Protein dynamics during presynaptic-complex assembly on individual single-stranded DNA molecules

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Homologous recombination is a conserved pathway for repairing double-stranded breaks, which are processed to yield single-stranded DNA overhangs that serve as platforms for presynaptic-complex assembly. Here we use single-molecule imaging to reveal the interplay between Saccharomyces cerevisiae RPA, Rad52 and Rad51 during presynaptic-complex assembly. We show that Rad52 binds RPA–ssDNA and suppresses RPA turnover, highlighting an unanticipated regulatory influence on protein dynamics. Rad51 binding extends the ssDNA, and Rad52–RPA clusters remain interspersed along the presynaptic complex. These clusters promote additional binding of RPA and Rad52. Our work illustrates the spatial and temporal progression of the association of RPA and Rad52 with the presynaptic complex and reveals a new RPA–Rad52–Rad51–ssDNA intermediate, with implications for how the activities of Rad52 and RPA are coordinated with Rad51 during the later stages of recombination.

DNA double-strand breaks (DSBs) are among the most toxic forms of DNA damage, and they can lead to genomic rearrangements and other severe chromosomal abnormalities. Homologous recombination (HR) is a conserved pathway that can be used to repair these lesions through an error-free mechanism that relies on the presence of an undamaged homologous chromosome, which serves as a template for repair of the broken DNA1–4. Defects in HR are widely associated with genetic abnormalities and cancer, thus highlighting the importance of this pathway for maintaining genome integrity. During HR, the newly exposed DNA ends are processed through Exo1– or Sgs1–dependent 5′→3′ resection pathways yielding long 3′ single-stranded DNA (ssDNA) overhangs5–9. These ssDNA overhangs are then paired with homologous sequence elsewhere in the genome, and any missing sequence information is restored, with the homologous DNA serving as a template for replication. The replicated intermediate is then resolved, thus regenerating the continuity of the broken chromosome. HR requires a complex repertoire of proteins, which are responsible for sensing damage, recruiting repair factors and processing and repairing the damaged DNA. Many of the eukaryotic proteins involved in HR were identified as S. cerevisiae mutants defective in the repair of DNA damage caused by ionizing radiation, and they are collectively referred to as the RAD52 epistasis group, which includes Rad50, Rfa1, Rad52, Rad54, Rdh54 (Tid1), Rad55–Rad57, Rad59, Mre11 and Xrs2. In addition to these original members, there are now known to be more than 30 different proteins or protein complexes involved in HR1–3.

Replication protein A (RPA) is an abundant protein that participates in all aspects of eukaryotic DNA metabolism involving ssDNA intermediates10,11. During the early stages of homologous recombination, the processed ssDNA overhangs are bound by RPA, which is a heterotrimeric complex composed of Rfa1, Rfa2 and Rfa3 (refs. 5,8,10,12–14). RPA protects the ssDNA from enzymatic degradation, removes secondary structure, serves as a checkpoint signaling intermediate15,16 and recruits specific HR proteins5,8,11,17–20. Rad51 is a DNA recombinase that assembles into an extended helical filament on the RPA-coated ssDNA21,22, and the resulting presynaptic complex is a critical HR intermediate in all eukaryotes2–3,23–26. This presynaptic filament is responsible for aligning the processed ssDNA overhang with a homologous double-stranded DNA (dsDNA) template, and it also performs strand invasion whereby the ssDNA is paired with the complementary DNA strand from the homologous duplex.

Many other accessory factors are also essential for the successful completion of HR, and these proteins promote numerous events during recombination. For example, RPA can outcompete Rad51 for ssDNA binding; therefore, presynaptic-complex assembly is stimulated by mediator proteins that assist Rad51 loading2,23. Rad52 is a key mediator in S. cerevisiae27–30, and Rad52 is also required for the second-strand capture and strand-annealing reactions that take place during the later stages of recombination31–34. Rad52 colocalizes with RPA and Rad51 at induced DSBs in vivo17,35, and during S phase Rad52 also forms spontaneous foci reflecting HR-dependent repair of stalled replication forks25,36. The importance of Rad52 is revealed by the extreme susceptibility of yeast rad52 mutants to DNA damage34. Although unrelated in sequence, Rad52 is functionally similar to human Brca2 (refs. 37,38), and it has recently been shown that human cells deficient for both Rad52 and Brca2 exhibit extensive chromosome abnormalities37.
Despite the growing knowledge of the proteins involved in HR and their contributions to the final outcome of the repair processes, there remains relatively little information regarding how the macromolecular complexes involved in HR are assembled and disassembled and how the individual protein components influence one another during the course of a reaction. Here we use two-color single-molecule imaging and ssDNA curtains to reveal the interplay between RPA, Rad52 and Rad51 during presynaptic-complex assembly. We show that individual Rad52 complexes bind tightly to an RPA-coated ssDNA, which mimics the physiologically relevant substrate present at the free ends of processed DSBs. The initial Rad52 complexes serve as nucleation sites allowing for association of additional Rad52 molecules, which spread along the RPA–ssDNA. RPA binds very tightly to ssDNA when free RPA is absent from the surrounding solution, but the bound proteins remain poised for very rapid concentration-dependent turnover through a mechanism involving a microscopically dissociated intermediate, thus enabling free RPA present in solution to compete for transiently exposed patches of ssDNA. Remarkably, Rad52 suppresses RPA turnover through a mechanism requiring direct protein-protein contacts between RPA and Rad52. This finding highlights an unanticipated regulatory influence of Rad52 on RPA dynamics during the early stages of HR. Rad51 binding causes rapid extension of the ssDNA, and small Rad52–RPA clusters remain interspersed between extended tracts of Rad51. These clusters serve as nucleation sites for the binding of additional molecules of RPA and Rad52. Together, our work illustrates the spatial and temporal progression of the association of RPA and Rad52 with the presynaptic complex throughout the early stages of HR and reveals the existence and assembly pathway of a new RPA–Rad52–Rad51–ssDNA presynaptic intermediate.

RESULTS

Visualizing Rad52-RPA-ssDNA interactions

We used ssDNA curtains to mimic the temporal progression of events thought to take place during the early stages of recombination (Fig. 1a). We injected S. cerevisiae RPA-mCherry (80 pM) into a microfluidic sample chamber containing ssDNA curtains and used ssDNA substrates generated by rolling-circle replication, using a biotinylated oligonucleotide and circular M13 ssDNA as a template. Then we anchored the resulting ssDNA to a lipid bilayer on the surface of a microfluidic sample chamber through a biotin–streptavidin linkage and aligned anchored ssDNA molecules along the leading edge of zigzag-shaped nanofabricated chromium (Cr) barriers through application of hydrodynamic force. The ssDNA remains compacted in the absence of RPA but quickly unravels when incubated with RPA. Once extended, the downstream ends of the RPA–ssDNA complexes become anchored to exposed Cr pedestals. We could then visualize the resulting double-tethered RPA–ssDNA complexes with total internal reflection fluorescence microscopy (TIRFM) even in the absence of buffer flow (Fig. 1b).

RPA–ssDNA represents the first HR intermediate expected to form after resection of a DSB. We mimicked the next stage of the presynaptic complex by injecting SNAP-tagged S. cerevisiae Rad52 labeled with either Alexa 488 or Alexa 546 (SNAP₄₈₈-Rad52 and SNAP₅₄₆-Rad52; Supplementary Figs. 1 and 2) and then asked whether these fluorescent versions of Rad52 could bind to the RPA–ssDNA. Fluorescent Rad52 colocalized with both RPA-mCherry (Fig. 1b) and unlabeled wild-type RPA (Fig. 1c). At low protein concentrations (50 pM), individual fluorescent Rad52 complexes were readily resolved (Fig. 1c), whereas Rad52 coated the RPA–ssDNA at higher protein concentrations (1 nM) (Fig. 1c). Although Rad52 could bind to naked ssDNA in our assays, these ssDNA molecules remained highly compacted (data not shown); we did not pursue characterization of the Rad52–ssDNA complexes in the absence of RPA because the RPA–ssDNA complex is the physiologically relevant substrate.

Mechanism of Rad52 assembly on RPA–ssDNA

As viewed in real time, Rad52 bound to numerous different locations along the length of the RPA–ssDNA, the bound molecules of Rad52 remained at fixed positions, and Rad52 could accumulate along the entire length of the RPA–ssDNA (Fig. 2a–b). Control reactions confirmed that there was no Rad52 binding to regions of the sample-chamber surface lacking RPA–ssDNA (Fig. 2c). There was no evidence that RPA was displaced from ssDNA upon association of Rad52 (Fig. 2d and Supplementary Fig. 3), results in agreement with previous studies. Rad52 forms a heptameric ring in solution, and the individual Rad52 complexes displayed uniform signal intensities (59.2 ± 10.3 arbitrary units (a.u.); n = 591; Fig. 2e), consistently with expectations for association events involving discrete, well-defined complexes. Moreover, quantitation of the Rad52

Figure 1 Single-stranded DNA curtain assay for presynaptic-complex assembly. (a) Schematic of ssDNA curtains. (b) Wide-field images of RPA-mCherry (magenta) bound to ssDNA in the absence (top) and presence of 50 pM SNAP₄₈₈-Rad52 (green; bottom). (c) Wide-field images of 50 pM SNAP₄₈₈-Rad52 (top) or 1 nM SNAP₄₈₈-Rad52 (bottom) bound to wild-type (unlabeled) RPA on ssDNA. The 5′→3′ orientation of the ssDNA is indicated in this and all subsequent figures.
binding distributions revealed no discernible pattern of preferential binding sites (Fig. 2f).

We next asked whether we could detect evidence of Rad52-Rad52 interactions on the RPA–ssDNA. For this, we conducted two-color pulse-chase experiments to determine whether differentially labeled Rad52 complexes would preferentially associate with one another on the DNA (Fig. 3a). We first injected SNAP<sub>488</sub>-Rad52 (50 pM) and allowed it to bind to the RPA–ssDNA and then chased immediately with an injection of SNAP<sub>546</sub>-Rad52 (50 pM; Fig. 3b). These experiments revealed a modest preference for incoming molecules of Rad52 to bind to the RPA–ssDNA at sites already occupied by preexisting Rad52 (r = 0.43, P = 6 × 10<sup>−13</sup>, n = 262 pixels ssDNA, Pearson correlation analysis; Fig. 3c), with 64% of binding events (n = 69 Rad52 binding events) occurring at preexisting Rad52 complexes (Fig. 3d and Supplementary Fig. 4). The remaining events represented new binding to regions of the RPA–ssDNA lacking detectable SNAP<sub>488</sub>-Rad52; for brevity, we refer to these as new binding events (Fig. 3d). The existence of these new binding events is not surprising considering the overall length of the RPA–ssDNA (~13 µm, ~36,000 nt), so even after an initial Rad52 nucleation event the amount of RPA–ssDNA still vastly exceeds the small number of bound Rad52 complexes. Together, these findings suggested that at low protein concentrations Rad52 could bind at random locations along the RPA–ssDNA, and it also had a modest preference for self-association after binding to the RPA–ssDNA.

If Rad52 binding occurred through a cooperative mechanism, then at higher protein concentrations quantitation of the total Rad52 signal on an individual ssDNA molecule should reveal an initial slow phase, corresponding to slow nucleation events, followed by a more rapid growth phase (Fig. 3e). Moreover, for a cooperative binding mechanism the individual nucleation events might be expected to lead to lateral growth of the Rad52 complexes along the RPA–ssDNA. In contrast, if Rad52 bound through a noncooperative mechanism, then there should be a uniform increase in the Rad52 intensity all along the RPA–ssDNA. At higher concentrations of Rad52 (625 nM) binding appeared to exhibit an initial nucleation phase followed by a more rapid growth phase (Fig. 3f), thus suggesting that assembly did involve cooperative association of Rad52. Owing to their low signal intensity relative to saturation, we did not detect the initial Rad52 binding events when the total Rad52 signal was integrated across the length of the ssDNA (Fig. 3f). However, the precise timing of initial binding events (i.e., nucleation) became readily apparent upon inspection of the corresponding kymographs (Fig. 3g), which revealed that the

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**Figure 2** Individual Rad52 complexes binding to RPA–ssDNA. (a) Kymographs showing the association of single SNAP<sub>488</sub>-Rad52 (50 pM) complexes with RPA-mCherry–ssDNA or (b) Wild-type (dark) RPA–ssDNA. (c) Kymograph confirming that SNAP<sub>488</sub>-Rad52 does not associate with a control surface lacking an ssDNA molecule. (d) Example of Rad52 bound to an RPA–ssDNA complex, highlighting that RPA is not selectively lost from sites bound by Rad52. (e) Histogram showing the uniform intensity distribution for individual SNAP-tagged Rad52 complexes bound to RPA–ssDNA (n = 591). (f) Distribution of Rad52 nucleation sites along the length of the RPA–ssDNA (n = 498). Error bars, s.d. from n bootstrap samples<sup>15</sup>.
initial binding events precede the more rapid growth phase of the reaction. In addition, these kymographs also revealed that the Rad52 individual nucleation events were followed by rapid (124 ± 41 nm s⁻¹, n = 8 Rad52 nucleation sites) growth in both the 5′→3′ and 3′→5′ directions as revealed by wedge-shaped patterns emanating outward from the initial binding sites (Fig. 3g). We conclude that Rad52 could bind RPA–ssDNA through a mechanism involving an initial nucleation event followed by more rapid bidirectional growth along the RPA–ssDNA.

Rad52 binds tightly to RPA–ssDNA
We have previously shown that RPA exhibits a lifetime >>2 h on ssDNA curtains, but this long lifetime is observed only when there is no free RPA present in solution (i.e., at the infinite dilution limit; described below)39. We next examined the stability of Rad52 bound to the RPA–ssDNA. For these experiments, we incubated fluorescent Rad52 with RPA–ssDNA, then flushed away the unbound proteins and monitored the resulting complexes to determine whether Rad52 remained bound or dissociated into free solution (Fig. 4a). We monitored the Rad52–RPA–ssDNA complexes over periods of either 10 min or 2 h (Fig. 4b); importantly, we shuttered the laser between each acquired image and adjusted the image acquisition rates such that the total time that each sample was exposed to laser illumination was identical for the 10-min and 2-h experiments. For experiments spanning either 10 min or 2 h, the loss of Rad52 fluorescence occurred by a gradual decrease in fluorescence signal, indicative of photobleaching of a multimeric Rad52 complex, rather than by an abrupt signal loss, which would be expected for protein dissociation. These findings indicated that Rad52 binds tightly to the RPA–ssDNA and remained stably associated with the RPA–ssDNA for ≥2 h. Experiments beyond 2 h are intractable due to stage drift and spontaneous breakage of the tethered ssDNA.

Rad52 regulates RPA turnover
RPA has an exceptionally slow off rate at the infinite dilution limit, yet it remains poised for rapid macroscopic dissociation though an unusual, newly recognized mechanism for the turnover of DNA-binding proteins, involving concentration-driven dissociation of a macroscopically dissociated intermediate38,49,50. In brief, ssDNA-bound RPA undergoes constant microscopic dissociation under all conditions but does not equilibrate with free solution39. These microscopic dissociation events are manifested as macroscopically detectable dissociation into free solution only when other ssDNA-binding proteins are present to compete with the transiently unbound species for exposed patches of ssDNA39,49,50.

The ability of RPA to exchange between free and bound states when free RPA is present in solution raises the question of what happens to Rad52 during RPA turnover. There are at least three possibilities: (i) RPA turnover might cause Rad52 dissociation; (ii) Rad52 could remain bound without affecting RPA turnover; or (iii) Rad52 might alter RPA turnover kinetics (Fig. 4c). To distinguish between these possibilities, we chased SNAP₄₈₈-Rad52–RPA-mCherry–ssDNA
Influence of Rad51 assembly on Rad52–RPA complexes

Assembly of the Rad51 filament coincides with the displacement of RPA from the ssDNA. The ability of Rad52 to act as a mediator of Rad51 assembly is well established. However, the fate of Rad52 during and after the assembly of Rad51–ssDNA filaments remains unclear. Rad51 physically displaces RPA from ssDNA raises questions regarding the fate of Rad52 bound to the RPA–ssDNA. This question is critical because, in addition to its role as a mediator, Rad52 is also required for second-strand capture and strand annealing, which take place during the later stages of recombination. The participation of Rad52 in these later stages of HR raises the possibility that it might remain bound to the presynaptic complex. Alternatively, Rad51 could provoke the dissociation of Rad52 from the RPA–ssDNA, in which case Rad52 would need to be recycled and to reassociate with the HR machinery at a later step in the pathway. Therefore we next asked what happens to Rad52 when Rad51 binds the ssDNA.

To determine the fate of Rad52 during presynaptic-complex assembly, we monitored the binding of unlabeled wild-type Rad51 to single-tethered ssDNA curtains prebound by RPA–mCherry and SNAP_{488}–Rad52 (ref. 39); single-tethered curtains allowed visual assessment of ssDNA extension by Rad51. As expected, Rad51 binding coincided with a rapid increase in the contour length of the ssDNA (~138%; Fig. 5b), which arises from physical extension of the ssDNA and the greater stiffness of the Rad51–ssDNA filament relative to RPA–ssDNA. Remarkably, Rad52 remained bound to the ssDNA during presynaptic-complex assembly, yielding the appearance of small protein clusters separated by long tracts of dark Rad51–ssDNA (Fig. 5b). The remaining Rad52 clusters always colocalized with small patches of fluorescent RPA (r = 0.79, P = 0, n = 187; Fig. 5b–d), thus revealing the continued presence of both proteins within the presynaptic complex. The distances between Rad52–RPA clusters followed a Poisson distribution (1.6 ± 0.79 μm, n = 346) reflecting the stochastic nature of the underlying molecular event(s) dictating the lengths of the Rad51 filaments or the dispersion of the Rad52–RPA clusters within the filaments (Fig. 5e). Assuming that the Rad51–ssDNA molecules are extended to near their full contour lengths, and that they are stretched by ~50% relative to an equivalent length of B-form dsDNA, then a 1.6-μm Rad51–ssDNA filament would correspond to ~1,000 molecules of Rad51. We conclude that small clusters of Rad52–RPA remained embedded between long Rad51–ssDNA filaments after assembly of the presynaptic complex.
likely source of these new proteins was Rad52 complexes that had dissociated from ssDNA upstream of the viewing area. These observations suggested the possibility that the Rad52–RPA clusters embedded within the Rad51 presynaptic filaments might serve as nucleation sites allowing the binding of additional Rad52. Therefore, we next asked whether newly added Rad52 could bind preassembled presynaptic complexes. To address this issue, we assembled presynaptic filaments composed of Rad51–RPA–SNAP488-Rad52 and then chased these complexes with an injection of SNAP546-Rad52 (Fig. 5f). These experiments confirmed that additional Rad52 could indeed associate with the presynaptic complex, revealing this pathway as a secondary entryway for recruitment of Rad52 to the presynaptic filament (Fig. 5g,h). Moreover, the spatial distribution of the newly bound Rad52 coincided primarily with the positions of the prebound Rad52–RPA clusters, thus indicating that these locations served as sites for more extensive association of Rad52 (Fig. 5h). There was more limited accumulation of new Rad52 at locations lacking detectable Rad52–RPA clusters, and this suggested the possibility that Rad52 might be able to bind directly to Rad51–ssDNA, albeit to a lesser extent than the binding observed at the site of preexisting Rad52–RPA. This differential binding pattern suggested the existence of two kinetically distinct pathways, as might be expected for association mechanisms involving distinct sets of protein-protein contacts (for example, lower-affinity Rad52–Rad51 interactions versus higher-affinity Rad52–Rad52 interactions).

We have previously shown that the Rad51 filaments are highly stable when ATP is present in the surrounding buffer and that RPA does not bind to these Rad51–ssDNA filaments. If ATP is removed from the buffer, then Rad51 is quickly replaced with RPA, which coincides with a decrease in the ssDNA extension. Together, these findings show that RPA alone does not bind extensively to the preassembled Rad51 filaments. We next asked whether co-injection of Rad52 and RPA could lead to more extensive binding to the preassembled Rad51–Rad52–RPA–ssDNA complexes and, if so, whether the newly bound molecules exhibited a distinct spatial association pattern (Fig. 6a). Remarkably, co-injection of both Rad52 and RPA led to extensive association of both proteins with the presynaptic complex (Fig. 6b); bead pulldown assays provided additional evidence supporting the ability of Rad51, Rad52 and RPA to co-occupy ssDNA (Supplementary Fig. 6). Moreover, inspection of the spatial and temporal distribution of the binding events revealed that Rad52 binding initiated at the preexisting clusters of Rad52–RPA and then spread outward along the length of the presynaptic complex (Fig. 6b–d). Interestingly, the newly added RPA bound to the preexisting Rad52–RPA cluster and spread outward along the presynaptic complex, although association and spreading of RPA was slightly delayed relative to that of Rad52 (Fig. 6c,d). These observations suggested a temporal order of association beginning with the binding of Rad52 at preexisting Rad52–RPA clusters and followed

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**Figure 6** Assembly of Rad51–Rad52–RPA–ssDNA presynaptic intermediates. (a) Experimental schematic for detecting Rad52 and RPA binding to a preassembled Rad51–Rad52–RPA–ssDNA complex. (b) Kymographs showing wild-type Rad51 filament assembly on Rad52–RPA–ssDNA, with co-injection of additional Rad52 and RPA (as indicated). Top shows a two-color overlay, and middle and bottom show the individual red and green channels, as indicated. (c,d) Spatially distinct binding kinetics of newly added RPA (c) and Rad52 (d) along the lengths of the preassembled Rad51–Rad52–RPA–ssDNA presynaptic filament. Green arrowheads indicate the locations of the preexisting Rad52–RPA clusters within the Rad51 filaments.

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**Figure 7** Model for RPA and Rad52 dynamics during presynaptic-complex assembly. (a) Assembly pathway for the Rad51–Rad52–RPA–ssDNA presynaptic complex. (b) Influence of Rad52–RPA on strand invasion and second-strand capture during the later stages of homologous recombination. Details of the models are presented in Discussion.
by the more gradual association of RPA. Importantly, the observed contour length of the presynaptic complexes did not change significantly (<4%), even upon extensive association of the newly added Rad52 and RPA (Fig. 6b–d). When taken together, our results suggest that Rad51 remained bound directly to the ssDNA as an extended helical filament within the context of these higher-order macromolecular complexes.

**DISCUSSION**

Here we have used ssDNA curtains to visualize the spatial and temporal progression of the molecular events accompanying the assembly of *S. cerevisiae* presynaptic complexes. Together, our experiments reveal the existence and assembly pathway of a new Rad51–Rad52–RPA–ssDNA presynaptic intermediate (Fig. 7a). We demonstrate that Rad52 binds directly to the RPA–ssDNA as discrete units, results consistent with expectations for a well-defined oligomer, and at higher protein concentrations Rad52 spreads along the RPA–ssDNA. We have previously shown that in the absence of free RPA, ssDNA-bound RPA undergoes continuous microscopic dissociation without equilibrating into solution35. Microscopic dissociation from the ssDNA is manifested as macroscopic dissociation into bulk solution only when free RPA is available to compete for partially exposed patches of ssDNA; importantly, this mechanism allows for extremely high binding affinities as well as rapid exchange kinetics39,49,50. Rad52 remarkably suppresses the concentration-driven turnover of RPA. The ability of Rad52 to restrict RPA exchange implies that Rad52 restricts microscopic dissociation of RPA from the ssDNA, and this in turn prevents macroscopic dissociation into free solution. When considering that Rad52 acts as a mediator of Rad51 assembly, it might intuitively be expected that Rad52 should destabilize the RPA–ssDNA complex, as opposed to having the stabilizing effect revealed in our assays. However, an important consideration is that the concentration-driven exchange of RPA between free and bound states is not specific to RPA, as previously revealed by the finding that *Escherichia coli* SSB can readily drive the dissociation of RPA from ssDNA and vice versa39. We propose that the suppression of RPA turnover by Rad52 may be necessary to prevent the inappropriate access of non-HR proteins to ssDNA recombination intermediates while at the same time directing Rad51 to the RPA-coated ssDNA.

Rad51 assemblies into extended filaments on the RPA–Rad52–ssDNA, and these long Rad51 filaments are punctuated by small clusters of Rad52–RPA, which are retained as a stable component embedded within the Rad51 presynaptic filaments. These clusters serve as nucleation sites enabling more extensive binding of Rad52 and RPA (Fig. 7a). Importantly, the Rad51–ssDNA remains in a highly extended configuration, even after extensive association of late-arriving Rad52 and RPA. This finding is consistent with only a model in which Rad51 remains bound to the ssDNA, thus suggesting that the newly bound RPA and Rad52 are associated with the surface of the Rad51–ssDNA filament through protein–protein contacts; additional studies will be essential for establishing the molecular details of the protein–protein and protein–ssDNA contacts responsible for assembly of these macromolecular complexes. The resulting Rad52–RPA–Rad51–ssDNA presynaptic intermediate has not previously been identified, but its existence has important mechanistic implications for how the activities of RPA and Rad52 are coordinated with those of Rad51 during the downstream steps in homologous recombination. Namely, this new intermediate places Rad52 and RPA in the right location to immediately stabilize the noncomplementary ssDNA strand as it is displaced by Rad51 during strand invasion (Fig. 7b)31–34. Moreover, the continued presence of RPA and Rad52 within the presynaptic complex may also help coordinate strand invasion with the capture and annealing of the second processed DNA end31–34. Finally, the finding that Rad52 and RPA can both stably coexist within the Rad51 presynaptic complex provides a possible explanation for *in vivo* observations showing that RPA and Rad52 arrive at DSBs before Rad51 and remain at these sites during and after the arrival of Rad51 (refs. 35,52–54). Future studies using ssDNA curtains may help reveal how other HR proteins interact with and influence the properties of presynaptic complexes and may also shed new light on later stages of homologous DNA recombination.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

B.G. and L.F.Y. cloned, expressed and purified RPA and Rad52, conducted the single-molecule experiments and analyzed the resulting data. Y.K. and H.N. expressed and purified Rad51, and Y.K. conducted bulk biochemical experiments. E.C.G. supervised the project. B.G. and E.C.G. wrote the manuscript with input from all coauthors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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1. Mazon, G., Mimitou, E.P. & Symington, L.S. SnapShot: homologous recombination in DNA double-strand break repair. Cell 142, 645–646.e1 (2010).
2. San Filippo, J., Sung, P. & Klein, H. Mechanism of eukaryotic homologous recombination. Annu. Rev. Biochem. 77, 229–257 (2008).
3. Krogh, B.O. & Symington, L.S. Recombination proteins in yeast. Annu. Rev. Genet. 48, 233–271 (2004).
4. Cromie, G.A., Connelly, J.C. & Leach, D.R. Recombination at double-strand breaks and DNA ends: conserved mechanisms from phage to humans. Mol. Cell 8, 1163–1174 (2001).
5. Cejka, P. et al. DNA end resection by Dna2–Sgs1–RPA and its stimulation by Top3–Rmi1 and Mre11–Rad50–Xrs2. Nature 467, 112–116 (2010).
6. Chen, X. et al. The Fun30 nucleosome remodeler promotes resection of DNA double-strand break ends. Nature 489, 576–580 (2012).
7. Mimitou, E.P. & Symington, L.S. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature 455, 770–774 (2008).
8. Niu, H. et al. Mechanism of the ATP-dependent DNA end-resection machinery from *Saccharomyces cerevisiae*. Nature 467, 108–111 (2010).
9. Zhu, Z., Chung, W.H., Shim, E.Y., Lee, S.E. & Ira, G. Sgs1 helicase and two nucleases Dna2 and Exo1 rescue DNA double-strand break ends. Cell 134, 981–994 (2008).
10. Wold, M.S. Replication protein A: a heterotrimetric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu. Rev. Biochem. 66, 61–92 (1997).
11. Broderick, S., Rehmet, K., Concannon, C. & Nashuever, H.P. Eukaryotic single-stranded DNA binding proteins: central factors in genome stability. Subcell. Biochem. 50, 143–163 (2010).
12. Mimitou, E.P. & Symington, L.S. DNA end resection: unraveling the tail. DNA Repair (Amst.) 10, 344–348 (2011).
13. Listby, M. & Rothstein, R. Choreography of recombination proteins during the DNA damage response. DNA Repair (Amst.) 8, 1068–1076 (2009).
14. Symington, L.S. & Gautier, J. Double-strand break end resection and repair pathway choice. Annu. Rev. Genet. 45, 247–271 (2011).
ONLINE METHODS

Proteins and DNA. RPA and Rad51 were purified as described35. RPA-mCherry was cloned, expressed in E. coli, and purified over Ni resin (Qiagen) and a Mono Q column (GE Healthcare). Concentrations of RPA-mCherry were determined on the basis of absorbance at 587 nm (ε_{587 nm} = 72,000 cm^{-1} M^{-1}).

S. cerevisiae Rad52 has multiple potential start codons, but the protein expressed in yeast corresponds to codons 34–504, yielding a 472-amino acid protein56. Therefore, Rad52 codons 34–504 were cloned with an N-terminal His6 and SNAP tag, and a C-terminal intein/chitin-binding domain; for brevity we refer to this construct as SNAP-Rad52. As an alternative, Rad52 codons 1–50 were cloned with an N-terminal His6 and SNAP tag, and a C-terminal intein/chitin-binding domain. Use of this longer reading frame provides an additional 34 amino acids placed between the SNAP tag and the N terminus of Rad52; we refer to this construct as SNAP-34-Rad52. Bacteria (E. coli Rosetta) were grown at 37 °C, induced overnight in 18 °C with 0.5 mM IPTG, and lysed by sonication. The clarified lysate was purified over a chitin column (NEB), washed with buffer A (50 mM Tris-HCl, pH 7.5, and 600 mM NaCl) plus 1 mM EDTA, and eluted overnight in buffer A plus 1 mM EDTA and 30 mM DTT. The eluate was dialyzed into buffer A plus 0.1 mM DTT and 10 mM imidazole, loaded onto a Ni-NTA column, washed sequentially with buffer A containing 60 mM and 125 mM imidazole, and eluted in buffer A plus 250 mM imidazole. Proteins were concentrated with a Vivaspin concentrator (50-kDa MWCO; GE Healthcare), exchanged into a storage buffer (40% glycerol, 40 mM Tris-HCl, pH 7.4, 600 mM NaCl, and 1 mM DTT), and stored at −80 °C.

The strand-annealing and mediator activities of the fluorescently tagged versions of Rad52 were determined by comparison of the percentage of product formed to reactions with unlabeled Rad52 at the same protein concentrations (Supplementary Figs. 1 and 2). Biochemical assays for strand-annealing activity revealed that TAMRA-Rad52, SNAP-Rad52, and SNAP-34-Rad52 displayed an average of ~69%, ~70%, and ~72% of the strand-annealing activity of unlabeled Rad52, respectively (Supplementary Fig. 1). The biochemical assays for mediator activity revealed that TAMRA-Rad52, SNAP-Rad52, and SNAP-34-Rad52 displayed an average of ~93%, ~41%, and ~67% mediator activity relative to reactions with unlabeled Rad52, respectively (Supplementary Fig. 2). SNAP-Rad52 was used for detailed quantitation and figure preparation; however, all three different fluorescently tagged versions of Rad52 gave qualitatively similar results in the single-molecule assays (data not shown).

Bead pulldown assays. Pulldown assays were used to verify co-occupancy of Rad52 and RPA on ssDNA (Supplementary Fig. 3). Each reaction sample contained 20 µl streptavidin magnetic beads (Life Technologies, Dynabeads M-280 Streptavidin), which were bound to an excess of ssDNA oligonucleotide (5′-biotin-TIRFM experiments and data analysis. Single-molecule experiments were conducted with a custom-built total internal reflection fluorescent microscope and

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ssDNA curtains, as described previously. Single-stranded substrates were generated by rolling-circle replication of circular M13mp18 ssDNA (7,249 nt; New England Biolabs). All single-molecule experiments began with the addition of either 100 pM WT RPA or 100 pM RPA–mCherry, as indicated. The ssDNA substrates remain highly compacted in the absence of RPA–mCherry and cannot be visualized by TIRF microscopy. Experiments with just RPA and Rad52 were conducted with double-tethered DNA curtains at flow rates of 0.1–0.2 ml min⁻¹; experiments involving Rad51 used single-tethered DNA curtains and continuous buffer flow (0.1 ml min⁻¹). Reactions were conducted at 30 °C in buffer containing 30 mM Tris-acetate, pH 7.5, 5 mM Mg-acetate, 50 mM KCl, 1 mM DTT, and 200 µg ml⁻¹ BSA. The reaction buffer was supplemented with 2.5 mM ATP for all experiments involving Rad51. Three fluorescently tagged versions of Rad52 were tested to ensure that experimental outcomes were not influenced by the labeling strategy: SNAP-Rad52, in which the SNAP domain was fused to the N terminus of Rad52; SNAP-34-Rad52, which bears an additional 34–amino acid linker between the SNAP domain and Rad52; and TAMRA-Rad52, in which Rad52 was labeled at the N terminus with 5(6) carboxytetramethylrhodamine (Supplementary Fig. 1). Use of the SNAP-tagged Rad52 constructs permits ready use of different color fluorophores; therefore, SNAP-Rad52 or SNAP-34-Rad52 was used for detailed quantitation and figure preparation. However, all three different versions of Rad52 gave qualitatively similar results in the single-molecule assays.

Image processing, fluorescence intensity and signal distribution measurements were made with NIH Image J 1.48c. Images (100-ms exposures) were collected from 10-min time-lapse experiments at 1-s intervals, and the lasers where shuttered in between frame acquisition to minimize photobleaching. For longer time-lapse experiments, images (100-ms exposures) were collected at 24-s intervals for 2 h, and the resulting data were corrected for stage drift. All data analysis was restricted to individual molecules of ssDNA identified by visual inspection of the RPA–mCherry or RPA–EGFP signal; overlapping ssDNA molecules were excluded from any further analysis or quantitation, and all conclusions were based on data collected from at least three separate experiments.

DNA curtains. Chromium (Cr) barriers were fabricated by e-beam lithography, as described previously. Bilayers were prepared with 91.5% DOPC, 0.5% biotinylated-DPPE, and 8% mPEG-550-DOPES. DNA substrates were generated with M13mp18 (7,249 bp; Invitrogen) as a template for rolling-circle replication. A biotinylated primer was annealed to the template, and reactions were initiated with addition of 629 DNA polymerase (100 nM) in buffer containing 50 mM Tris, pH 7.4, 2 mM DTT, 10 mM MgCl₂, 10 mM ammonium sulfate, and 200 µM dNTPs. The ssDNA molecules were aligned at the barriers with buffer flow, as described previously.

TIRF microscopy. Experiments were performed with a prism-type TIRF microscope (Nikon) with two back-illuminated iXon EMCCDs (Andor Technology). Illumination was provided by a 200-mW, 488-nm laser and a 150-mW, 561-nm laser (Coherent). Intensity at the prism face was ~14 mW and ~25 mW for the two spectrally separated fluorophores, respectively. Fluorescence signals were separated by a filter cube equipped with a dichroic mirror (ZT561rdc), band-pass filter (ET525/50m), and long-pass filter (ET757lp) (Chroma Technology).

Rad52 binding to RPA–ssDNA. All RPA and Rad52 single-molecule experiments were conducted at 30 °C in buffer containing 30 mM Tris-acetate, pH 7.5, 5 mM Mg-acetate, 50 mM KCl, 1 mM DTT, and 200 µg ml⁻¹ BSA. Rad52 binding measurements were made with preassembled RPA (wild type or mCherry tagged, as indicated)–ssDNA filaments in a double-tethered DNA-curtain format; unless otherwise indicated, free RPA was flushed from the sample chamber before the injection of Rad52. Single Rad52 particle fluorescence intensity measurements were made from wide-field images collected from reactions performed in the presence of 100 pM WT RPA and 50 pM SNAP⁴⁸⁸-Rad52 after free proteins had been flushed from the sample chamber. Rad52 nucleation–site distributions were based upon data collected from ssDNA bound by RPA–mCherry after the injection of 50 pM SNAP⁴⁸⁸-Rad52. Two-color cooperative Rad52 binding measurements were conducted with WT RPA–ssDNA complexes and an initial injection of 50 pM SNAP⁴⁸⁸-Rad52. Unbound protein was flushed from the sample chamber, and this was followed by a second injection of 50 pM SNAP⁴⁸⁸-Rad52. Colocalization of the two differentially labeled proteins was determined by Pearson correlation analysis of the overlap between preexisting SNAP⁴⁸⁸-Rad52 clusters with the binding distributions of the newly injected SNAP⁴⁸⁸-Rad52. New SNAP⁴⁸⁸-Rad52 binding events that occurred at sites occupied by preexisting molecules of SNAP⁴⁸⁸-Rad52 were manually scored by inspection of the kymographs, and new SNAP⁴⁸⁸-Rad52 binding events were considered overlapping if they were occurred within ± 1 pixel of the center position of a preexisting peak of SNAP⁴⁸⁸-Rad52.

Rad52 nucleation and growth measurements were made by injection of 625 nM SNAP⁴⁸⁸-Rad52 into sample chambers containing preassembled WT RPA–ssDNA complexes. Nucleation sites and bidirectional growth were identified by visual inspection of the resulting data, and growth rates were estimated from the linear expansion of the accumulating Rad52 fluorescence moving outward from the initiating site of nucleation. Analysis of the growth rates was restricted to nucleation events that were well resolved from the nearest neighboring events, and the rates were estimated only until the Rad52 fluorescence signal merged with the signal of adjacent complexes. Rad52 binding lifetime measurements were made with 100 pM RPA–mCherry (100 pM) and 50 pM SNAP⁴⁸⁸-Rad52. After initial binding, free proteins were flushed from the sample chamber, and data were acquired for either 10-min or 2-h intervals, with frame acquisition frequencies of either 1 frame per second or 1 frame per 24 s, respectively; the lasers were shuttered in between frame acquisition so that the total time that each sample was exposed to laser illumination was identical for each of the two different time courses. RPA chase experiments were conducted with preassembled RPA–mCherry–ssDNA complexes or RPA–mCherry–SNAP⁴⁸⁸-Rad52–ssDNA complexes made with 100 pM RPA–mCherry and 1 nM SNAP⁴⁸⁸-Rad52 or 0.6–1.0 nM wild-type Rad52, as indicated. Free proteins were flushed from the sample chamber and then were rapidly replaced with buffer containing 100 nM WT RPA. The kymographs in Figure 4d were collected under identical illumination conditions and camera gain settings, and the image contrast of the RPA–mCherry signal for the WT RPA chase was adjusted identically for reactions in either the presence or absence of SNAP⁴⁸⁸-Rad52. RPA–mCherry and SNAP⁴⁸⁸-Rad52 colocalization analysis after chasing with 100 nM WT RPA was made with normalized pixel-intensity profiles generated from separate kymographs for each of the two spectrally separated fluorophores, and the resulting data were quantified by Pearson correlation analysis.

Presynaptic-complex assembly. Reactions involving Rad51 were conducted at 30 °C in buffer containing 30 mM Tris-acetate, pH 7.5, 5 mM Mg-acetate, 50 mM KCl, 1 mM DTT, 2.5 mM ATP and 200 µg ml⁻¹ BSA. RPA–mCherry–SNAP⁴⁸⁸-Rad52–ssDNA complexes were assembled onto single-tethered ssDNA curtains, as described above, with the exception that the reactions used 150 pM SNAP⁴⁸⁸-Rad52. Presynaptic-complex assembly was then initiated by injection of 1–3 pM Rad51 under continuous buffer flow (0.2 ml min⁻¹) while images were acquired at 1-s intervals for approximately 10 min. Control reactions confirmed that Rad51 filament formation on the ssDNA was ATP dependent. The length of the protein-bound ssDNA was measured as a function of time tracking the location of the most 3'-distal detectable complex of SNAP⁴⁸⁸-Rad52. The dark Rad51–bound ssDNA may extend beyond the most distal Rad52 complex, but it would not be detectable because neither the ssDNA nor the Rad51 is fluorescently tagged; this possibility does not affect the interpretation of our results. The total relative amount of SNAP⁴⁸⁸-Rad52 bound to the ssDNA before and during the injection of Rad51 was calculated on the basis of the integrated signal intensity across the full length of each individual ssDNA molecule over the course of the reactions. The resulting data were then calculated as a percentage decrease in each fluorescence signal over time. The apparent Rad51 filament length distributions are reported on the basis of the experimentally observed center-to-center distances between the small isolated clusters of RPA–mCherry that remained bound to the ssDNA after Rad51 filament formation.

Rad52 and RPA binding to preassembled presynaptic complexes. Presynaptic complexes were assembled exactly as described above, with RPA–mCherry (100 pM), SNAP⁴⁸⁸-Rad52 (150 pM), and WT Rad51 (1–3 µM). Free proteins were then flushed out of the sample chamber with buffer containing 30 mM Tris-acetate, pH 7.5, 5 mM Mg-acetate, 50 mM KCl, 1 mM DTT, 2.5 mM ATP and 200 µg ml⁻¹ BSA. Control experiments demonstrated that the Rad51 filaments remained stable in the presence of ATP, and all subsequent buffers contained 2.5 mM ATP to prevent dissociation of the Rad51 filaments. The preassembled presynaptic
complexes were then chased with SNAP546-Rad52, and data were acquired at 1-s intervals for 10 min, as described above. SNAP546-Rad52 binding kinetics were quantified by spatial segregation of sections of the presynaptic complex that were either bound by WT Rad51 or by SNAP488-Rad52–RPA-mCherry complexes on the basis of visual inspection, and the increase in integrated SNAP546-Rad52 signal intensity (a.u.) for each region was determined as a function of time.

56. Antúnez de Mayolo, A. et al. Multiple start codons and phosphorylation result in discrete Rad52 protein species. *Nucleic Acids Res.* **34**, 2587–2597 (2006).

57. Galletto, R., Amitani, I., Baskin, R.J. & Kowalczykowski, S.C. Direct observation of individual RecA filaments assembling on single DNA molecules. *Nature* **443**, 875–878 (2006).

58. Krejci, L. et al. Interaction with Rad51 is indispensable for recombination mediator function of Rad52. *J. Biol. Chem.* **277**, 40132–40141 (2002).

59. Busygina, V. et al. Hed1 regulates Rad51-mediated recombination via a novel mechanism. *Genes Dev.* **22**, 786–795 (2008).

60. Kwon, Y., Zhao, W. & Sung, P. Biochemical studies on human Rad51-mediated homologous recombination. *Methods Mol. Biol.* **745**, 421–435 (2011).

61. Greene, E.C., Wind, S., Fazio, T., Gorman, J. & Visnapuu, M.L. DNA curtains for high-throughput single-molecule optical imaging. *Methods Enzymol.* **472**, 293–315 (2010).