.** (2014). Human MUS81-EME2 can cleave a variety of DNA structures including intact Holliday junction and nicked duplex. *Nucleic Acids Res.* **42**: 5846–5862.
- Andreassen, P.R., D’Andrea, A.D., and Taniguchi, T.** (2004). ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes Dev.* **18**: 1958–1963.
- Argueso, J.L., Wanat, J., Gemici, Z., and Alani, E.** (2004). Competing crossover pathways act during meiosis in *Saccharomyces cerevisiae*. *Genetics* **168**: 1805–1816.
- Armstrong, S.** (2013). Spreading and fluorescence in situ hybridization of male and female meiocyte chromosomes from *Arabidopsis thaliana* for cytogenetical analysis. *Methods Mol. Biol.* **990**: 3–11.
- Armstrong, S.J., Caryl, A.P., Jones, G.H., and Franklin, F.C.** (2002). Asy1, a protein required for meiotic chromosome synapsis, localizes to axis-associated chromatin in *Arabidopsis* and *Brassica*. *J. Cell Sci.* **115**: 3645–3655.
- Berchowitz, L.E., and Copenhaver, G.P.** (2008). Fluorescent *Arabidopsis* tetrads: a visual assay for quickly developing large crossover and crossover interference data sets. *Nat. Protoc.* **3**: 41–50.
- Berchowitz, L.E., Francis, K.E., Bey, A.L., and Copenhaver, G.P.** (2007). The role of AtMUS81 in interference-insensitive crossovers in *A. thaliana*. *PLoS Genet.* **3**: e132.
- Blanco, M.G., and Matos, J.** (2015). Hold your horSSEs: controlling structure-selective endonucleases MUS81 and Yen1/GEN1. *Front. Genet.* **6**: 253.
- Boisvert, R.A., and Howlett, N.G.** (2014). The Fanconi anemia ID2 complex: dueling axes at the crossroads. *Cell Cycle* **13**: 2999–3015.
- Carballo, J.A., Panizza, S., Serrentino, M.E., Johnson, A.L., Geymonat, M., Borde, V., Klein, F., and Cha, R.S.** (2013). Budding yeast ATM/ATR control meiotic double-strand break (DSB) levels by down-regulating Rec114, an essential component of the DSB-machinery. *PLoS Genet.* **9**: e1003545.
- Castor, D., Nair, N., Déclais, A.C., Lachaud, C., Toth, R., Macartney, T.J., Lilley, D.M., Arthur, J.S., and Rouse, J.** (2013). Cooperative control of holliday junction resolution and DNA repair by the SLX1 and MUS81-EME1 nucleases. *Mol. Cell* **52**: 221–233.
- Ceccaldi, R., Sarangi, P., and D’Andrea, A.D.** (2016). The Fanconi anaemia pathway: new players and new functions. *Nat. Rev. Mol. Cell Biol.* **17**: 337–349.
- Chaudhury, I., Sareen, A., Raghunandan, M., and Sobek, A.** (2013). FANCD2 regulates BLM complex functions independently of FANCI to promote replication fork recovery. *Nucleic Acids Res.* **41**: 6444–6459.
- Chelysheva, L., Gendrot, G., Vezon, D., Doutriaux, M.P., Mercier, R., and Grelon, M.** (2007). Zip4/Spo22 is required for class I CO formation but not for synapsis completion in *Arabidopsis thaliana*. *PLoS Genet.* **3**: e83.
- Chelysheva, L., Vezon, D., Chambon, A., Gendrot, G., Pereira, L., Lemhemdi, A., Vrielynck, N., Le Guin, S., Novatchkova, M., and Grelon, M.** (2012). The *Arabidopsis* HEI10 is a new ZMM protein related to Zip3. *PLoS Genet.* **8**: e1002799.
- Chelysheva, L., et al.** (2005). AtREC8 and AtSCC3 are essential to the monopolar orientation of the kinetochores during meiosis. *J. Cell Sci.* **118**: 4621–4632.
- Ciccia, A., McDonald, N., and West, S.C.** (2008). Structural and functional relationships of the XPF/MUS81 family of proteins. *Annu. Rev. Biochem.* **77**: 259–287.
- Collis, S.J., Barber, L.J., Ward, J.D., Martin, J.S., and Boulton, S.J.** (2006). *C. elegans* FANCD2 responds to replication stress and functions in interstrand cross-link repair. *DNA Repair (Amst.)* **5**: 1398–1406.
- Copenhaver, G.P., Housworth, E.A., and Stahl, F.W.** (2002). Crossover interference in *Arabidopsis*. *Genetics* **160**: 1631–1639.
- Crismani, W., Girard, C., Froger, N., Pradillo, M., Santos, J.L., Chelysheva, L., Copenhaver, G.P., Horlow, C., and Mercier, R.** (2012). FANCM limits meiotic crossovers. *Science* **336**: 1588–1590.
- Culligan, K., Tissier, A., and Britt, A.** (2004). ATR regulates a G2-phase cell-cycle checkpoint in *Arabidopsis thaliana*. *Plant Cell* **16**: 1091–1104.
- Dangel, N.J., Knoll, A., and Puchta, H.** (2014). MHF1 plays Fanconi anaemia complementation group M protein (FANCM)-dependent and FANCM-independent roles in DNA repair and homologous recombination in plants. *Plant J.* **78**: 822–833.

- de los Santos, T., Hunter, N., Lee, C., Larkin, B., Loidl, J., and Hollingsworth, N.M. (2003). The Mus81/Mms4 endonuclease acts independently of double-Holliday junction resolution to promote a distinct subset of crossovers during meiosis in budding yeast. *Genetics* **164**: 81–94.
- de Massy, B. (2013). Initiation of meiotic recombination: how and where? Conservation and specificities among eukaryotes. *Annu. Rev. Genet.* **47**: 563–599.
- Duroc, Y., Lemhemdi, A., Larchevêque, C., Hurel, A., Cuacos, M., Cromer, L., Horlow, C., Armstrong, S.J., Chelysheva, L., and Mercier, R. (2014). The kinesin AtPSS1 promotes synapsis and is required for proper crossover distribution in meiosis. *PLoS Genet.* **10**: e1004674.
- Fanconi, G. (1967). Familial constitutional panmyelocytopenia, Fanconi's anemia (F.A.). I. Clinical aspects. *Semin. Hematol.* **4**: 233–240.
- Francis, K.E., Lam, S.Y., and Copenhaver, G.P. (2006). Separation of Arabidopsis pollen tetrads is regulated by QUARTET1, a pectin methylesterase gene. *Plant Physiol.* **142**: 1004–1013.
- Francis, K.E., Lam, S.Y., Harrison, B.D., Bey, A.L., Berchowitz, L.E., and Copenhaver, G.P. (2007). Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **104**: 3913–3918.
- Garcia, V., Gray, S., Allison, R.M., Cooper, T.J., and Neale, M.J. (2015). Tel1(ATM)-mediated interference suppresses clustered meiotic double-strand-break formation. *Nature* **520**: 114–118.
- Garcia, V., Bruchet, H., Comesças, D., Granier, F., Bouchez, D., and Tissier, A. (2003). AtATM is essential for meiosis and the somatic response to DNA damage in plants. *Plant Cell* **15**: 119–132.
- Garner, E., and Smogorzewska, A. (2011). Ubiquitylation and the Fanconi anemia pathway. *FEBS Lett.* **585**: 2853–2860.
- Girard, C., Crismani, W., Froger, N., Mazel, J., Lemhemdi, A., Horlow, C., and Mercier, R. (2014). FANCM-associated proteins MHF1 and MHF2, but not the other Fanconi anemia factors, limit meiotic crossovers. *Nucleic Acids Res.* **42**: 9087–9095.
- Hartung, F., Suer, S., Bergmann, T., and Puchta, H. (2006). The role of AtMUS81 in DNA repair and its genetic interaction with the helicase AtRecQ4A. *Nucleic Acids Res.* **34**: 4438–4448.
- Herrmann, N.J., Knoll, A., and Puchta, H. (2015). The nuclease FAN1 is involved in DNA crosslink repair in *Arabidopsis thaliana* independently of the nuclease MUS81. *Nucleic Acids Res.* **43**: 3653–3666.
- Higgins, J.D., Armstrong, S.J., Franklin, F.C., and Jones, G.H. (2004). The Arabidopsis MutS homolog AtMSH4 functions at an early step in recombination: evidence for two classes of recombination in Arabidopsis. *Genes Dev.* **18**: 2557–2570.
- Higgins, J.D., Buckling, E.F., Franklin, F.C., and Jones, G.H. (2008a). Expression and functional analysis of AtMUS81 in Arabidopsis meiosis reveals a role in the second pathway of crossing-over. *Plant J.* **54**: 152–162.
- Higgins, J.D., Sanchez-Moran, E., Armstrong, S.J., Jones, G.H., and Franklin, F.C. (2005). The Arabidopsis synaptonemal complex protein ZYP1 is required for chromosome synapsis and normal fidelity of crossing over. *Genes Dev.* **19**: 2488–2500.
- Higgins, J.D., Vignard, J., Mercier, R., Pugh, A.G., Franklin, F.C., and Jones, G.H. (2008b). AtMSH5 partners AtMSH4 in the class I meiotic crossover pathway in *Arabidopsis thaliana*, but is not required for synapsis. *Plant J.* **55**: 28–39.
- Hilliers, K.J., and Villeneuve, A.M. (2003). Chromosome-wide control of meiotic crossing over in *C. elegans*. *Curr. Biol.* **13**: 1641–1647.
- Hollingsworth, N.M., and Brill, S.J. (2004). The Mus81 solution to resolution: generating meiotic crossovers without Holliday junctions. *Genes Dev.* **18**: 117–125.
- Holloway, J.K., Booth, J., Edelman, W., McGowan, C.H., and Cohen, P.E. (2008). MUS81 generates a subset of MLH1-MLH3-independent crossovers in mammalian meiosis. *PLoS Genet.* **4**: e1000186.
- Houghtaling, S., Timmers, C., Noll, M., Finegold, M.J., Jones, S.N., Meyn, M.S., and Grompe, M. (2003). Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev.* **17**: 2021–2035.
- Hunter, N. (2007). Meiotic recombination. In *Molecular Genetics of Recombination*, A. Aguilera and R. Rothstein, eds (Berlin, Heidelberg: Springer), pp. 381–442.
- Jacquemont, C., and Taniguchi, T. (2007). The Fanconi anemia pathway and ubiquitin. *BMC Biochem.* **8** (suppl. 1): S10.
- Jones, G.H., and Franklin, F.C. (2006). Meiotic crossing-over: obligation and interference. *Cell* **126**: 246–248.
- Joyce, E.F., Pedersen, M., Tiong, S., White-Brown, S.K., Paul, A., Campbell, S.D., and McKim, K.S. (2011). Drosophila ATM and ATR have distinct activities in the regulation of meiotic DNA damage and repair. *J. Cell Biol.* **195**: 359–367.
- Kee, Y., and D'Andrea, A.D. (2012). Molecular pathogenesis and clinical management of Fanconi anemia. *J. Clin. Invest.* **122**: 3799–3806.
- Keeney, S., Giroux, C.N., and Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* **88**: 375–384.
- Klein Douwel, D., Boonen, R.A., Long, D.T., Szybowska, A.A., Raschle, M., Walter, J.C., and Knipscheer, P. (2014). XPF-ERCC1 acts in unhooking DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/SLX4. *Mol. Cell* **54**: 460–471.
- Kleinboelting, N., Huel, G., Kloetgen, A., Viehoveer, P., and Weisshaar, B. (2012). GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. *Nucleic Acids Res.* **40**: D1211–D1215.
- Knoll, A., Higgins, J.D., Seeliger, K., Reha, S.J., Dangel, N.J., Bauknecht, M., Schröpfer, S., Franklin, F.C., and Puchta, H. (2012). The Fanconi anemia ortholog FANCM ensures ordered homologous recombination in both somatic and meiotic cells in Arabidopsis. *Plant Cell* **24**: 1448–1464.
- Kotemann, M.C., and Smogorzewska, A. (2013). Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. *Nature* **493**: 356–363.
- Kratz, K., Schöpf, B., Kaden, S., Sandoel, A., Eberhard, R., Lademann, C., Cannavó, E., Sartori, A.A., Hengartner, M.O., and Jiricny, J. (2010). Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. *Cell* **142**: 77–88.
- Kurzbauer, M.T., Uanschou, C., Chen, D., and Schögelhofer, P. (2012). The recombinases DMC1 and RAD51 are functionally and spatially separated during meiosis in Arabidopsis. *Plant Cell* **24**: 2058–2070.
- Lachaud, C., Moreno, A., Marchesi, F., Toth, R., Blow, J.J., and Rouse, J. (2016). Ubiquitinated Fancd2 recruits Fan1 to stalled replication forks to prevent genome instability. *Science* **351**: 846–849.
- Lam, S.Y., Horn, S.R., Radford, S.J., Housworth, E.A., Stahl, F.W., and Copenhaver, G.P. (2005). Crossover interference on nucleolus organizing region-bearing chromosomes in Arabidopsis. *Genetics* **170**: 807–812.
- Lange, J., Pan, J., Cole, F., Thelen, M.P., Jasin, M., and Keeney, S. (2011). ATM controls meiotic double-strand-break formation. *Nature* **479**: 237–240.
- Liang, C.C., Li, Z., Lopez-Martinez, D., Nicholson, W.V., Vénien-Bryan, C., and Cohn, M.A. (2016). The FANCD2-FANCI complex is recruited to DNA interstrand crosslinks before monoubiquitination of FANCD2. *Nat. Commun.* **7**: 12124.
- Liu, T., and Huang, J. (2016). DNA end resection: facts and mechanisms. *Genomics Proteomics Bioinformatics* **14**: 126–130.
- Liu, T., Ghosal, G., Yuan, J., Chen, J., and Huang, J. (2010). FAN1 acts with FANCI-FANCD2 to promote DNA interstrand cross-link repair. *Science* **329**: 693–696.

- López, E., Pradillo, M., Oliver, C., Romero, C., Cuñado, N., and Santos, J.L. (2012). Looking for natural variation in chiasma frequency in *Arabidopsis thaliana*. *J. Exp. Bot.* **63**: 887–894.
- Lynn, A., Soucek, R., and Börner, G.V. (2007). ZMM proteins during meiosis: crossover artists at work. *Chromosome Res.* **15**: 591–605.
- MacKay, C., Déclais, A.C., Lundin, C., Agostinho, A., Deans, A.J., MacArtney, T.J., Hofmann, K., Gartner, A., West, S.C., Helleday, T., Lilley, D.M., and Rouse, J. (2010). Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. *Cell* **142**: 65–76.
- Manhart, C.M., and Alani, E. (2016). Roles for mismatch repair family proteins in promoting meiotic crossing over. *DNA Repair (Amst.)* **38**: 84–93.
- Martini, E., Diaz, R.L., Hunter, N., and Keeney, S. (2006). Crossover homeostasis in yeast meiosis. *Cell* **126**: 285–295.
- McMahill, M.S., Sham, C.W., and Bishop, D.K. (2007). Synthesis-dependent strand annealing in meiosis. *PLoS Biol.* **5**: e299.
- Michl, J., Zimmer, J., and Tarsounas, M. (2016). Interplay between Fanconi anemia and homologous recombination pathways in genome integrity. *EMBO J.* **35**: 909–923.
- Mohibullah, N., and Keeney, S. (2017). Numerical and spatial patterning of yeast meiotic DNA breaks by Tel1. *Genome Res.* **27**: 278–288.
- Murina, O., von Aesch, C., Karakus, U., Ferretti, L.P., Bolck, H.A., Hänggi, K., and Sartori, A.A. (2014). FANCD2 and CtIP cooperate to repair DNA interstrand crosslinks. *Cell Reports* **7**: 1030–1038.
- Muyt, A.D., Mercier, R., Mézard, C., and Grelon, M. (2009). Meiotic recombination and crossovers in plants. *Genome Dyn.* **5**: 14–25.
- Nakanishi, K., Yang, Y.G., Pierce, A.J., Taniguchi, T., Digweed, M., D'Andrea, A.D., Wang, Z.Q., and Jasin, M. (2005). Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. *Proc. Natl. Acad. Sci. USA* **102**: 1110–1115.
- Oke, A., Anderson, C.M., Yam, P., and Fung, J.C. (2014). Controlling meiotic recombinational repair - specifying the roles of ZMMs, Sgs1 and Mus81/Mms4 in crossover formation. *PLoS Genet.* **10**: e1004690.
- Pace, P., Mosedale, G., Hodkinson, M.R., Rosado, I.V., Sivasubramaniam, M., and Patel, K.J. (2010). Ku70 corrupts DNA repair in the absence of the Fanconi anemia pathway. *Science* **329**: 219–223.
- Park, W.H., Margossian, S., Horwitz, A.A., Simons, A.M., D'Andrea, A.D., and Parvin, J.D. (2005). Direct DNA binding activity of the Fanconi anemia D2 protein. *J. Biol. Chem.* **280**: 23593–23598.
- Phadnis, N., Hyppa, R.W., and Smith, G.R. (2011). New and old ways to control meiotic recombination. *Trends Genet.* **27**: 411–421.
- Pizzolato, J., Mukherjee, S., Schärer, O.D., and Jiricny, J. (2015). FANCD2-associated nuclease 1, but not exonuclease 1 or flap endonuclease 1, is able to unhook DNA interstrand cross-links in vitro. *J. Biol. Chem.* **290**: 22602–22611.
- Preuss, D., Rhee, S.Y., and Davis, R.W. (1994). Tetrad analysis possible in *Arabidopsis* with mutation of the QUARTET (QRT) genes. *Science* **264**: 1458–1460.
- Rinaldo, C., Bazzicalupo, P., Ederle, S., Hilliard, M., and La Volpe, A. (2002). Roles for *Caenorhabditis elegans* rad-51 in meiosis and in resistance to ionizing radiation during development. *Genetics* **160**: 471–479.
- Robert, T., Nore, A., Brun, C., Maffre, C., Crimi, B., Bourbon, H.M., and de Massy, B. (2016). The TopoVIB-Like protein family is required for meiotic DNA double-strand break formation. *Science* **351**: 943–949.
- Robinson, S.J., et al. (2009). An archived activation tagged population of *Arabidopsis thaliana* to facilitate forward genetics approaches. *BMC Plant Biol.* **9**: 101.
- Ross, K.J., Fransz, P., and Jones, G.H. (1996). A light microscopic atlas of meiosis in *Arabidopsis thaliana*. *Chromosome Res.* **4**: 507–516.
- Sanchez-Moran, E., Armstrong, S.J., Santos, J.L., Franklin, F.C., and Jones, G.H. (2002). Variation in chiasma frequency among eight accessions of *Arabidopsis thaliana*. *Genetics* **162**: 1415–1422.
- Sanchez Moran, E., Armstrong, S.J., Santos, J.L., Franklin, F.C., and Jones, G.H. (2001). Chiasma formation in *Arabidopsis thaliana* accession Wassileskija and in two meiotic mutants. *Chromosome Res.* **9**: 121–128.
- Sato, K., Toda, K., Ishiai, M., Takata, M., and Kurumizaka, H. (2012). DNA robustly stimulates FANCD2 monoubiquitylation in the complex with FANCI. *Nucleic Acids Res.* **40**: 4553–4561.
- Smogorzewska, A., Desetty, R., Saito, T.T., Schlabach, M., Lach, F.P., Sowa, M.E., Clark, A.B., Kunkel, T.A., Harper, J.W., Colaiácovo, M.P., and Elledge, S.J. (2010). A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. *Mol. Cell* **39**: 36–47.
- Sobeck, A., Stone, S., and Hoatlin, M.E. (2007). DNA structure-induced recruitment and activation of the Fanconi anemia pathway protein FANCD2. *Mol. Cell. Biol.* **27**: 4283–4292.
- Thacker, D., Mohibullah, N., Zhu, X., and Keeney, S. (2014). Homologue engagement controls meiotic DNA break number and distribution. *Nature* **510**: 241–246.
- Unno, J., Itaya, A., Taoka, M., Sato, K., Tomida, J., Sakai, W., Sugasawa, K., Ishiai, M., Ikura, T., Isobe, T., Kurumizaka, H., and Takata, M. (2014). FANCD2 binds CtIP and regulates DNA-end resection during DNA interstrand crosslink repair. *Cell Rep.* **7**: 1039–1047.
- Vignard, J., Siwec, T., Chelysheva, L., Vrielynck, N., Gonord, F., Armstrong, S.J., Schlögelhofer, P., and Mercier, R. (2007). The interplay of RecA-related proteins and the MND1-HOP2 complex during meiosis in *Arabidopsis thaliana*. *PLoS Genet.* **3**: 1894–1906.
- Vrielynck, N., Chambon, A., Vezon, D., Pereira, L., Chelysheva, L., De Muyt, A., Mézard, C., Mayer, C., and Grelon, M. (2016). A DNA topoisomerase VI-like complex initiates meiotic recombination. *Science* **351**: 939–943.
- Wang, S., Zickler, D., Kleckner, N., and Zhang, L. (2015). Meiotic crossover patterns: obligatory crossover, interference and homeostasis in a single process. *Cell Cycle* **14**: 305–314.
- Wang, X., and D'Andrea, A.D. (2004). The interplay of Fanconi anemia proteins in the DNA damage response. *DNA Repair (Amst.)* **3**: 1063–1069.
- Ward, J.O., Reinholdt, L.G., Motley, W.W., Niswander, L.M., Deacon, D.C., Griffin, L.B., Langlais, K.K., Backus, V.L., Schimenti, K.J., O'Brien, M.J., Eppig, J.J., and Schimenti, J.C. (2007). Mutation in mouse hei10, an e3 ubiquitin ligase, disrupts meiotic crossing over. *PLoS Genet.* **3**: e139.
- Whitby, M.C. (2005). Making crossovers during meiosis. *Biochem. Soc. Trans.* **33**: 1451–1455.
- Wyatt, H.D., and West, S.C. (2014). Holliday junction resolvases. *Cold Spring Harb. Perspect. Biol.* **6**: a023192.
- Yamamoto, K.N., Kobayashi, S., Tsuda, M., Kurumizaka, H., Takata, M., Kono, K., Jiricny, J., Takeda, S., and Hirota, K. (2011). Involvement of SLX4 in interstrand cross-link repair is regulated by the Fanconi anemia pathway. *Proc. Natl. Acad. Sci. USA* **108**: 6492–6496.
- Yeo, J.E., Lee, E.H., Hendrickson, E.A., and Sobeck, A. (2014). CtIP mediates replication fork recovery in a FANCD2-regulated manner. *Hum. Mol. Genet.* **23**: 3695–3705.
- Youds, J.L., Barber, L.J., and Boulton, S.J. (2009). *C. elegans*: a model of Fanconi anemia and ICL repair. *Mutat. Res.* **668**: 103–116.
- Zhang, L., Kim, K.P., Kleckner, N.E., and Storlazzi, A. (2011). Meiotic double-strand breaks occur once per pair of (sister) chromatids and, via Mec1/ATR and Tel1/ATM, once per quartet of chromatids. *Proc. Natl. Acad. Sci. USA* **108**: 20036–20041.