Homology-directed repair involves multiple strand invasion cycles in fission yeast

Amanda Vines, Kenneth Cox, Bryan Leland, and Megan King

Corresponding author(s): Megan King, Yale School of Medicine

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Megan,

Thank you submitting this interesting manuscript, with reviews and your revisions to the Molecular Biology of the Cell. I am very pleased to inform you that I find the manuscript to be of high significance and nicely done.

Please complete the remaining revisions you proposed and return the paper to us. I expect to be able to acceptance the paper for publication in MBOC at that point.

Sincerely,
Kerry Bloom
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. King,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

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Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org
Response to Reviewers: Vines et al.

We are grateful to the Reviewers for their thoughtful and helpful assessment of our work. Below we include a point-by-point response to the Reviewers’ critiques concerning the interpretation of our results and the power of our system to elucidate key dynamics of fission yeast homology-directed repair (HDR). We appreciate that the Reviewers judged our assay to be a valuable new tool for studying DSB repair in *S. pombe*. In general, the Reviewers also felt that our data provides new insights into homology search during HDR in fission yeast, including 1) that multiple DSB-donor encounters often precede repair and 2) that the activity of the helicase Rqh1, which dissolves strand invasion structures, alters the kinetics and efficiency of HDR in *S. pombe*. The Reviewers also raised several concerns with regards to 1) some technical aspects of the experimental approach, 2) the display of the data, and 3) the interpretation of the data. The Reviewers requested additional experiments to address the efficacy of our 5 minute observational time window and the rate of spontaneous damage in the Rqh1 null background, which we have provided in a resubmission. We have also clarified experimental details that the Reviewers found confusing as worded in the original text. Lastly, the Reviewers highlighted minor needed adjustments to the figures that we have incorporated.

**Reviewer #1** (Evidence, reproducibility and clarity (Required)):

Vines et al adapted a system that has been used in *S. cerevisiae* to study the homology search and homologous recombination repair events by live cell imaging. The authors utilized a system they set up in a fission yeast strain that has a fluorescently tagged endonuclease induced DSB site and monitored RAD52 focus formation in both haploid and diploid cells. The main findings presented are that multiple strand invasion events occur during DSB repair and the role of Rqh1 in promoting these multiple events. For example, cells with Rqh1 loss either have a single strand invasion event that quickly leads to repair or a very long extensive repair time. Overall the results are intriguing with new insight into DSB repair being presented.

We appreciate the Reviewer’s recognition that our work provides new insights into homology-directed repair (HDR) in fission yeast.

*The manuscript would benefit from having another system to help to support or validate the key findings and/or the use of some mutants to help uncouple the different roles of Rad51 and/or Rqh1.*

While we agree with the Reviewer that using orthogonal approaches is always desirable, it is not clear what other experimental platform can address the dynamic events with single cell resolution that underlie our observations here; indeed, this was the motivation behind designing our novel approach. However, we have provided additional, detailed context to support our findings in the revised manuscript that highlights how orthogonal experimental strategies (e.g. DSB repair outcome assays) already in the literature (e.g. Hope et al., *PNAS*, 2006) are consistent with our findings. Importantly, however, there is no other population-based system we are aware of that could demonstrate, for example, that Rqh1 shows two different behaviors in individual cells (repair failure and more rapid repair). See more in response to comment 7, below.
**Major comment:**

1) In Figure 1C, and also Figure 2D, the RAD52 focus observed does not appear in the same location as the LacO cassette. I assume this is because of the way the images are cropped. It would be nice if the authors are saying that the RAD52 focus co-localizes with the inducible DSB location for this to be more readily apparent in the representative images.

Co-localization events, indicated with the yellow circles, are assessed within raw 3D data that is then flattened for representation in 2D in the figures. For Figure 1C, the two events in the example cell indeed overlap in 3D space. However, in Figure 2D (cells lacking Rad51) we do not observe any colocalization events due to the defect in strand invasion.

2) In Figure 3A, the authors claim that the mean time to repair an endonuclease induced DSB is 50 min +/- 20 min. It is unclear whether or not this experiment is done in a diploid strain.

We apologize if we were not clear. All experiments presented in the manuscript are carried out in diploid cells. What varies is whether there is a lac operator integrated at one copy of Chr II (all experiments except Fig. 2A-B and “2 lacO at mmf1” data in Fig. 2C, E, F) or on both copies (only Fig. 2A-B and “2 lacO at mmf1” data in Fig. 2C, E, F). This is clarified in the revised text.

3) In Figure 3, whether or not this experiment represents asynchronous cells can greatly influence the timing of DSB repair, as the cell cycle is a huge contributor to HDR repair.

We agree with the Reviewer - the cell cycle has a critical influence on DSB repair mechanism. The diploid fission yeast in which we induced and observed DSBs are indeed asynchronous. However, in fission yeast, which spend over 80% of their cell in G2, we can assess cell cycle by morphology; cytokinesis coincides with the beginning of G2, which then persists until mitotic entry (which is also very obvious from the nuclear shape as visualized by Rad52-mCherry). Moreover, we previously found that HO endonuclease only induces DSBs during S phase (Leland et al., eLife, 2018), so on an individual cell basis we observe site-specific DSBs beginning in late S and early G2 phases. These observations are further validated by the observation that an HO-induced DSB undergoes very high rates of gene conversion in fission yeast (Prudden et al, EMBO J., 2003).

4) In Figure 3D, since a major finding of the paper is that there are multiple invasion events, it would be nice to show some representative images of a few cells where multiple pairings occur.

In Figure 3E, we provide an example of a cell with multiple encounters between the DSB and donor. This is more clearly highlighted in the revised text.

5) It is known from Eric Greene’s work that RAD51 mediated homology search can do multiple samplings of 8-9 nucleotide segments. Have the authors considered the area around the DSB site and how many potential pairing sites there might be in this region? Is it possible that having
a LAC array with repeated segments might be influencing this the pairing since there would be multiple templates?

We acknowledge that the homology of the region surrounding the DSB is important for faithful recognition of a homologous donor and acknowledge that there could be many pairing sites surrounding our induced DSB after end resection. Such local sampling, however, would not be discernible due to the resolution of the light microscope (~0.2µm). We address this noteworthy point during our discussion in the revision. Importantly, we placed the lacO array over 3 kb away from the locus where the HO recognition site is integrated on the homologous chromosome to avoid exactly the Reviewer’s concern.

6) It would aid the reader if there were some picture schematics of what the authors think is occurring throughout the paper in the Figures. Since this is a results/discussion, this approach would be appropriate in lieu of a model figure at the end (which would also be very nice).

We agree that diagrams would aid in communication of our hypotheses, and these are included in the revision.

7) Since the multiple strand invasion events is a major finding of the paper, it is important to test the hypothesis that multiple strand invasion events are occurring a different way. A few ideas would be to examine Lorraine Symington’s work on BIR where she observes multiple template switching events (Smith, CE, Llorente, B, Symington, LS (2007) Nature, 447(7140): 102-105) or something analogous to Wolf Heyer’s recent study in Cell on template switching that the authors already cited. Another idea is to try a RAD51 mutant. For example, Doug Bishop’s group has created a RAD51 mutant that uncouples the homology search from strand exchange, Rad51-II3A mutant (Cloud, V et al (2012) Science, 337(6099): 1222). Perhaps a mutant like this might be able to further support the key finding here.

While our findings share parallels with the works raised by the Reviewer, we would argue that there is a fundamental difference between BIR-type assays and the one we present here, namely that we are visualizing multiple strand invasion events at the homologous chromosome in a normal, high fidelity repair event rather than multiple strand invasion events during BIR, which frequently results in translocations. Moreover, as the two chromosomes are perfectly homologous in our assay, we cannot leverage sequencing to reveal past strand invasion events that took place during HDR. We also cannot, unfortunately, access multiple simultaneous strand invasion events due to the diffraction limit of the light microscope. We concede that it would be informative to further dissect strand invasion using tools such as the Rad51-II3A mutant described in budding yeast in work referenced above by Reviewer #1 and developed in fission yeast by Sarah Lambert’s group (Ait Saada et al., Mol. Cell, 2017). However, with the present limitations on our laboratory access, we feel this is currently beyond the scope of this work.

8) It is surprising that Rqh1 doesn’t have a role in DNA end resection since this is a conserved function from budding yeast to man. Would similar results to what is observed in Figure 4 be observed in a Dna2 or Exo1 mutant?
We acknowledge that Rqh1 orthologs in other organisms (BLM/Sgs1/etc.) have been shown to contribute to DSB end resection. However, previous work from our group indicates that Rqh1 is entirely dispensable for long-range resection in fission yeast (Leland et al., *eLife*, 2018). Interestingly, in this work we also demonstrated that it is only upon loss of either the 53BP1/Rad9 orthologue Crb2 or Rev7 that Rqh1 is able to compensate for loss of Exo1. It remains unclear whether this is a peculiarity of fission yeast (perhaps because they rely heavily on HR due to extensive time in G2) or if it is a direct consequence of the long G2 itself. Regardless, we demonstrated that cells lacking Exo1 cannot generate sufficient ssDNA tracts to load visualizable Rad52-mCherry (Leland et al., *eLife*, 2018). Given this, we cannot address this mutant in this assay. The essential role for Dna2 in replication has also precluded its analysis.

**Minor comment:**

1) As mentioned in the first line of the abstract, HDR is generally considered error-free as opposed to a pathway that "can be" error-free.

We acknowledge that HDR (and more specifically HR) is often error-free, but there are notable exceptions such as when a non-homologous donor is utilized for repair or when the polymerases engaged during repair incorporate errors (work from Haber and colleagues). We clarify this sentence in the revision.

2) In Figure 2D, it is unclear whether this experiment is done in diploid cells. The rest of the figure is in diploid cells but two LacO cassette are not present past the first frame. Please clarify in the legend and/or figure panel. As mentioned above, this is also confusing in Figure 3.

As above, we monitored repair events in diploid cells only – this is clarified in the revised text.

Reviewer #1 (Significance (Required)):

The most important advancement in this paper is that multiple strand invasion events occur during homologous recombination and the role of the Rqh1 in this process. Rqh1 is important protein whose mutation is implicated in human disease such as Bloom syndrome and cancer. In addition, misregulation of double-strand break repair and particularly of Rad51 is associate with cancer. Therefore, understanding the basic mechanisms of how Rad51 mediates double-strand break repair and the role of Rqh1 in this process is critical for understanding fundamental aspects of cancer development.

We appreciate the Reviewer’s assessment of the impact of this work.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In this study, Vines et al developed a microscopy-based assay to determine the kinetics of a site-specific interhomolog repair event, in living fission yeast cells. They detect efficient homology search and homology-directed repair in the system. They also observe that repair is likely to involve multiple site-specific and Rad51-dependent co-localization events between the
DSB and donor sequence, suggesting that efficient inter-homologue repair involves multiple strand invasion events. Loss of the RecQ helicase Rqh1 leads to repair through a single strand invasion event. However, failure to repair is more frequent in rqh1 mutants, which could reflect increased strand invasion at non-homologous sites.

Overall, I find the approach to investigate homology search and homology-directed repair using live cell imaging interesting and potentially very informative. The ability to observe the process in living cells, and with high temporal resolution, complements a variety of previous studies that employ more indirect approaches to invoke similar models. In particular, previous work by the Heyer, Lichten and Hunter laboratories, in budding yeast, has established that Sgs1 promotes non-crossover recombination by acting as a quality control in the maturation of HR intermediates. In this sense, while newly described here for fission yeast, it is not unexpected that homology-directed repair involves multiple strand invasion cycles. In my opinion, the strength of the work is the method/approach, rather than the specific conclusions made (even though I think that it is important to know how fission yeast cells perform homology search).

We thank the Reviewer for their appreciation of the value that cell biology can bring to the study of homology-directed repair. We wholeheartedly agree that this work is consistent with prior work on Sgs1. With regards to multiple strand invasion cycles, while we agree that there may be many in the field who would expect this result, we would argue that 1) demonstrating this by direct visualization of individual DNA repair invents is inherently valuable and 2) many studying homology search itself (or who have modeled homology search in silico, for example) do not incorporate multiple strand invasion cycles in their thinking. Thus, we would argue that this work goes beyond a technical feat and will have impact beyond the approach.

However, for the reasons detailed below, my general impression is that it isn't clear how robust the method is at delivering unambiguous information on the important questions asked:

1) The authors state that they have developed a system to monitor the 'dynamics and kinetics' of an engineered, inter-homologue repair event. With this in mind, I was expecting a more detailed exploration of the process of homology search. For example, what happens at shorter time scales? Is it possible that by imaging at every 5 minutes many of the events are missed? Could the authors be missing very transient events (especially in rqh1 mutants) by using an inappropriate time scale?

We acknowledge that it would be ideal to observe DSB repair across a range of time scales in our system. For practical reasons we found it most valuable to choose the 5 minute time window since it was most amenable to observing the entire course of repair as often as possible in an asynchronous cell population (see our response to Reviewer #1’s comment 3 above) while mitigating photobleaching. However, we recognize that we sacrificed time resolution between frames in order to do this. Like the Reviewer, we were also concerned that we were missing transient events due to an inappropriate timescale.

To address this, we have acquired additional data in WT with greater time resolution with a focus on encounter frequency rather than time to repair (as the overall length of the usable
movie that we can obtain is shorter). When imaging WT cells with a site-specific DSB at 2 minute intervals (2.5 times more frequently), we do observe a shift (of ~1 encounter per 30 minute window) toward more colocalization events with the donor sequence. We acknowledge that more sampling will also lead to an increase in random encounters as revealed by analysis of the two lacO control strain as described in the manuscript. These data are included in the revision (Supplementary Figure 3A-B) and suggest that we may be missing some transient encounter events while using 5 minute time points. As noted by Reviewer #2, this could account for repair in the subset of WT and Rqh1-null cells in which we observed no encounters. We acknowledge these caveats in the revision but would argue that our data support the conclusion that loss of Rqh1 decreases the number and/or lifetime of strand invasion events.

2) Another point relates to the Rad52 signal/foci, which is central to the study. While it is clear to me what the authors consider to be a focus of Rad52, I am not sure how to interpret what happens when Rad52 is as enriched throughout the entire nucleus as it is in the repair focus in the still before. For example, Figure 1C, 40 min vs 45 min. How do the authors interpret what is being visualised? Similarly, is the level of colocalization at 90 min really reflecting a specific enrichment of Rad52 at the DSB site? Much more of the Rad52 signal is away from the DSB. In other words, are quantitative criteria being used to assign colocalization events?

As described in our Methods and the text, we used specific criteria to define 1) whether DSBs are site-specific and 2) whether they are colocalized with the donor site. In the images indicated as “contrast adjusted” we have scaled each panel time point individually with respect to the pixel intensities (that is, the least and most intense pixels have been set the same value for each). This strategy allows us to convey relatively dim Rad52-mCherry foci, particularly early after DSB end resection. A consequence of this is that the apparent background for panels in which there is not a strong Rad52-mCherry focus will appear higher, while the background will appear relatively less at time points with a strong Rad52-mCherry focus. For this reason we also present the raw image (found above). It is important to emphasize that when we are applying co-localization criteria, we do so within a 3D stack of images to ensure that the Rad52-mCherry signal and lacO array GFP signal coincide. In 2D representation, however, we understand that this may appear less clear.

In the particular case of the colocalization in Figure 1C at 90 minutes that the Reviewer points out, it is more evident in the 3-D Z stacks that the surrounding mCherry signal apart from the colocalization with the lacO array is due to inhomogeneity in the background signal. Another contribution is that the lacO array signal often becomes delocalized during colocalization events (as evident in that 90 minute time point). Although this is an interesting observation, we are still investigating what activity may explain this response. We address the caveats of our colocalization analysis more fully in the revision.

3) In the system described here, Rad52 foci form in only ~15% of cells. I think it would be important to rationalise this low number in the manuscript. Moreover, G2 Rad52 foci still form at considerable rates in cells without HO. I think it would be important that the authors provide some explanation on what this might reflect.
There are several considerations that we believe contribute to this observation, which we also documented previously in haploid cells (Leland et al., *eLife*, 2018). First and foremost, this assay is quite different from endpoint assays that involve induction of HO nuclease because we analyze only those events that happen immediately after additional of uracil to elevate HO endonuclease expression under the control of the *urg1* promoter. Combined with the efficient repair of any DSB induced by leaky HO expression (taking less than an hour according to our data), we likely miss events that have already taken place or would take place later in other assay systems. Lastly, it is established that nucleosomes can prevent HO cleavage in its intrinsic role in budding yeast (Laurenson and Rine, *Microbiol. Rev.*, 1992; Haber, *Ann. Rev. Genet.*, 1998); we cannot rule out that cleavage at this particular site is less efficient due to intrinsic nucleosome stability. With respect to spontaneous DNA damage, most of this is short-lived and occurs in S-phase, likely due to replication stress, although we occasionally observe long-lived Rad52 foci in a sub-population of cells – this is in line with previous publications (Coulon et al., *MBoC*, 2006; Lorenz et al. *Mol. Cell Biol.*, 2009; Sanchez et al., *Mol. Cell Biol.*, 2012; Schonbrun et al., *J Biol. Chem.*, 2013). We provide a greater explanation of the observed induction rate in the revision.

**Other issues to consider:**

4) In Figure 2D, the overlay does not show any green. It is possible that the green channel was not overlaid with the pink?

We apologize for this error and very much appreciate the Reviewer noticing that it is missing from the merged image. This is corrected in the revision.

5) In Figure 2D, the unadjusted images for Rad52 are very sharp. Did the authors perform contrast adjustment in the top panels? If so, this should be indicated. My current impression is that the data was duplicated by mistake.

The Rad52-mCherry data in Figure 2D was labelled correctly and not duplicated. Because cells lacking Rad51 accumulate extensively resected DSBs (and therefore abnormally high levels of Rad52 loading), the intensity of Rad52-mCherry is very high. For simplicity, we have removed the contrast-adjusted Rad52-mCherry images for this panel in the revision.

6) I don't understand why is the time since nuclear division different is every single figure. For simplicity, it would be much better to start every figure at T=0.

We agree with the Reviewer. In the revision we have normalized all kymographs to begin at t=0 with the exception of Fig. S1D (where we are visualizing the subsequent division) and Fig. 3E (where the unobserved time of nuclear division is estimated based on time of cytokinesis).

Reviewers #2 (Significance (Required)): 
Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In this manuscript, the authors describe a system to monitor an inducible site-specific double-strand break (DSB) and the undamaged homologous locus during homology-directed repair in *S. pombe* cells. The authors show that the Rad52 focus on the induced DSB is more persistent than spontaneous Rad52 foci that form throughout the cell cycle. The persistent Rad52 focus intermittently colocalizes with the donor sequence labeled with LacI-GFP, reflecting multiple strand invasion events, and this colocalization requires the Rad51 recombinase. The authors report that the time to repair is dependent on the number of strand invasion events (colocalization of Rad52 and homolog), and that the initial distance between the induced DSB and the homolog predicts the time to their first contact, but does not predict the time to repair. Lastly, the authors claim that repair in *rqh1Δ* cells is bimodal, either failing to repair within the experimental time frame, or being more efficient than WT cells (which often involves a single colocalization event).

**These claims are supported by the data:**

1) Rad52 focus on the induced DSB is more persistent than spontaneous Rad52 foci that form throughout the cell cycle.

2) Multiple colocalization events between Rad52 focus and the donor sequence are frequent, and this colocalization is dependent on Rad51, which reflects multiple strand invasion events.

3) *rqh1Δ* cells have a lower rate of productive repair compared to WT cells.

The key concern I have for this section is the noise in Rad52 images. For example, in Fig. 1C at 15 minutes, it looks like there is a Rad52 focus both before and after adjustment but the time point is labeled as not having a Rad52 focus. Conversely, in Fig. 2D at 60 minutes, it looks like there isn't a Rad52 focus but the time point is labeled as having a Rad52 focus. How did the authors determine the presence of a Rad52 focus? Additionally, it is difficult to assess colocalization of Rad52 and LacI-GFP in merged images (hard to see Rad52 focus in Fig. 1C merged and LacI-GFP in Fig. 2D merged).

The criteria that we established to indicate a Rad52-mCherry focus (as annotated by a pink circle and as explained in the Methods) is that it persists for at least three frames (>15 minutes). This was chosen because it is a characteristic of the HO-induced DSB but not of spontaneous DNA damage that occurs frequently during S-phase. Indeed, the numerous, small, and short-lived foci at the 15 minute time point in Fig. 1C occurring just 15 minutes after nuclear division is perfectly characteristic of replication stress that is independent of HO endonuclease expression. Thus, the pink circles indicate a specific type of Rad52-mCherry focus that is relevant for the assay. We agree that the Rad52-mCherry focus in Fig. 2D at ~60 minutes is poorly visualized in
the flattened image, but would like to emphasize that we assess the foci in the true 3D volume. With regards to the merged images, we have adjusted the individual signals to make it easier for the reader to assess colocalization in the revision.

**These claims are supported by weak data:**

1) The initial distance between the induced DSB and donor sequence predicts the time to their first physical encounter (Line 60).

   We agree with the Reviewer that our word choice (“predicts”) suggests a stronger relationship than is supported by the data. However, we also argue that there is nonetheless a meaningful correlation. We believe this is an important point to make because it supports prior work in budding yeast suggesting that relative position affects donor choice preference. We edited this language in the revised text.

2) Repair efficiency is dictated by the number of strand invasion events (Line 61-62). Figures 3E and 3F technically have positive correlations that support the authors’ claims but there is a lot of noise. I think the data needs to be more robust, especially considering the strong wording used to describe the data. A minor comment on Fig. 3F: why is there a data point with 3.5 encounters?

   Again, we agree with the Reviewer that our word choice (“dictate”) is too strong given the data and we have edited the text accordingly. We thank the reviewer for noticing the error in Fig. 3F, which is corrected in the revision (Fig. 4F).

**These claims are not supported by the data:**

1) In the absence of Rqh1, successful repair requires a single strand invasion event (Line 63).

   We acknowledge that this is too strong a claim to make based on our data and have amended this language in the revision text. Specifically, and as outlined in our response to Reviewer #2 with regards to our imaging frequency, we have revised the manuscript to state that cells lacking Rqh1 are more likely to repair without a visualized colocalization event and/or they possess shorter lived strand invasion events. Importantly, repair outcome assays indicate that cells lacking Rqh1 display elevated gene conversion rates rather than non-HDR-mediated repair (Hope et al., PNAS, 2006). Thus, we do not expect that the lack of colocalization reflects NHEJ but rather our inability to “catch” the colocalization event with the temporal resolution we can achieve.

2) rqh1Δ cells that complete repair are more efficient than WT cells and often involve a single colocalization event (Line 178-179).

   As for the above, we agree that our claim that rqh1Δ cells “often” involve a single colocalization event is too strong a claim based on our data. We have amended this language in the revised text.
Fig. 4A shows an example of a rqh1Δ cell with productive repair but without any colocalization with the homolog, which contradicts the statement that successful repair requires a single strand invasion event in the absence of Rqh1. If the authors interpreted the single continuous presence of Rad52 focus during time-lapse as evidence of a single strand invasion event, then it would nullify using multiple colocalization events as evidence for multiple strand invasion events. In other words, the data in Fig. 3D that clearly displays multiple colocalization events in individual cells during repair can no longer be evidence of multiple strand invasion events since those cells all had one continuous presence of Rad52 focus.

We believe that we understand the confusion that the Reviewer is articulating in their comment and apologize that we have not been clearer in explaining our interpretation. For this site-specific DSB to be repaired, we expect that it must either 1) engage with the homologous chromosome to be repaired by HR or 2) be repaired through an alternative pathway – at this non-repetitive, resected locus this would likely have to be a microhomology-mediated (alt-) NHEJ mechanism. However, prior analysis of repair outcome in a model of interhomologue repair in the absence of Rqh1 (Hope et al., PNAS, 2006) demonstrates an increase in cross-over HR events rather than end joining events, arguing that interhomologue HR still dominates (and with increased CO to NCO frequency). We interpret the continuous presence of a Rad52 focus to only reflect a DSB has been subjected to resection and has not yet been repaired. Taking these two points together, within the lifetime of a Rad52-loaded DSB it can either 1) never colocalize with the donor sequence and fail to repair (as in cells lacking Rad51, Fig. 2D-F) or 2) undergo strand invasion (and therefore colocalization) at least one time (but possibly multiple times) to allow for HR to occur. However, we agree (and have clarified in the revision) that we often infer that at least one strand invasion event has taken place to support successful HDR when we do not capture the event at our experimental time resolution. Based on the additional data at shorter timescales that we will add to the revised manuscript (as outlined in the response to Reviewer 2, point 1), which demonstrates that we may in some cases be undercounting relevant colocalization events that are too brief to be accurately captured with 5 minute time resolution, we think the most parsimonious explanation is that cells lacking Rqh1 spend less time with the DSB and donor sequence colocalized prior to repair. We agree with the Reviewer, however, that we cannot say whether this reflects a shorter duration of interactions and/or a fewer number of interactions. We have therefore revised the manuscript to acknowledge this point.

Regarding the second claim, I think Fig. 4D only shows rqh1Δ cells with successful repair (since the longest repair time is 55 minutes, but it is not clear from the figure legend). It is not shown how many colocalization events these cells had in Fig. 4D, but there are 16 cells in Fig. 4D while there are only 2 cells with a single encounter (shown in Fig. 4F). With these numbers, it seems like rqh1Δ cells that complete repair are more efficient than WT cells but only few of these cells involve a single colocalization event.

The Reviewer is correct, Figure 4D does indeed show only rqh1Δ cells with the site-specific DSB that successfully repair – this is clarified in the revision text. As described above in our response to Reviewer #2’s comment 1, it may be that we are missing colocalization events in
rqh1Δ DSB cells. However, we would argue that our data do support that, for cells lacking Rqh1 that execute repair, there are fewer and/or shorter-lived colocalization events.

Also, how often do Rad52 foci form spontaneously in rqh1Δ cells and what is the duration? This data was provided for WT but not for rqh1Δ.

We do find an increase in spontaneous Rad52-mCherry focus formation in rqh1Δ cells. However, our criteria to exclude such spontaneous damage from our analysis remains valid. This data is included in the revision.

All of the data would have been more supported if the homologous chromosome would have been tagged. Such a configuration would really have helped the interpretation of the rqh1Δ data.

We agree that in theory it would be advantageous to have both copies of the chromosome tagged. Indeed, we attempted to leverage a different version of this experimental system with lacO arrays on both copies while inducing a DSB. However, the complexity of monitoring (and keeping the identity clear) for the two copies presented major challenges. Better would be two distinct arrays – an approach that has been used in budding yeast. However, to date many groups, including ours, have been unable to get TetO-TetR arrays to perform well in fission yeast.

Reviewer #3 (Significance (Required)):

The significance of this work is the conceptual advance in the field of DNA repair. Homology search is an important process in homology-directed repair and is not fully understood. This study reports time-lapse data on the interaction between a DSB and its donor template during repair and provides insight into the kinetics of homology search. The audience for this manuscript is the field of DNA repair, and to a lesser extent, field of live-cell imaging.
Dear Megan,

Thank you for your thoughtful response to the reviewers comments. I am very pleased to inform you that the manuscript is now suitable for publication in the Molecular Biology of the Cell. Thank you for submitting this interesting work.

Sincerely,
Kerry Bloom

Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. King:

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