Spontaneous self-segregation of Rad51 and Dmc1 DNA recombinases within mixed recombinase filaments

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Abstract

During meiosis, the two DNA recombinases Rad51 and Dmc1, form specialized presynaptic filaments that are adapted for performing recombination between homologous chromosomes. There is currently a limited understanding of how these two recombinases are organized within the meiotic presynaptic filament. Here, we used single molecule imaging to examine the properties of presynaptic complexes comprised of both Rad51 and Dmc1. We demonstrate that Rad51 and Dmc1 have an intrinsic ability to self-segregate, even in the absence of any other recombination accessory proteins. Moreover, we found that the presence of Dmc1 stabilizes the adjacent Rad51 filaments, suggesting that crosstalk between these two recombinases may affect their biochemical properties. Based upon these findings, we describe a model for the organization of Rad51 and Dmc1 within the meiotic presynaptic complex, which is also consistent with in vivo observations, genetic findings and biochemical expectations. This model argues against the existence of extensively intermixed filaments, and we propose that Rad51 and Dmc1 have intrinsic capacities to form spatially distinct filaments, suggesting that additional recombination cofactors are not required to segregate the Rad51 and Dmc1 filaments.

Introduction

Meiosi s is characterized by a single round of replication followed by two rounds of cell division to yield haploid gametes from a diploid precursor (1-6). During meiosis, the endonuclease Spo11 initiates programmed double-strand breaks (DSBs), which are then repaired by homologous recombination between homologs (1-6). Recombination during meiosis is required for chromosome segregation and also generates new combinations of parental alleles (1-6).

Most eukaryotes have two Rad51/RecA family recombinases: Rad51, which is expressed throughout the cell cycle; and Dmc1, which is expressed only during meiosis (7). The reasons why eukaryotes have these two recombinases remain unknown (1,6). Rad51 and Dmc1 share ~45% amino acid identity (6,8,9), they assemble into structurally similar right-handed helical filaments on single-stranded DNA (ssDNA) (10-12), and they have similar, albeit not identical, biochemical properties (1,6). Despite these many commonalities, Dmc1 is the recombinase responsible for performing strand invasion during meiosis (13). In contrast, Rad51 is actively down-regulated by Hed1- and Mek1-mediated inhibition of its interactions with Rad54, which is a cofactor that is required for Rad51 strand invasion activity (11,14-17). However, Rad51 is required for normal progression through meiosis and promotes Dmc1 foci formation, consistent with a role for Rad51 as a Dmc1 accessory factor (6,7,13,18).
Interesting, a number of *in vivo* studies have suggested that Rad51 and Dmc1 form closely associated, yet separate, homo-polymorphic filaments at the ends of meiotic DSBs (1,6). Indeed, Rad51 and Dmc1 appear as partially offset foci in many organisms (18-20), which led to the proposal of an asymmetric loading model in which Rad51–only filaments formed on one end of a meiotic DSB, and Dmc1–only filaments formed on the other end of the same DSB (6,19,20). More recently, super-resolution microscopy experiments have shown that *S. cerevisiae* Rad51 and Dmc1 co-occupy both ends of meiotic DSBs, arguing against the asymmetric loading model, and instead suggesting a model in which Rad51 and Dmc1 form separate filaments on the same ssDNA (21).

It is not known how Rad51 and Dmc1 might segregate into separate filaments. The two recombinases have distinct mediators, Rad52, which promotes assembly of Rad51 filaments (22,23), and Mei5/Sae3, which promotes assembly of Dmc1 filaments (24). So, one possibility is that Rad52 and Mei5/Sae3 direct Rad51 and Dmc1 filament assembly at discrete locations. Alternatively, the recombinases might form separate filaments with no need for assistance from additional regulatory cofactors. To test these hypotheses, here, we assembled presynaptic complexes using mixtures of *S. cerevisiae* Rad51 and Dmc1, and ask whether in the absence of mediator proteins the recombinases assemble into randomly intermixed filaments, or whether they form homo-polymorphic filaments. We find that Dmc1 and Rad51 can spontaneously self-segregate into discrete homo-polymorphic filaments on ssDNA, yielding a side-by-side arrangement reminiscent of the organization of Rad51 and Dmc1 observed *in vivo* (1,6). This finding supports a model for the meiotic presynaptic filament wherein Rad51 and Dmc1 have an intrinsic capacity to form spatially distinct filaments, suggesting that other cofactors may not be required to direct segregation of the Rad51 and Dmc1 filaments.

**Results**

*Models for mixed recombinase filaments* – Rad51 and Dmc1 are both required for timely progression through meiosis (1,6), and the two proteins form closely associated foci at sites of meiotic DSBs *in vivo* (18-21). However, there have been a very limited number of *in vitro* studies designed to test the properties of presynaptic complexes comprised of both Rad51 and Dmc1 (13), and to our knowledge there is no experimental data suggesting how mixed filaments might assemble.

We considered two hypothetical outcomes (Figure 1A). One possibility is that the recombinases might form highly intermixed heterotypic complexes in which the Rad51 and Dmc1 monomers were simply randomly dispersed within the filament. Such a scenario could be readily envisioned if, for instance, the interfaces formed between Rad51 and Dmc1 monomers were similar to the monomer-monomer interfaces within each separate homo-polymer. This scenario is not unreasonable given that the two proteins are closely related and share ~45% sequence identity (6,8,9). Alternatively, the two recombinases might not interact with one another, in which case homotypic tracts of Rad51 or Dmc1 might be expected to form on the ssDNA, as has been suggested from microscopic observations of recombination foci or filaments during meiosis (Figure 1A) (19-21). We present these two opposing scenarios for simplicity, with the understanding that the actual situation might lie somewhere between these two ends of the spectrum.

*Presynaptic complex assembly kinetics* – To test these hypotheses, we next sought to establish an experimental system for examining the spatial organization of Rad51 and Dmc1 within the context of a mixed presynaptic complex assembled *in vitro*. For this, we used ssDNA curtains and TIRF microscopy (Figure 1B), which we have used previously to examine the properties of presynaptic complexes assembled with either Rad51 alone or Dmc1 alone, but never the two recombinases together (33,36).

In these experiments, the ssDNA is first extended using *S. cerevisiae* RPA-mCherry, and the free RPA-mCherry is then flushed from the sample chamber. *S. cerevisiae* Rad51 only (2 µM), Dmc1 only (2 µM), or equimolar ratios of Rad51 and Dmc1 (1:1; 2 µM total protein) were injected into the flow cell (Figure 1C,D). We then measured the rate and extent of presynaptic complex assembly by quantitating the RPA-mCherry fluorescence signal intensity, which was integrated over the entire length of the ssDNA.
molecules, as described (25,33,34,37). From these experiments, we found the mean rates of presynaptic complex assembly to be $0.55 \pm 0.06$ sec$^{-1}$, $0.51 \pm 0.06$, $0.57 \pm 0.06$ sec$^{-1}$ (mean ± s.d.), for the Rad51 only, Dmc1 only and mixed Rad51 plus Dmc1 filaments respectively (Figure 1D,E). These results indicate that the rates of filament assembly in our assays are similar regardless of recombinase identity. In contrast, the assembly reactions plateau at normalized RPA-mCherry intensity values, integrated over the entire length of the ssDNA, of $0.29 \pm 0.02$ a.u., $0.15 \pm 0.02$ a.u., and $0.33 \pm 0.02$ a.u. (mean ± s.d.), for the Rad51 only, Dmc1 only and mixed filaments, respectively (Figure 1F). This result indicates that under our reaction conditions Dmc1 displaces more RPA-mCherry from the ssDNA compared to Rad51, and that this effect is reduced in equimolar mixtures of Rad51 and Dmc1. Together, these kinetic data indicate that the assembly characteristics of Dmc1 and Rad51 are similar, but not identical.

**Visualization of mixed recombinase presynaptic complexes** – To clearly distinguish between the two models presented in Figure 1A, it would be necessary to label one of the two recombinases such that we could measure its spatial distribution within the resulting presynaptic complexes. We cannot yet directly label either recombinase: GFP-tagged Rad51 is not biologically viable (38), we presume that GFP-tagged Dmc1 would be similarly problematic, and we have not succeeded in generating yeast Rad51 (or Dmc1) that is labeled with small organic dyes and still retains activity. Therefore, as an alternative strategy, we used GFP-tagged Hed1 to detect distribution of Rad51 with the mixed filaments. Genetic and biochemical data have demonstrated that Hed1 interacts specifically with Rad51 (15,39), and Hed1-GFP complements a hed1Δ strain, indicating that the fluorescent protein retains function (14). Consistent with these findings, we can readily detect Hed1-GFP binding to Rad51, but we are unable to detect any GFP-Hed1 association with Dmc1 (Figure 2A)(40). Therefore, we consider Hed1-GFP to be good proxy for defining the location of Rad51 within the presynaptic complex.

Given that Hed1-GFP binds uniformly to Rad51-ssDNA filaments, but does not bind to Dmc1-ssDNA, we can make some predictions for the outcome of Hed1-binding experiments based upon the models presented in Figure 1A. Namely, if Rad51 and Dmc1 formed highly intermixed filaments, then this would be revealed as a gradual reduction of overall Hed1-GFP signal intensity, but the distribution of Hed1-GFP should remain relatively uniform (within optical resolution). In contrast, if Rad51 and Dmc1 segregated into separate filaments, then the overall Hed1-GFP signal should decrease, but the pattern of Hed1-GFP binding should become highly punctate instead of uniform.

Using the experimental strategy outlined above, we next sought to visualize presynaptic complexes comprise of both Rad51 and Dmc1. Mixtures of Rad51 and Dmc1 (3:1, 1:1, or 1:3) were then injected into the sample chamber to initiate presynaptic complex assembly. We then injected Hed1-GFP to determine where Rad51 was located within the presynaptic complex. Our expectation was that as we altered the ratio of Rad51 to Dmc1, the number of Hed1 binding sites would change proportionally. We measured this by titrating Hed1-GFP into presynaptic complexes assembled with 1:0, 3:1, 1:1, 1:3 ratios of Rad51:Dmc1 (2 μM Rad51 plus Dmc1 total). As we reduced the ratio of Rad51 to Dmc1, the appearance of GFP-Hed1 transitioned from uniform binding patterns (e.g. 3:1 Rad51:Dmc1; Figure 2B) to highly punctate patterns when more Dmc1 was included in the reactions (e.g. 1:1 Rad51:Dmc1; Figure 2B). These findings indicated that S. cerevisiae Rad51 and Dmc1 self-segregated into a side-by-side arrangement of homo-polymeric filaments on the same ssDNA molecules to an extent that is readily detectable at optical resolution.

To estimate the amount of Rad51 present in the mixed filaments, we measured the integrated Hed1-GFP signal intensity over the entire ssDNA, and evaluated the resulting data by non-linear regression to obtain a value for the maximal signal level ($B_{max}$) for each of the different Rad51:Dmc1 ratios. This data was then plotted and used as a standard curve to estimate the actual amount of Rad51 bound to the ssDNA relative to the input ratio of Rad51 to Dmc1 (Figure 2B). These experiments suggested the presynaptic complexes assembled at 3:1, 1:1 and 1:3 molar ratios of Rad51:Dmc1 were actually comprised of ~50, ~12.5, and ~10% Rad51 bound to the ssDNA. The
finding that Rad51 is underrepresented in the bound fraction of recombinase, relative to the concentrations of injected proteins, despite the observation that both recombinases display similar assembly rates (Figure 1E), suggests that Dmc1 might exhibit greater cooperativity than Rad51. This interpretation is also consistent with the observation that Dmc1 displaces more RPA from the ssDNA relative to what was observed for Rad51 (Figure 1F).

We next examined the spatial distributions of Hed1-GFP foci for mixed recombinase filaments assembled at a 1:1 ratio of Rad51 to Dmc1. Visual inspection of these filaments suggested that Hed1-labeled Rad51 filaments were randomly distributed along the ssDNA (Figure 3A,B). Further support for this conclusion was obtained by analyzing the position distribution of a larger number of Hed1-labeled Rad51 filaments (N=340; Figure 3C). Moreover, the average center-to-center distance between adjacent Hed1-labeled Rad51 foci was 1.7 ± 0.2 μm (Figure 3D), which would correspond to an average estimated distance of ~3,600 nucleotides, or ~1,200 recombinase monomers, between each of the observed Hed1-labeled Rad51 foci. We speculate that the center-to-center distances between adjacent Hed1-labeled Rad51 foci represent the average distances between individual Rad51 filament nucleation events in these mixed recombinase reactions (see Discussion).

**Dmc1 stabilizes adjacent Rad51 filaments** – A prevailing hypothesis on the role of Rad51 in meiotic filaments is to stabilize Dmc1 filaments (6). This hypothesis is supported by the observation that Dmc1 foci formation is compromised in vivo when Rad51 is absent (7,18), the finding that Rad51 is downregulated by Hed1 and Mek1 during meiosis (14,16,17), and the finding that Rad51 strand invasion activity is not required during meiosis (13). We wanted to test the possibility that Dmc1 might also exert some influence over Rad51 by comparing the disassembly rates of Rad51 only, Dmc1 only, and mixed filaments containing both recombinases. For this comparison, we also took advantage of the fact that when calcium is present, Rad51 dissociates from ssDNA upon ATP depletion, but Dmc1 does not (Figure 4A)(40).

We anticipated that the stability of Rad51 within the mixed recombinase filaments could be independently assessed by depletion of ATP. Presynaptic complex disassembly was quantitated by measuring the rate and extent of RPA-GFP rebinding, after ATP depletion, as previously described (28,33,37,41,42). We began by comparing the stability of presynaptic complexes comprised of Rad51 only and Dmc1 only, to complexes comprised of 9:1, 3:1, and 1:1 mixtures of Rad51 to Dmc1 (Figure 4A,B). At a 9:1 ratio of Rad51 to Dmc1, the filaments disassembled completely upon depletion of ATP, indicating that they were comprised primarily of Rad51 (Figure 4B). However, at a 3:1 ratio of Rad51 to Dmc1, only 25% of the bound protein dissociated upon ATP depletion (Figure 4A,B). This result was surprising, because, based upon Hed1-GFP labeling, presynaptic complexes assembled with at a 3:1 ratio of Rad51 to Dmc1 should be comprised of ~50% Rad51 (Figure 2B). The finding that a larger than expected fraction of recombinase remained bound to the ssDNA upon ATP depletion suggested that Dmc1 might be stabilizing Rad51.

We also quantitated the rate of Rad51 dissociation upon ATP depletion within the context of mixed recombinase filaments, using the reappearance of RPA-GFP as a proxy for Rad51 dissociation. Dissociation rates obtained from this analysis were then plotted against the amount of Rad51 present in the reaction, which revealed that the rate decreased in direct proportion to the amount of Dmc1 present in the reaction (Figure 4C). We conclude that the presence of Dmc1 reduces the rate and extent of dissociation for Rad51 filaments bound to the same ssDNA molecules.

**Rad51 remains bound within mixed recombinase filaments upon ATP depletion** – The findings presented above suggested that the presence of Dmc1 stabilized bound Rad51 against dissociation from the ssDNA upon depletion of ATP. Based on this observation, we predicted that some fraction of the Rad51 likely remained bound to the ssDNA upon ATP depletion when Dmc1 was present. To test this prediction, we sought to determine whether any Rad51 remained bound to the mixed recombinase filaments after ATP depletion by using Hed1-GFP to detect Rad51. Importantly, our
previous experiments have shown that Hed1-GFP does not bind to either RPA-ssDNA or Dmc1-ssDNA, and presence of bound Hed1 is strictly dependent upon the integrity of the Rad51-ssDNA (40). Therefore, Hed1-GFP would only be able to bind to the presynaptic complexes if Rad51 remained present after ATP depletion. Therefore, we tested for the presence of Rad51 by first assembling mixed recombinase filaments, then depleting ATP from the reaction buffer, followed by injection of Hed1-GFP and RPA-mCherry (Figure 5A). Control reactions using presynaptic complexes comprised of only Rad51, confirmed that we could readily detect RPA-mCherry binding after ATP depletion, but there was no evidence of Hed1-GFP binding (Figure 5B,F). Additional, control experiments using presynaptic filaments containing only Dmc1 revealed no evidence of Hed1-GFP binding and minimal re-binding of RPA-mCherry, consistent with the expectations that Dmc1 filaments were resistant to ATP depletion and that that Hed1-GFP was unable to bind to Dmc1-ssDNA (Figure 5C,F). In contrast, we could detect both Hed1-GFP and RPA-mCherry binding after ATP depletion in experiments using filaments assembled at a 4:1 ratio of Dmc1 to Rad51 (Figure 5D,F). This observation confirmed that some, but not all, of the Rad51 dissociated from the mixed recombinase filaments.

We next sought to confirm how much Rad51 remained bound to the ssDNA upon ATP depletion based upon the integrated signal intensity of the bound Hed1-GFP. For mixed filaments prepared at a 4:1 ratio of Rad51 to Dmc1, we anticipated that up to ~50% of the bound Rad51 may remain on the ssDNA after ATP depletion (Figure 4B). Based upon the titration curve for Hed1-GFP binding to Rad51-ssDNA (Figure 2B), the integrated Hed1-GFP signal intensity prior to ATP depletion should yield a value of ~2.3 x 10^6 (Figure 5E). Therefore, if ~50% Rad51 remained within the mixed filaments upon depletion of ATP, we anticipated that the integrated Hed1-GFP signal should be ~1.2 x 10^6 (Figure 5E). Consistent with this prediction, the integrated Hed1-GFP signal intensity after ATP depletion for filaments assembled at a 4:1 ratio of Dmc1 to Rad51 yielded a value of ~0.98 x10^6, which was within error of the predicted value (Figure 5E). Together, these findings support the conclusion that ~50% of the ssDNA-bound Rad51 became resistant to ATP depletion when Dmc1 is also present in the reactions.

Discussion
Our findings support a model for the meiotic presynaptic complex wherein Rad51 and Dmc1 have an intrinsic ability to form spatially distinct recombinase filaments bound to the same ssDNA molecule, and argues against the existence of highly intermixed filaments (Figure 1A). We do not rule out the possibility that there may be some intermixing of the recombinases in our assays, but if intermixing is taking place, then it does not represent the majority of the population, and is not detected in our Hed1-GFP binding assays. Importantly, the side-by-side arrangement of homotypic Rad51 and Dmc1 filaments observed in our experiments is strikingly similar to in vivo microscopic observations of Rad51 and Dmc1 foci (19,20), and recent in vivo super-resolution microscopy data that indicate Rad51 and Dmc1 form at discrete regions on meiotic filaments (21). In addition, our data are also consistent with genetic experiments that had previously suggested Rad51 and Dmc1 might not form intermixed filaments (43,44).

Our data suggests that the information required to create self-segregated recombinase filaments is encoded with in the recombinase enzymes themselves, with no need for contributions from other accessory factors. This argues against the possibility that mediator proteins, such as Rad52 and Mei5-Sae3 (22-24), are responsible for directing the assembly of segregated Rad51 and Dmc1 filaments that are observed in vivo. Instead, mediators may enhance loading of recombinase proteins, help reinforce segregation of the recombinases, and/or help regulate the length of the resulting recombinase filaments. This later point may prove important, given that the filaments we are examining in our biochemical assays are much longer than those observed in vivo (21), and one could readily envision that changes in nucleation frequency could be used as a regulatory mechanism for modulating filament lengths. Rather than requiring accessory factors to direct the assembly of separate Rad51 and Dmc1 filaments, we suggest that assembly of segregated recombinase filaments

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could result from differences in the monomer-monomer interfaces for Rad51 and Dmc1, or it could arise from differences in the ssDNA binding surfaces, or a combination of both. Future studies will be essential to determine which protein surfaces and amino acids might contribute to the assembly of separate Rad51 and Dmc1 filaments.

We also provide biochemical evidence for communication between recombinase filaments. This communication is revealed by the enhanced stabilization of Rad51 to ATP depletion when Dmc1 is present, and increased dissociation of Rad51 when Dmc1 is removed. Rad51 is required for efficient formation of Dmc1 foci during meiosis (18,44), but Rad51 strand exchange activity is not required (13), leading to a model wherein Rad51 functions primarily to stimulate assembly of Dmc1 filaments (6,13). Thus, our data suggest that this stimulatory effect can also work in the reverse direction, with Dmc1 increasing the stability of the adjacent Rad51 filaments.

Rad51/RecA family recombinases assemble through a well-defined pathway involving a rate limiting nucleation step, followed by more rapid spreading along the DNA (45-53). Within the context of this model, we can consider two possible mechanisms that might lead to segregated Rad51 and Dmc1 filaments (Figure 6A,B). First, random nucleation of separate Rad51 and Dmc1 filaments might allow for assembly of spatially segregated filaments, which eventually merge with one another as they spread along the ssDNA (Figure 6A). We favor this model, given that Rad51 and Dmc1 have similar assembly rates under our experimental conditions, which suggests that they also have similar nucleation rates. Second, it is also possible that Rad51 subunits might occasionally be incorporated into the growing ends of the Dmc1 filaments, or vice versa, such that continued filament growth favored incorporation of the heterotypic recombinase (Figure 6B). These two models are not necessarily mutually exclusive. Moreover, we note that accessory factors that affect Rad51 or Dmc1 filament assembly may affect how recombinase monomers incorporated into the mixed filaments.

Our data also provide evidence that the side-by-side filaments may be able to communicate with one another, perhaps through allosteric interactions. This conclusion is supported by our observation that the presence of Dmc1 stabilizes Rad51 against dissociation upon depletion of ATP. Rad51, and other Rad51/RecA family recombinases, dissociate from DNA through an end-dependent mechanism, which is also dependent upon ATP-hydrolysis (41,51,54-56). Therefore, we speculate that Dmc1 may cap one or both ends of the Rad51 filaments causing allosteric changes that reduce the rate of ATP-hydrolysis and correspondingly increase the stability of the Rad51 filaments.

An important question for future studies will be to establish the functional significance of the ability of Rad51 and Dmc1 to self-segregate. One possibility, is that the ability of Rad51 and Dmc1 to self-segregate may help ensure that the Dmc1 recombinase filaments responsible for strand invasion during meiosis are not randomly interspersed with inactivated Rad51 recombinases, which might otherwise hinder the homology search, strand invasion, or both processes. Alternatively, segregation of Rad51 and Dmc1 may be essential to control the organization of other regulatory factors during meiotic recombination.

Experimental Procedures
Proteins – *S. cerevisiae* RPA, Rad51, Dmc1, GFP-RPA, mCherry-RPA were all purified as previously described (25-27). GST-Hed1-6xHis-mCherry, GST-Hed1-6xHis-GFP were purified as follows. pGEX plasmids were transformed into *E. coli* Rosetta(DE3) cells (Novagen). Cell were grown to an OD of 0.6-0.8 at 37°C, cultures were then shifted to 16°C and induced overnight with 0.1 mM IPTG. After overnight expression cells were harvested and resuspended in 20 mL cell lysis buffer (50 mM Tris-C1 [pH 7.5], 700 mM KCl, 1 mM EDTA, 10 mM βME, Protease inhibitor cocktail (Roche, Cat. No. 05892988001), 10% Glycerol, and 1 mM PMSF) per 1 L of cell culture. Cells were lysed with lysozyme and sonication. The lysate was clarified by ultracentrifugation for 45 min at 35,000 rpm. Clarified extract was incubated in batch with Glutathione resin (GE Healthcare, Cat. No. 17-0756-01) for 1 hour at 4°C. The supernatant was then removed, and the resin washed with 2x 10 column volumes (CV) with Buffer K1000 (20 mM Tris-C1 [pH 7.5], 1 M KCl, 10 mM βME (β-mercaptoethanol), 1mM PMSF, 10% Glycerol, 2.5 mM Imidazole). Resin was then washed with 0.1 M 2-Mercaptoethanol (β-mercaptoethanol) and buffer K1000. Probes were then washed with 0.1 M 2-Mercaptoethanol (β-mercaptoethanol) and buffer K1000 and eluted with 10mM 2-Mercaptoethanol (β-mercaptoethanol) in buffer K1000.
Buffer K 300 (20 mM Tris-Cl [pH 7.5], 1 M KCl, 10 mM BME, 1 mM PMSF, 10% Glycerol, 2.5 mM Imidazole). Protein was eluted with Buffer K300 + 25 mM Glutathione. Peak fractions were bound to cOmplete Nickel Resin (Roche, Cat. No. 05893682001) for 1 hour at 4°C. Resin was then washed 2 x 5 CV of Buffer K1000, followed washing with 2 x 5 CV of Buffer K300. Protein was then eluted with Buffer K300 plus 100 mM Imidazole. Peak fractions were pooled and dialyzed against Buffer K 150 (20 mM Tris-Cl [pH 7.5], 150 mM KCl, 10 mM βME, 1 mM PMSF, 10% Glycerol). Proteins were quantified by absorbance at 280 nm, and in the case of GFP and mCherry protein concentrations were quantified by measuring the absorbance of the chromophores at 488 nm (ε488 nm = 55,000 cm⁻¹M⁻¹) or 587 nm (ε587 nm = 72,000 cm⁻¹M⁻¹), respectively. Samples were flash frozen and stored at -80°C.

**TIRFM experiments** – All experiments were conducted with a custom-built prism type total internal reflection fluorescence (TIRF) microscope (Nikon) equipped with 488 nm laser (Coherent Sapphire, 200 mW) and a 561-nm laser (Coherent Sapphire, 200 mW), as previously described (28,29). For all two-color images, we use a custom-built shuttering system to avoid the bleed through from the green into the red channel during image acquisition. With this system, images from the green (GFP) and the red (mCherry) channels are recorded independently, these recordings are offset by 100 milliseconds such that when one camera records the red channel image, the green laser is shuttered off, and vice versa (30-32).

Flow cells were constructed by deposition of chrome barriers on quartz microscope slides via electron beam lithography and thermal evaporation, as described (28,29). Bilayers were prepared with 91.5% DOPC (1,2-dioleoyl-sn-glycerol-3-phosphocholine), 0.5% biotinylated-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl)), and 8% mPEG 2000-DOPE (18:1 PEG2000: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]. Bilayers were deposited through sequential injections of a lipid master mix in lipid buffer (20 mM Tris-Cl pH 7.5, 100 mM NaCl), as described (28,29). The surface was then blocked with Dmc1 buffer (30 mM Tris-Cl [pH 7.5], 100 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 0.2 mg/mL BSA, 1 mM DTT) and conjugated to streptavidin to provide attachment points for tethering the biotinylated-ssDNA (28). The ssDNA substrate was prepared by rolling circle replication using phi29 DNA polymerase with a biotinylated primer annealed to M13 circular single stranded DNA as a template (33,34). The ssDNA was then deposited onto the bilayer, and the flow cell attached to the microfluidic system (28).

**Rad51 and Dmc1 filament assembly** – The ssDNA molecules were aligned along the diffusion barriers at a flow rate of 0.5 mL/min in Dmc1 buffer plus RPA (30 mM Tris-Cl [pH 7.5], 100 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 0.2 mg/mL BSA, 1 mM DTT, 0.1 nM RPA-GFP or RPA-mCherry). Once molecules were aligned, the flow rate was adjusted to 1.0 mL/min and 0.5 mL of 7 M Urea was injected into the flow cell to disrupt any remaining secondary structure. The sample chamber was then flushed with Dmc1 buffer plus RPA-GFP or RPA-mCherry (0.1 nM) at 1.0 mL/min for 10 min. After 10 min, Dmc1 buffer plus ATP (30 mM Tris-Cl [pH 7.5], 100 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 0.2 mg/mL BSA, 1 mM DTT, plus 2.5 mM ATP) at 1.0 mL/min for 3 min. Either Rad51, Dmc1, mixed ratios of the two were injected (2 μM) into the flow cell, buffer flow was terminated, and the reaction was incubated at 30°C for 15 minutes to allow filament assembly. The RPA fluorescence signal was then monitored to verify filament assembly. Following this 15-minute incubation, free recombinase was flushed from the sample chamber with Dmc1 buffer plus ATP.

**Data Acquisition and Analysis** – All data was collected with 100 millisecond integration time, and laser shuttering was varied to minimizing photo-bleaching depending on the need of the particular experiment. Images were collected using Nikon software, and images were exported as individual TIFF images. TIFF stacks were imported into ImageJ (Fiji)(35). For two-color imaging, the two channels were first corrected for stage drift and then merged into TIFF images, which were then converted to TIFF stacks. All TIFF stacks were then corrected for stage drift using the registration/translation function within Fiji. For each time course experiment, kymographs
were generated from the TIFF image stacks by defining a 1 pixel wide region of interest (ROI) along the axis of each individual ssDNA molecule, and these ROIs were extracted from each image within the TIFF stack. All of the slices corresponding to one ssDNA molecule were then aligned to yield a kymograph representing the entire experimental time course, and this process was repeated for each ssDNA molecule that was analyzed. For ssDNA intensity analysis, a 39-pixel ROI was taken from individual DNA molecules and the pixel intensity was summed over the length of the DNA to generate an intensity value. A separate 39-pixel ROI was also taken from a region with no DNA molecules, and the intensity measured. This was subtracted from all DNA samples as background, and the resulting background subtracted values were averaged.

Predicted Hed1-GFP and RPA-mCherry signal intensities – The predicted Hed1-GFP signal intensity value for the +ATP column for Figure 5E was obtained by comparison of the integrated Hed1-GFP signal intensity values for ssDNA molecules bound at a 4:1 ratio of Rad51 to Dmc1 to the Hed1-GFP standard curve in Figure 2B. This standard curve was obtained by determining the integrated signal intensity values for Hed1-GFP binding obtained at 0.1, 0.3, 1.0, 3.0, 10, or 30 nM Hed1 under different ratios of recombinases (Figure 2B). These data were fit by non-linear regression, and the B_{max} values calculated under each condition. These values were used to generate a standard curve that could be used to predict the amount of Hed1-GFP that should bind under a given recombinase ratio. In Figure 5, for ratio of 4:1 Rad51 to Dmc1, the predicted Hed1 signal intensity (~2.3 x 10^6 a.u.) in the presence of ATP is based on where this ratio falls in the standard curve in Figure 2B.

The predicted Hed1-GFP signal intensity for the –ATP column for Figure 5E was obtained by comparison to the disassembly experiments shown in Figure 4B. These disassembly experiments indicated that for a 3:1 or 1:1 ratio of Dmc1 to Rad51, only half of the bound Rad51 (25% of the total bound recombinase) would be susceptible to dissociation upon ATP depletion. Therefore, from this observation, we predicted that for a ratio of 4:1 Rad51 to Dmc1 roughly 25% of the bound Rad51 would also remain bound to the ssDNA upon ATP depletion. Comparison of this value to the Hed1-GFP standard curve (Figure 2B), yields a predicted value for the integrated Hed1-GFP signal after ATP depletion of ~1.2 x 10^6 (a.u.), which is the value reported in the –ATP column for Figure 5E.

Likewise, the predicted RPA-mCherry signal intensity for the Rad51:Dmc1 (4:1) column for Figure 5F was obtained by comparison to the disassembly experiments shown in Figure 4B. As indicated above, the disassembly experiments shown in Figure 4B revealed that for a 3:1 or 1:1 ratio of Dmc1 to Rad51, only half of the bound Rad51 (25% of the total bound recombinase) would be susceptible to dissociation upon ATP depletion. Therefore, we predicted that for an input ratio of 4:1 Rad51 to Dmc1, ~25% of the bound Rad51 would also remain bound to the ssDNA upon ATP depletion. Therefore, if 25% of the Rad51 dissociated upon depletion of ATP, then it would be replaced by a predictable amount of RPA-mCherry. We then measured the integrated signal intensity of RPA-mCherry binding to ssDNA after depletion of ATP for Rad51 only filaments (e.g. Figure 5B, left panel), which yielded a value of 0.65 x 10^6 a.u., which corresponds to the complete (100%) replacement of Rad51 with RPA-mCherry. Based on this value for the RPA-mCherry signal upon complete replacement of Rad51, we predicted that if only 25% of the Rad51 were replaced with RPA-mCherry, then we should obtain an integrated RPA-mCherry signal value of 0.14x 10^6 (a.u.), which we report as this predicted RPA-mCherry signal intensity in Figure 5F.

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Author Contributions: JBC designed, executed and analyzed all of the single molecule experiments. KK and YK provided purified proteins. ECG supervised the project. JBC and ECG wrote the manuscript with input from KK, YK, and PS.

Conflict of Interest: The Authors declare they have no conflict of interest with the contents of this article.

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FIGURE LEGENDS
Figure 1. Assembly of mixed recombinase filaments containing both Rad51 and Dmc1.
A, Schematic diagram proposing how Rad51 (blue) and Dmc1 (orange) can potentially form mixed meiotic filaments, illustrated as homotypic filaments, or randomly intermixed heterotypic filaments. B, Diagram of an ssDNA curtain experiment illustrating strategy for visualizing presynaptic complex assembly by monitoring the displacement of fluorescent (GFP- or mCherry-tagged) RPA. C, Kymograph showing the assembly of a mixed recombinase filament. In this example, equimolar amounts of Rad51 (1 μM) and Dmc1 (1 μM) were injected into a sample chamber containing ssDNA curtains bound by RPA-mCherry (magenta). D, Quantification of normalized average signal intensities for Rad51 only (N = 86), Dmc1 only (N = 123), and Rad51:Dmc1 (1:1) mixed filaments (N = 48). This analysis was performed for each observed ssDNA molecule, and the resulting data were fit with single exponential decays, and the reaction rate and plateau were determined from the fit. E, Distribution of assembly rates (min⁻¹) for determined for each ssDNA molecule from these experiments. F, Distribution of reaction plateaus based on the integrated RPA-mCherry signal intensity (a.u.) for each ssDNA molecule measured in these experiments.

Figure 2. Hed1-GFP binding to Rad51 with mixed recombinase filaments.
A, Wide-field TIRFM images of Rad51-ssDNA or Dmc1-ssDNA filaments bound by 0.1, 0.3, 1.0, 3.0, 10, or 30 nM Hed1-GFP (green), as indicated. B, Wide-field TIRFM images of 0.1, 0.3, 1.0, 3, 10, or 30 nM Hed1-GFP (green), as indicated, bound to mixed recombinase filaments comprise of either 3:1, 1:1, or 1:3 molar ratios of Rad51 to Dmc1. The graph (lower right panel) was generated by measuring the integrated Hed1-GFP signal intensity (a.u.) for at least 30 ssDNA molecules at each titration point, fitting those values by non-linear regression, and then plotting the maximum signal intensity for each ratio tested. The data was fit by an exponential function and the error bars represent a 95% confidence interval in the fit.

Figure 3. Distribution of Rad51 filaments within mixed recombinase filaments.
A, Magnified image showing three typical mixed recombinase filaments assembled at a 1:1 ratio of Rad51 to Dmc1 and bound by Hed1-GFP (10 nM, green). B, Examples of line graphs illustrating the signal intensity (a.u.) versus the position (μm) of the Hed1-GFP foci bound to single ssDNA molecules bound by Rad51 and Dmc1 (1:1), and shown in A; from these types of line graphs, we measured the distribution and peak-to-peak distance measurements for the Hed1-GFP foci. C, Binding distribution histogram for Hed1-GFP labeled Rad51 foci within mixed recombinase filaments (1:1, Rad51:Dmc1; N = 340). Error bars were generated by bootstrapping the data with a custom Python script, as described (28,41). D, Histogram illustrating the distribution of peak-to-peak distances between adjacent Hed1-GFP foci measured for mixed recombinase filaments (1:1, Rad51:Dmc1; N = 275). The resulting data were fit by a Gaussian distribution to determine the mean distance between adjacent Hed1-GFP foci.

Figure 4. Dmc1 renders Rad51 resistant to dissociation upon ATP depletion.
A, Example kymographs showing RPA-GFP (green) rebinding upon the depletion of ATP followed by the depletion of CaCl₂ for Rad51 only, Dmc1 only, and mixed recombinase filaments (3:1 Rad51:Dmc1). The arrowheads and dashed lines demarcate the time points of ATP and CaCl₂ depletion, respectively. B, Quantification of mean RPA-GFP normalized signal intensity (a.u.) integrated over the entire length of the ssDNA for Rad51 alone, Dmc1 alone, and mixed filaments at 9:1, 3:1, 1:1 ratios of Rad51 to Dmc1, as indicated. Error bars represent the standard deviation for at least 25 individual DNA molecules for each ratio tested. C, The RPA-GFP rebinding data in B was fit by single-phase association curves from the
point of ATP depletion to the point of CaCl$_2$ depletion, and a rate was determined from this fit; we refer to this data as $k_{off}$ (min$^{-1}$, ATP-dependent), because we take the binding of RPA-GFP as a proxy for the dissociation of Rad51 from the ssDNA, and because rate only reflects population of recombinase that dissociates when ATP is depleted. The error bars represent the 95% confidence interval for the fit of the data.

**Figure 5. Some Rad51 remains bound to the ssDNA after ATP depletion when Dmc1 is present.**

A, Schematic illustrating the experimental design used to determine if a population of Rad51 is resistant to ATP depletion remained bound to the ssDNA in presence of Dmc1. Recombinase filaments were first assembled in the presence of 2.5 mM ATP, free recombinase was flushed from the sample chamber in buffer containing 2.5 mM ATP and 0.1 nM RPA-mCherry (magenta). After 10 min, ATP was omitted from the sample buffer to allow for Rad51 disassembly. After an addition 15 min, Hed1-GFP (green; 10 nM) was injected into the sample chamber and TIRFM images were collected. B, Wide-field image showing the effect of ATP depletion on filaments comprised of only Rad51. In this case, Rad51 has dissociated from the ssDNA, as evidenced by the bright RPA-mCherry signal (red channel), and the absence of detectable Hed1-GFP (green channel). C, Wide-field TIRFM image showing the effect of ATP depletion on filaments comprised of a 4:1 mixture of Rad51 to Dmc1. In this case, some Rad51 has dissociated from the ssDNA, as evidenced by the presence of RPA-mCherry signal (red channel), but some Rad51 remains bound, as evidenced by the presence of Hed1-GFP (green channel). Note, that the contrast of the red channel was scaled to show RPA-mCherry binding, which was considerably lower than the experiments performed with Rad51 alone, as in B; the data shown in panel F below provide a direct measure of the amount of RPA-mCherry bound to the ssDNA under each of the different reaction conditions. D, Wide-field TIRFM image showing the effect of ATP depletion on filaments comprised of only Dmc1. Here, Dmc1 remains bound to the ssDNA based on the low RPA-mCherry signal and the absence of detectable Hed1-GFP. E, Graph representing the predicted Hed1-GFP binding intensity in the presence of ATP (the prediction is based on the titration data from Figure 2B), the measured integrated signal intensity for Hed1-GFP (N = 40) after ATP depletion, and the predicted Hed1-GFP signal after ATP depletion for mixed Rad51:Dmc1 (4:1) filaments (the prediction is based on the dissociation data from Figure 4C). The error bars represent the 95% confidence interval for the predicted value, and the standard deviation of individual ssDNA molecules for the observed value. F, Quantititation of the integrated RPA-mCherry signal intensity after ATP depletion for individual ssDNA molecules for experiments performed with Rad51 only (N = 13), mixed recombinase filaments made with 4:1 Rad51 to Dmc1 (N = 40), and Dmc1 only (N = 25). Error bars represent the standard deviation between individual DNA molecules. Also shown in the predicted RPA-mCherry signal for the mixed recombinase filaments based on the dissociation data from Figure 4C.

**Figure 6. Models for mixed recombinase filament assembly and disassembly.**

A, Random nucleation by either Rad51 or Dmc1, followed by homotypic elongation, allowing for eventual merger of the different filaments. B, Random nucleation by either Rad51 or Dmc1, followed by homotypic elongation, but with occasional heterologous incorporation events, which allow then for growth of the alternate filament. C, Models of the effects of ATP depletion for Rad51 only, Dmc1 only, and mixed recombinase filaments, illustrating filament “capping” as a possible mechanism of Dmc1-meditated stabilization of Rad51 to the effects of ATP-depletion within the context of mixed recombinase filaments. Additional details of these models are presented in the Discussion.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Spontaneous self-segregation of Rad51 and Dmc1 DNA recombinases within mixed recombinase filaments

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