Rev7 and 53BP1/Crb2 prevent RecQ helicase-dependent hyper-resection of DNA double-strand breaks

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Abstract

Poly(ADP ribose) polymerase inhibitors (PARPi) target cancer cells deficient in homology-directed repair of DNA double-strand breaks (DSBs). In preclinical models, PARPi resistance is tied to altered nucleolytic processing (resection) at the 5' ends of a DSB. For example, loss of 53BP1 or Rev7/MAD2L2/FANCV derepresses resection to drive PARPi resistance, although the mechanisms are poorly understood. Long-range resection can be catalyzed by two machineries: the exonuclease Exo1, or the combination of a RecQ helicase and Dna2. Here, we develop a single cell microscopy assay that allows the distinct phases and machineries of resection to be interrogated simultaneously in living *S. pombe* cells. Using this assay, we find that the 53BP1 orthologue and Rev7 specifically repress long-range resection through the RecQ helicase-dependent pathway, thereby preventing hyper-resection. These results suggest that "rewiring" of BRCA1-deficient cells to employ an Exo1-independent hyper-resection pathway is a driver of PARPi resistance.

Introduction

DNA repair is an essential process conserved throughout evolution and commonly disrupted in tumor cells (Jeggo et al., 2016). Many cancer treatments, including poly(ADP ribose) polymerase (PARP) inhibitors (PARPi), target DNA repair pathways to kill rapidly dividing, repair-deficient cells (Farmer et al., 2005; Fojo and Bates, 2013; Lord et al., 2015; Mateo et al., 2015). 5' end resection, which generates tracts of single-strand DNA (ssDNA) at DNA double-strand break (DSB) ends dictates repair pathway choice: blocking resection promotes canonical non-homologous end joining (typically in G1), while initiating resection commits a DSB to repair by homologous recombination (HR), usually in S/G2 (Chapman et al., 2012; Hustedt and Durocher, 2016; Symington, 2016). The resection machinery is tightly controlled at both the step of resection initiation (involving Ctp1/Sae2/CtIP and the MRN/X complex) and during long-range resection, which is mediated by two parallel pathways catalyzed by either the exonuclease Exo1 or the combination of a RecQ helicase and Dna2 (Chen et al., 2012; Croteau et al., 2014; Nimonkar et al., 2011; Tkáč et al., 2016; Zimmermann et al., 2013). Additional accessory factors play key roles as modulators of resection; for example, loss of Rev7/MAD2L2/FANCV, a small, multifunctional HORMA domain protein, (Bluteau et al., 2016; Rosenberg and Corbett, 2015) derepresses resection inhibition (Boersma et al., 2015; Xu et al., 2015), thereby allowing HR-deficient, *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells to become resistant to PARPi. In human cells, Rev7 appears to act in concert with another inhibitor of resection, 53BP1(Chapman et al., 2013; Ochs et al., 2016; Zimmermann et al., 2013), loss of which is also sufficient to drive PARPi resistance (Boersma et al., 2015; Bouwman et al., 2010; Jaspers et al., 2013; Xu et al., 2015). Importantly, the mechanisms by which Rev7 and 53BP1 inhibit DSB end resection remain poorly understood. To gain insights into how resection is controlled, we have developed a single-cell microscopy-based assay capable of quantitatively measuring DSB end resection rates in the facile genetic model, *S. pombe*. Leveraging this assay, we find that Rev7 and the 53BP1 orthologue, Crb2, specifically inhibit the RecQ-helicase-dependent long-range resection pathway. Moreover, through derepression of RecQ helicases, *rev7*Δ or *crb2*Δ cells can achieve very fast resection rates (>20 kb/hr) – approximately twice as fast as Exo1-dependent long-range resection. As BRCA1 activity has been tied to Exo1-dependent long-range resection (Tomimatsu et al., 2012), our findings suggest that PARPi resistance can be driven by compensation through derepression of the RecQ-helicase-dependent resection pathway.

Results

A microscopy-based assay to measure the rate of long-range resection in single cells

In order to quantitatively measure initial steps in DSB processing in single, living cells, we developed a microscopy-based DSB end resection assay (Figure 1A). In this system, an ectopic 10.3 kb, 256-copy LacO array and adjacent HO endonuclease cut

site are engineered at a euchromatic (but intergenic) region near *Mmf1* (Figure 1–figure supplement 1). A single, site-specific DSB is generated by regulating the expression of the HO endonuclease under the control of the Ura-inducible $P_{urg1lox}$ RMCE system (Watson et al., 2008; 2011). The timing of on-target DSB events is visualized by the appearance of a Rad52(Rad22)-mCherry focus that co-localizes with Mmf1:LacO/LacI-GFP (Figure 1B). By tracking cell lineages, we see that HO endonuclease induction produces on-target Rad52-mCherry foci in S/G2 (G1 is very short in *S. pombe*) when the repair machinery is primed for HR (Symington and Gautier, 2011) (Figure 1B). Importantly, we do not observe loading of Rad52-mCherry at the LacO/LacI-GFP array in the absence of HO endonuclease expression (on-target Rad52 foci in < 0.2% of uninduced cells, n=657), suggesting that the LacO array is not sufficient to create a "fragile site" in *S. pombe* (Jacome and Fernandez-Capetillo, 2011; Saad et al., 2014). As resection proceeds, the LacO repeats become single stranded, disrupting LacI-GFP binding and causing the intensity of the GFP focus to progressively decrease (Bell and Lewis, 2001) (Figure 1B, Figure 1–figure supplement 2). To verify that loss of GFP focus intensity reflects DSB end resection, we analyzed cells lacking Exo1, which catalyzes the majority of long-range resection in WT *S. pombe*, with the Rqh1/Sgs1/BLM and Dna2 resection pathway playing a secondary role (Langerak et al., 2011). As expected, cells lacking Exo1 show a persistent LacO/LacI-GFP focus over many hours even after Rad52-mCherry loads (Figure 1C). Importantly, loss of Exo1 does not influence the induction of the site-specific DSB as measured by loss of a PCR product across the cut site (Figure 1–figure supplement 3A).

This assay only visualizes DSB foci after resection initiation by Ctp1/Sae2/CtIP and MRN/MRX (~100 nt of resection), as Rad52 loads at DSBs by exchanging with RPA on resected ssDNA ends (Jensen and Russell, 2016; Lisby et al., 2004; Ma et al., 2015; Mimitou and Symington, 2008). Using quantitative imaging with calibration strains, we estimate that visualization of the Rad52-mCherry focus requires loading of ~30 copies (see Methods), representing a Rad52 filament equivalent to at least 90 nt (but likely several hundred nts, see below) of resected ssDNA on each sister in G2 (Gibb et al., 2014; Grimme et al., 2010; Kagawa et al., 2002; Singleton et al., 2002; Swartz et al., 2014; Wu and Pollard, 2005). To test the frequency with which resection initiation leads to visible Rad52 foci in this assay, we compared the timing of Rad52-mCherry foci formation within the cell population to an independent measure of resection using a quantitative PCR (qPCR) assay in which resection protects from digestion at an ApoI cut site 168 nt downstream of the HO cut site (Langerak et al., 2011) (Figure 1–figure supplement 3B). Using this qPCR based assay, we observe similar DSB induction frequency and kinetics for ApoI protection and Rad52 focus formation, with an apparent 30-60 minute delay between ApoI protection (qPCR) and Rad52-mCherry loading, which likely represents the time required for RPA loading and exchange to Rad52 (Lisby et al., 2004) (Figure 1–figure supplement 3C). As the visibility of the LacO array is an important endpoint for this assay, we estimate that the LacI-GFP focus is visible down to a LacO array length of <500bps under these conditions, based on our ability to robustly

detect the focus from a 1kb LacO array integrated into *S. pombe* (Figure 1-figure supplement 3D-G).

By measuring the time interval between Rad52-mCherry focus formation and LacO/LacI-GFP focus disappearance, we can determine the time it requires to resect through the full LacO array in individual cells (Figure 1B, blue boxes, Figure 1–figure supplement 2). For example, for the two daughter cells in Figure 1B arising from cell division, the time interval from Rad52-mCherry loading until loss of the LacO/LacI-GFP focus is 150 minutes for the upper cell ("1"), and 140 minutes for the lower cell ("2"). The progressive loss of LacI-GFP intensity and gain in Rad52-mCherry intensity during the duration of resection is further illustrated by quantitative image analysis (Figure 1E-F). As we know the genomic separation of the LacO array and the HO cut site (13.9 kb, Figure 1–figure supplement 1), we can calculate the resection rate from the resection duration. For example, the calculated resection rates for the two cells in Figure 1B is very similar at 5.55 and 5.94 kb/hr. Across all WT cells, we detect a median, long-range resection rate of 7.6 kb/hr (Figure 2B). This rate is somewhat faster than the resection rates measured by previous population-based assays using qPCR in *S. pombe* (4 kb/hr) or Southern blot in *S. cerevisiae* (4.4 kb/hr) (Langerak et al., 2011; Zhu et al., 2008). As this assay isolates the process of long-range resection after Rad52 loading one possibility is that resection rates that measure both resection initiation and long-range resection give rise to slower rates. Importantly, using the qPCR approach to compare resection upstream of the DSB (which contains the LacO array) and downstream of the DSB (which does not) demonstrates that any influence of the LacO array on resection rate is minor (Figure 1–figure supplement 3H).

As resection in the absence of Exo1 is very inefficient (Figure 1C), the rate cannot be determined using this assay. However, we can infer an upper bound of the long-range resection rate of ~2.8 kb/hr for *exo1*Δ cells. We also note a strong inhibition of resection (comparable to *ctp1*Δ cells) as close as 300 nts from the DSB in cells lacking Exo1 as detected by qPCR (Figure 1–figure supplement 3I); this correlates with a defect in Rad52-mCherry loading in the imaging-based resection assay (Figure 1–figure supplement 3J). This observation confirms that the extent of resection required to form visible Rad52 foci in this assay discussed above (~30 molecules; <300 nt) partially requires Exo1-dependent resection in addition to MRN/MRX- and Ctp1/Sae2/CtIP-dependent resection initiation (Symington, 2016), consistent with a study in budding yeast suggesting a specific requirement for Exo1 in the early phase of resection post-initiation (Saad et al., 2014).

53BP1 (in human cells) and its orthologue Rad9 (in budding yeast) repress resection initiation (Chapman et al., 2012; Ferrari et al., 2015; Symington, 2016); in budding yeast, loss of Rad9 also increases resection efficiency (Bonetti et al., 2015). Applying the live cell resection assay to fission yeast lacking the orthologous Crb2, we observe a strong increases the median rate of resection to (13.9 kb/hr), with some individual cells demonstrating very fast (~40 kb/hr) resection rates (Figure 1F). Thus, we can readily

assess factors that positively and negatively influence long-range resection rate using this new LacO-based assay.

Rev7 inhibits long-range resection

Next, we examined how loss of Rev7 influences long-range resection at DSBs during S/G2. The duration of LacO array resection is shorter in *rev7*Δ cells than in WT cells (80 minutes or less, Figure 2A-B and Figure 2–figure supplement 1). In the population the median long-range resection rate for *rev7*Δ cells is similar to cells lacking Crb2 (10.4 kb/hr, Figure 2C). As Rev7 also functions with Rev3 as part of the polymerase ζ complex in translesion synthesis, we also confirmed that Rev3 does not affect resection (Figure 2C), consistent with previous data showing that repressing resection during HR is a distinct function of Rev7 (Boersma et al., 2015; Rosenberg and Corbett, 2015; Xu et al., 2015). Using the orthogonal qPCR approach (Figure 1–figure supplement 3B), we confirm that in *rev7*Δ cells, more chromosomes with DSBs have undergone 3kb and 13kb of resection than WT between 90 and 180 minutes after HO induction, respectively (Figure 2D). Again, loss of Rev7 has no influence on the rate of DSB induction (Figure 2E).

The RecQ helicase, Rqh1, rather than Exo1, drives hyper-resection in the absence of Crb2 and Rev7

We next asked if Crb2/Rad9/53BP1 and Rev7 inhibit long-range resection through the Exo1 pathway, the RecQ helicase (Rqh1)-Dna2 pathway, or both (Symington and Gautier, 2011). Interestingly, we find that the rapid long-range resection rate observed in *crb2*Δ or *rev7*Δ cells is entirely Exo1-independent (Figure 3A-C, Figure 3–figure supplement 1). In stark contrast, the rapid rate of resection in a *rev7*Δ single mutant is entirely dependent on the presence of Rqh1 (Figure 3A). Consistent with these observations, we find that loss of Rev7 is able to rescue the severe growth defect of *exo1*Δ cells on rich media plates containing camptothecin, consistent with a derepression of Rqh1-dependent resection in the absence of a functional Exo1 pathway (Figure 3D). When considering only precisely determined resection events, the average long-range resection rate in *crb2*Δ cells is not statically less than that of *crb2*Δ*rqh1*Δ cells when correcting for multiple comparisons ($p = 0.16$) (Figure 3A). However, in many *crb2*Δ*rqh1*Δ cells, resection durations extend beyond the timeframe of data acquisition, suggesting that rapid *crb2*Δ resection also requires Rqh1 (Figure 3–figure supplement 2).

Taken together, these results strongly suggest that loss of either Rev7 or Crb2 drives a shift in resection pathway mechanism from Exo1 to the RecQ helicase-dependent pathway. Unlike Exo1-driven resection that dominates in WT cells, *crb2*Δ or *rev7*Δ cells in which the RecQ helicase is derepressed are capable of resection rates in excess of 20 kb/hr, indicating that the RecQ helicases are more capable of to drive hyper-resection of DSBs than the Exo1-dependent pathway. The strong inhibition of RecQ helicase-dependent resection by Crb2 and Rev7 also explains why Rqh1 is not a major player in WT *S. pombe* resection, since Rqh1 can be derepressed to such a large extent

(by loss of Crb2 or Rev7) that Exo1 becomes dispensable for long-range resection in *crb2*Δ*exo1*Δ and *rev7*Δ*exo1*Δ cells (Figure 3A).

Discussion

Here, we demonstrate a microscopy-based assay capable of quantitatively measuring DSB end resection in living cells, specifically the long-range phase of resection catalyzed by Exo1 or Rqh1/Dna2. By allowing for the visualization of resection in single cells, this assay reveals individual long-range resection rates, which will provide access to information about the variability in resection efficiency within cell populations. Moreover, as this assay allows the tracking of the location of DSB lesions within the nucleus, it will be able to uniquely interrogate the role of intranuclear architecture on resection rates and pathways in the future.

By leveraging the advantages of this LacO-based assay, we show that Crb2/Rad9/53BP1 and Rev7 both act as specific inhibitors of RecQ helicase-mediated long-range resection of DSBs (Figures 2 and 3), supporting a model in which loss of these resection inhibitors drives a change in resection pathway rather than boosting Exo1-dependent resection. This is consistent with a previous study that identified specific mutations in the budding yeast RecQ helicase, Sgs1, that can disrupt inhibition by the Crb2 orthologue, Rad9, leading to a gain in Sgs1-dependent resection (Bonetti et al., 2015), as well as a recent study highlighting the ability of Rad9 to antagonize resection at stalled replication forks by repressing a Dna2-dependent pathway (Villa et al., 2018). Integrating these findings together with this study, we suggest that Crb2/Rad9 and Rev7 enforce Exo1-dependent long-range resection in yeasts, which prevents the hyper-resection that we find is characteristic of the RecQ helicase/Dna2 pathway (Figure 3 and Bonetti et al., 2015). In support of this model, we find that loss of Rev7 is able to restore cell viability of *exo1Δ* cells on media containing camptothecin, suggesting that derepression of Rqh1 can substitute for loss of Exo1, consistent with the gain in resection rate in cells lacking both Rev7 and Exo1 (Figure 3).

The ability of Rev7 to antagonize RecQ helicase/Dna2-dependent long-range resection in fission yeast is very likely to be conserved in mammalian cells. Indeed, indirect evidence suggests that Rev7 knock-down promotes a gain in resection, as loss of Rev7 rescues CtIP-dependent RPA and Rad51 loading at irradiation-induced DSBs in cells lacking BRCA1 (Xu et al., 2015). Importantly, the resection pathway responsible for the gain in repair factor loading was not investigated in this study, but our work suggests that BLM/DNA2 is a likely candidate. Loss of Rev7 does lead to longer 3' single-stranded G-rich overhangs at telomeres in cells with inactivated TRF2 shelterin component, TRF2; in this case, only a partial rescue was obtained by co-depletion of CtIP or Exo1 (Boersma et al., 2015). Again, we hypothesize that this finding likely reflects contributions of BLM/DNA2, although it remains possible that the impact of Rev7 on long-range resection during G2 figures more prominently in fission yeast.

Critically, our findings suggest a new mechanism by which either 53BP1 or Rev7 allows *BRCA1*$^{-/-}$ *p53*$^{-/-}$ cells to become resistant to PARP inhibitors (Jaspers et al., 2013; Xu et al., 2015). It has been proposed previously that the compromised CtIP- and Exo1-dependent resection in BRCA1-deficient cells can be restored by loss of 53BP1 or Rev7 (Boersma et al., 2015, Xu et al., 2015), thereby overcoming the increased DSB load caused by PARP inhibitors (Polato et al., 2014; Tomimatsu et al., 2012). Our study reveals that not just the efficiency, but also the molecular mechanism of resection is altered upon loss of Crb2/53BP1 or Rev7. We expect these insights to have several consequences. First, RecQ helicases, when paired with DNA2, are capable of exceptionally fast resection *in vitro* (Niu et al., 2010), which when derepressed by loss of 53BP1 or Rev7 could cause extended tracts of ssDNA several kb long, promoting further genome instability (Hicks et al., 2010; Ochs et al., 2016); indeed, our data point to such a hyper-resection phenotype upon loss of either Crb2 or Rev7 (Figure 2C). Second, our results suggest that inhibitors of RecQ helicases could potentially re-sensitize BRCA1-null cells to PARP inhibitors, as this would make them both Exo1- and RecQ helicase-deficient (Aggarwal et al., 2013; Yazinski et al., 2017).

Materials and Methods:

Cell culture, strain construction and verification

The strains used in this study are listed in Supplementary file 1. *S. pombe* were grown, maintained, and crossed using standard procedures and media (Moreno et al., 1991). Gene replacements were made by exchanging open reading frames with various MX6-based drug resistance genes (Bähler et al., 1998; Hentges et al., 2005). The 10.3 kb LacO array was inserted between *Mmf1* and *Apl1* on the right arm of chromosome II (Chr II: 3,442,981) using a modified two-step integration procedure that first creates a site-specific DSB to increase targeting efficiency of linearized plasmid pSR10_ura4_10.3kb (Leland and King, 2014; Rohner et al., 2008). A modified MX6-based hygromycin-resistance cassette containing the HO cut site was then inserted between *Apl1* and *Mug178* on chromosome II (Chr II: 3,446,249), 3.2 kb distal to the LacO insertion. The total distance between the HO cut site and the beginning of the 10.3 kb LacO array is 3.57 kb. As the LacO array can contract during the process of transformation, integrants were screened by HincII digest followed by Southern blot (Figure 1–figure supplement 1) using standard procedures, a biotin-conjugated LacO probe, and a Streptavidin-HRP chemilluminescent detection system (Thermo #N100 & #34096).

DSB induction using $P_{urg1lox}$-HO

We used the uracil-responsive $P_{urg1lox}$ expression system, with slight modifications, to induce HO endonuclease expression and create site-specific DSBs at the HO cut site (Watson et al., 2011; Watt et al., 2008). We performed a fresh integration of the *HO* gene at the endogenous *urg1* locus for each experiment in order to reduce long-term instability at the HO cut site or the development of HO resistance presumably due to insertion/deletion events caused by basal expression levels of HO. The pAW8E*Nde*I-HO plasmid (a gift from Tony Carr) was transformed into *S. pombe*, which were then plated onto EMM-leu+thi-ura plates (-leucine: plasmid selection; +thiamine: P_{nmt1}-*Cre* repression; -uracil: $P_{urg1lox}$-*HO* repression). After 4-5 days of growth at 30°C, 40-100 individual colonies were combined to obtain a reproducible plasmid copy number across the population. Cre-mediate *HO* gene exchange at the endogenous Urg1 locus (*urg1::RMCE*$_{bleMX6}$) was induced by overnight culture in EMM-thi-ura+ade+NPG media (-thiamine: expression of Cre from pAW8E*Nde*I-HO; -uracil: $P_{urg1lox}$-*HO* repression; +0.25mg/mL adenine: reduce autofluorescence; +0.1mM n-Propyl Gallate (NPG): reduce photobleaching in microscopy experiments, prepared fresh). The following day, site-specific DSBs were induced in log-phase cultures by the addition of 0.50 mg/ml uracil. This induction strategy resulted in ~15% of cells making a DSB within ~2 hrs (Figure 1C).

qPCR resection assay

Initiation of resection was assessed using a previously described qPCR assay where ssDNA produced by resection causes protection from ApoI digestion (Langerak et al., 2011). ApoI cut site positions (relative to the HO cut cite (Chr II: 3446192)) and PCR primer sets spanning each ApoI recognition site can be found in Supplementary file S3. Mock HincII digestions (do not affect qPCR products) and additional control primers at Ncb2 were used to normalize for ApoI digestion efficiency (see Supplementary Table S3).

Microscopy

All images were acquired on a DeltaVision widefield microscope (Applied Precision/GE) using a 1.2 NA 100x objective (Olympus), solid-state illumination, and an Evolve 512 EMCCD camera (Photometrics). Slides were prepared ~20 minutes after adding 0.50 mg/ml uracil to log-phase cultures to induce HO endonuclease expression and DSB formation. Cells were mounted on 1.2% agar pads (EMM +0.50 mg/ml uracil, +2.5 mg/ml adenine, +0.1 mM freshly prepared NPG) and sealed with VALAP (1:1:1 vaseline:lanolin:paraffin). Image acquisition began between 40 and 80 minutes after uracil addition. Imaging parameters for all resection assay data acquisition were as follows. Transmitted light: 35% transmittance, 0.015 sec exposure; mCherry: 32% power, 0.08 sec exposure; GFP: 10% power, 0.05 sec exposure. At each time point (every 10 min for 5-7 hrs), 25 Z-sections were acquired at 0.26 μm spacing. Identical imaging parameters were used to image a strain expressing endogenously tagged Sad1-mCherry (Sad1 forms a single focus at the spindle pole body that contains between 450 and 1,030 molecules) and relative mCherry foci intensities were used to determine that ~30 molecules of Rad52-mCherry are required to detect a visible focus with these imaging parameters (Wu and Pollard, 2005).

Image analysis

For the LacO resection assay, every cell cycle was tracked and quantified individually, including timing of nuclear division, cellular division, Rad52-mCherry focus formation, and LacO/LacI-GFP focus disappearance. Only on-target Rad52 foci (that co-localized with LacO/LacI-GFP for at least 2 frames) were considered, since many DSB events occur throughout the genome spontaneously, especially during S-phase. The number of cells and events used to generate the plots in all Figures is included as Supplementary file 2. The time between the first frame with an on-target Rad52-mCherry focus and the first frame with complete disappearance of the LacO/LacI-GFP focus is the duration of resection through 3.57 kb (between the HO cut site and the start of the LacO repeats) plus the full 10.3 kb LacO array. All fields from all genotypes were input into custom ImageJ macros that randomized the order of the fields/genotypes, blinded the images by remove the file names, set the contrast to be identical for every image, and numbered each cell lineage. Each blinded field was then manually assessed for

photobleaching of the LacO/LacI-GFP foci in cells without induced DSBs (>80% of all cells) to ensure that disappearance of any LacO/LacI-GFP foci in cells with on-target DSBs was due to resection through the LacO array rather than photobleaching of the GFP signal. Next, using the pre-determined contrast settings for mCherry and GFP channels (to maintain consistency across all images analyzed) individual cells which had on-target DSB events were manually identified, and scored for the first frame of Rad52-mCherry focus appearance and then the first frame in which the LacO/LacI-GFP focus had completely disappeared.

For focus intensity plots (e.g. Figure 1C,D), quantification was performed in ImageJ on the full 5D image stacks (not maximum intensity projections, which are shown in the image panels throughout for ease of viewing, e.g. Figure 1B). Subpixel measurements were made in a cylinder approximating the PSF surrounding the manually scored subpixel center of the focus. A cylindrical shell surrounding the focus was used for background subtraction in both the GFP and mCherry channels.

Raw data were processed, visualized, and analyzed using R, in particular packages dplyr, ggplot2, and broom. Raw data, complete code, and other supporting materials will be made publically available on github.com. During the review process, these materials can be found at: http://goo.gl/4fbtoj.

Growth assays

Cells were grown overnight in YE5S media. Concentrations for each culture were monitored by both OD_{600} and a Coulter Principle cell counter (Orflow Moxi Z). Cultures were diluted as needed to ensure identical numbers of cells were spotted for each genotype, starting with ~$4x10^6$ cell/mL and going down by 6-fold dilutions. Plates were prepared using standard procedures (Moreno et al., 1991), with the addition of 30μM camptothecin (Sigma; ≥95% HPLC purified) after autoclaving.

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Figure Legends

Figure 1. A microscopy-based assay to measure long-range resection in single cells.
(A) Design of the LacO resection assay in *S. pombe*. HO endonuclease cut cite (HOcs) and LacO integration at the *Mmf1* locus on Chr II allows live-cell measurements of resection rates. Rad52-mCherry loads on DSB ends after resection initiation, and LacI-GFP is displaced as resection creates long tracts of ssDNA through the LacO array.
(B) DSB resection events in two WT daughter cells. The majority of the *S. pombe* cell cycle is spent in G2, and all DSBs are observed in S/G2 based on the timing of mitosis and cell fission. Images shown are maximum intensity Z-projections acquired at 10-minute time intervals. Blue annotations denote the starting point of resection (first frame with a detectable Rad52 focus, which formation shortly after resection begins) and the end point (first frame with total loss of the LacO/LacI-GFP focus) of individual resection events. These start/end frames mark the total duration of resection through the 13.87 kb distance between the HOcs and the distal end of the repetitive LacO array, and are used to compute resection rate (kb/hr) for individual cells. (C) Representative resection-deficient *exo1*Δ cell that that loads on-target Rad52-mCherry but does not lose the LacO/LacI-GFP focus. Because resection of the LacO array is too slow to be completed during the window of data acquisition, we are not able to quantify the rate of resection in *exo1*Δ cells. (D and E) Quantification of LacO/LacI-GFP focus and Rad52-mCherry focus intensities over time for the cells shown in (B). The arrow and "M" show the time of mitotic division, which leads to a decrease in GFP intensity. Quantification used full Z-stack images (not maximum intensity projections shown in B) at subpixel resolution with background normalization. See Methods for more details. (F) Single-cell measurements of resection rate using the LacO resection assay. Horizontal red bars mark the median resection rate for each genotype. *exo1*Δ rates cannot be determined (N.D.) because resection through the LacO array does not complete within 5 hrs of data acquisition. p-values shown are from pairwise two-tailed t-tests, using a Bonferroni correction for multiple comparisons. Number of biological replicates and counts of analyzed cells can be found in Supplementary file 2.

Figure 1–figure supplement 1.
(A) Map of LacO array integration and HO cut cite integration in *S. pombe* (Chr II: 3,442,981 and Chr II: 3,446,249, respectively) drawn to scale (note: only 6 of the ~256 LacO repeats are shown).
(B) Verification of LacO array length after integration into *S. pombe* by HincII digest and Southern blot with a LacO-targeted probe. Circled lanes: (i) MKSP1381: the *S. pombe* strain with a complete 10.3 kb LacO array integration used to derive all LacO assay strains used in this study; (ii) MKSP1173: a strain where the LacO array contracted upon integration to < 5 kb (not used for LacO resection assay); (iii) pSR10_ura4_10.3kb and (iv) pSR10_ura4_5.6kb are plasmids with LacO arrays of known size. Linearized pSR10_ura4_10.3kb was integrated into *S. pombe*.

Figure 1–figure supplement 2.
(A-D) Additional examples of the LacO resection assay in individual WT cells. As in Figure 1B, the blue annotations denote the starting point of resection (first frame with a detectable Rad52 focus, which formation shortly after resection begins) and the end point (first frame with total loss of the LacO/LacI-GFP focus) of individual resection events. These start/end frames mark the total duration of resection through the 13.87 kb distance between the HOcs and the distal end of the repetitive LacO array, and are used to compute resection rate (kb/hr) for individual cells.
(A', B') Additional examples of the subpixel quantification of focus intensity for (A) and (B), respectively, as described in Figure 1C,D and in the Methods.

Figure 1–figure supplement 3.
(A) qPCR using primers spanning the HO cut site to monitor the efficiency DSB induction efficiency at 180 minutes after HO induction by Uracil addition. (B) Diagram of the qPCR assay. Resection at the HO-induced DSB generates ssDNA through the ApoI site 168 nt from the HO cut cite, protecting from ApoI digestion. (C) Kinetics of resection assessed by qPCR and Rad52 loading. Percentage of chromosomes that have undergone 168 nt of resection at the HO cut cite over time, measured by the ApoI-site protection-based qPCR assay (bars, left y-axis). Error bars show SEM for three technical qPCR replicates. Corresponding data for Rad52-mCherry focus formation in the LacO resection assay (red line, right y-axis). (D-G) Representative images showing that LacO arrays as short as 1kb in length are easily detectible under these imaging conditions. (C) is an image of LacO/LacI-GFP foci for a 10.3 kb LacO array (the same one used in the resection assay throughout). (D) is a comparision showing a still readily visible 1kb LacO array, whereas (E) is a cell with LacI-GFP expression but no LacO array, showing the pan-nuclear background signal if LacI-GFP is allowed to diffuse throughout the nucleus without LacO binding. (C-D) are maximum intensity Z projections with similar exposure and contrast. (F) Is a control strain without any LacI-GFP expression that is imaged under longer exposure conditions and shown as a single Z slice in the middle of the cells to highlight the very faint autofluorescence in the cytoplasm but absent in the nucleus that is characteristic of fluorescent microscopy in *S. pombe*. Scale bars = 5 μm. (H) The rate of resection is equivalent on both the LacO and non-LacO sides of the HOcs in WT cells. Average long-range resection at the population level assessed by an orthogonal qPCR assay that quantifies the percentage of chromosomes that have undergone resection through ApoI sites at various distances from the HOcs. Resection to single-stranded DNA through an ApoI site protects that site from digestion by the double-strand specific nuclease, ApoI. Error bars show 95% CIs for three technical qPCR replicates. (I) Resection rates, measured by a population-based qPCR assay, at various distances from the HOcs in *exo1*Δ and *ctp1*Δ cells 90 minutes after HO induction by Uracil addition. Resection to single-stranded DNA through an ApoI site at the indicated positions relative to the HO cut site protects that site from digestion by the double-strand specific nuclease, ApoI. Error bars show 95% CIs for three technical qPCR replicates. (J) On-target Rad52-mCherry foci form at reduced rates in *exo1*Δ cells. p-value shown is from pairwise two-tailed t-tests, using a

Bonferroni correction for multiple comparisons. Number of biological replicates and counts of analyzed cells can be found in Supplementary file 2.

Figure 2. Loss of Rev7 causes an increase in long-rage resection comparable to the loss of Crb2.
(A) Representative timeseries of a resection through the LacO array in a single *rev7*Δ cell. Blue annotations mark the beginning and end of individual resection events, as in Figure 1B.
(B) Subpixel quantification of focus intensity for the *rev7*Δ cell DSB event shown in (A). Quantification is as described in Figure 1C,D and in the Methods.
(C) Single-cell measurements of resection rate using the LacO resection assay. Horizontal red bars mark the median resection rate for each genotype. p-values shown are from pairwise two-tailed t-tests, using a Bonferroni correction for multiple comparisons. Number of biological replicates and counts of analyzed cells can be found in Supplementary file 2.
(D) The long-range rate of resection *rev7*Δ and WT, measured with an ApoI protection qPCR assay on a population level. ApoI cut site distances from the HOcs are indicated and shown in the diagram at right. Error bars show 95% CIs for at least three technical qPCR replicates across 2 or more biological replicates.
(E) Similar to D, qPCR primers spanning the HOcs itself are used to monitor the efficiency of the HO cutting to form DSBs at 180 minutes after HO induction by Uracil addition.

Figure 2–figure supplement 1.
(A-D) Additional examples of rapid long-range resection through the LacO array in *rev7*Δ cells. Blue annotations mark the beginning and end of individual resection events, as in Figure 1B.
(A'-D') Corresponding subpixel quantification of focus intensity, as described in Figure 1D,E and in the Methods.

Figure 3. Rev7 and Crb2 act through the RecQ helicase, Rqh1, and not Exo1, to inhibit long-range resection.
(A) Epistasis analysis of long-range resection rates from single-cell measurements. *exo1*Δ rates cannot be determined (N.D.) because resection through the LacO array does not complete within 5 hrs of data acquisition. Red bars show median resection rates and p-values are from pairwise two-tailed t-tests, using a Bonferroni correction for multiple comparisons (significant comparisons shown in green; see text).
(B,C) Very rapid resection through the 10.3 kb LacO array is common in *crb2*Δ*exo1*Δ (B) and *rev7*Δ*exo*Δ (C) cells, in contrast to *exo1*Δ single mutants that do not completely resect the LacO array within 5 hrs (see Figure 1D). (D) Growth assay on rich media with and without camptothecin. Loss of Rev7 can rescue the severe growth defect of *exo1*Δ cells.

Figure 3–figure supplement 1.

Additional examples of very rapid resection through the LacO array in individual *crb2*Δ*exo1*Δ (A) and *rev7*Δ*exo*Δ (B) cells.

Figure 3–figure supplement 2.
(A) Diagram of exactly and non-exactly determined resection events. The LacO resection assay is limited to 5 hrs due to photobleaching. Thus, many resection events will start before the first frame or end after the last frame of data acquisition (red). Longer durations (slower resection) are more likely to extend past the first or last frame, and the lower threshold for exactly determined resection rates is 2.8 kb/hr for 5 hrs of data acquisition.
(B) Duration of individual resection events through the 10.3 kb LacO array (secondary y-axis shows resection rate). Minimum possible durations are shown in red. Of particular note, many slow *crb2*Δ*rqh1*Δ events have longer durations (slower rates) and thus they cannot be exactly determined. See Supplementary file 2 for number of biological replicates and counts of analyzed cells.

Supplementary file S1: Strains used in this study. These strains were for all LacO resection assay experiments in Figures 1-4 unless otherwise noted. P_{Dis1}-GFP-LacI-NLS is derived from Shimada et al. 2003. LacO integrations were performed as described in Leland and King, 2014. Strains containing the RMCE $P_{urg1lox}$ expression system were derived from Watson et al., 2011.

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Strain	Description	Complete Genotype
MKSP1381	Original LacO integration used to generate all other strains in this study (Figure S1B)	*h- leu1-32 ura4-D18 his7+:P_{Dis1}-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Cut11-mCherry::natMX6*
MKSP1173	Example of LacO array contracting upon integration (Figure S1B)	*h- leu1-32 ura4-D18 his7+:P_{Dis1}-GFP-LacI-NLS Leu2::Ura4-LacO<5kb Rad26-mCherry::natMX6*
MKSP2123	WT LacO resection assay	*h- leu1-32 ura4-D18 his7+:P_{Dis1}-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-bleMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6*
MKSP1914	*exo1*Δ LacO resection assay	*h- leu1-32 ura4-D18 his7+:P_{Dis1}-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::natMX6 urg1::RMCE-kanMX6 exo1::bleMX6*
MKSP2501	*rqh1*Δ LacO resection assay	*h- leu1-32 ura4-D18 his7+:P_{Dis1}-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 rqh1::kanMX6*
MKSP2244	*crb2*Δ LacO resection assay	*h- leu1-32 ura4-D18 his7+:P_{Dis1}-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 crb2::kanMX6*

MKSP2476	*crb2*Δ*exo1*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 crb2::kanMX6 exo1::bleMX6*
MKSP2479	*crb2*Δ*rqh1*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 crb2::kanMX6 rqh1::kanMX6*
MKSP2149	*rev7*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 rev7::natMX6*
MKSP2392	*rev7*Δ*exo1*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 rev7::natMX6 exo1::bleMX6*
MKSP2456	*rev7*Δ*rqh1*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 rev7::natMX6 rqh1::kanMX6*
MKSP2245	*rev7*Δ*crb2*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 rev7::natMX6 crb2::kanMX6*
MKSP2262	*rev3*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 rev3::kanMX6*
MKSP2477	*ctp1*Δ LacO resection assay	*h+ leu1-32 ura4-D18 his7+:*P_{Dis1}*-GFP-LacI-NLS ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6 Rad52-mCherry::bleMX6 urg1::RMCE-bleMX6 ctp1::kanMX6*

Supplementary file S2: Number of biological replicates, cell cycles, and DSB events analyzed for each genotype. These counts pertain to all data from the resection assay in Figures 1-3.

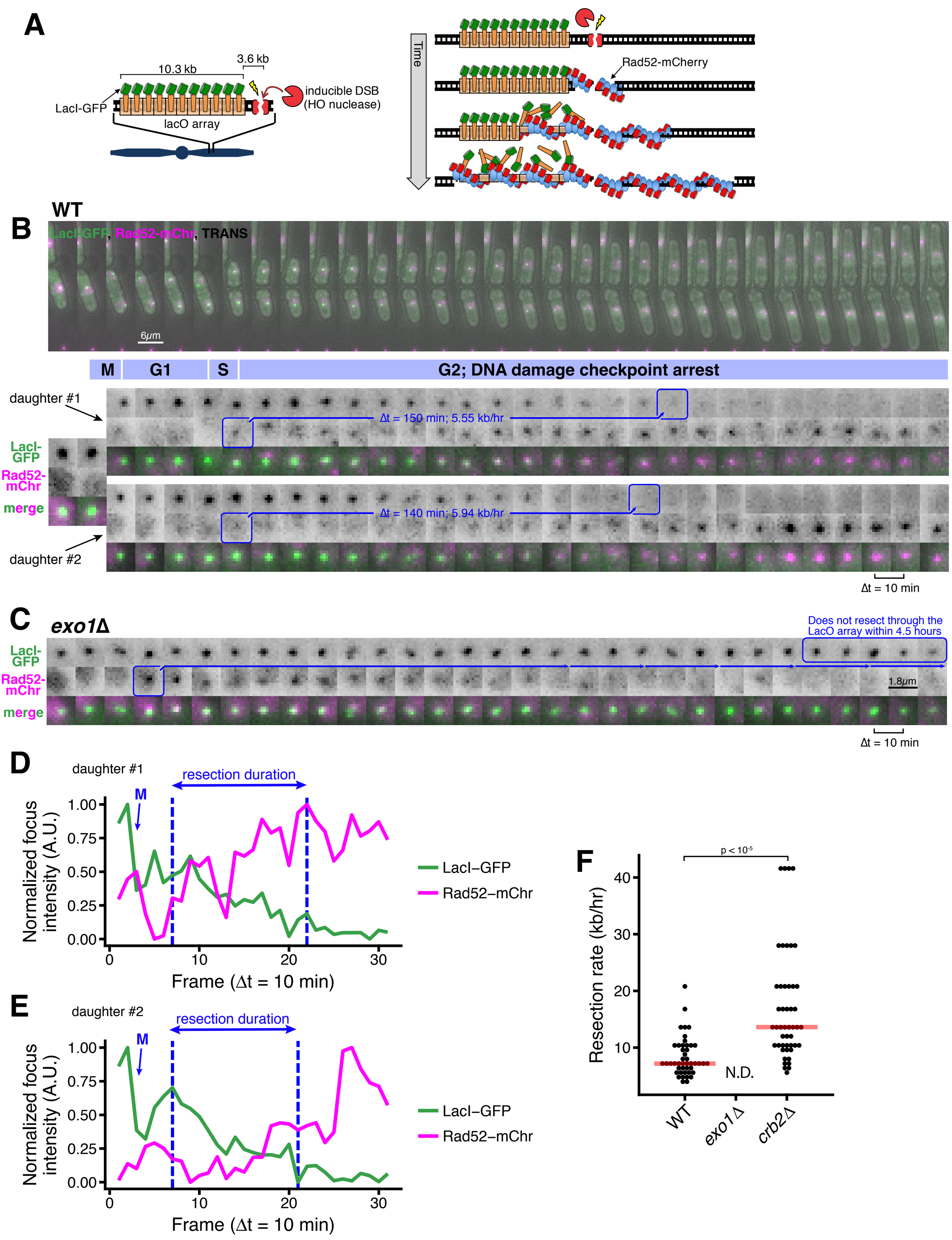
Genotype	Number of biological replicates	Total cell cycles observed	Number of dead or sick cells*	Number of daughter cell cycles whose parent had an on-target DSB*	Observed cell cycles valid for %Rad52 calculations	Number of Rad52 focus formation events	Number of resection events that are exactly determined
WT	6	1049	27	52	486	77	45
*exo1*Δ	4	945	13	2	508	8	0
*rqh1*Δ	6	991	61	2	556	49	32
*crb2*Δ	5	1045	78	164	425	62	48
*crb2*Δ*exo1*Δ	4	1122	42	128	579	64	44
*crb2*Δ*rqh1*Δ	3	1207	73	70	714	83	27
*rev7*Δ	5	3182	190	24	1851	112	54
*rev7*Δ*exo1*Δ	8	1251	70	2	702	26	11
*rev7*Δ*rqh1*Δ	7	1311	57	10	809	130	50
*rev7*Δ*crb2*Δ	3	1122	43	88	477	65	42
*rev3*Δ	2	637	21	24	275	43	31

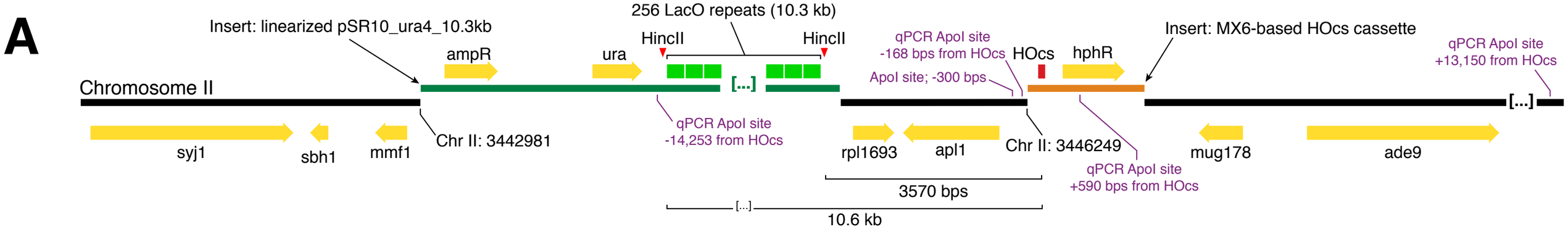
* Cell cycles that were excluded from the analyses of resection rate in Figures 1F, 2C, 3A.

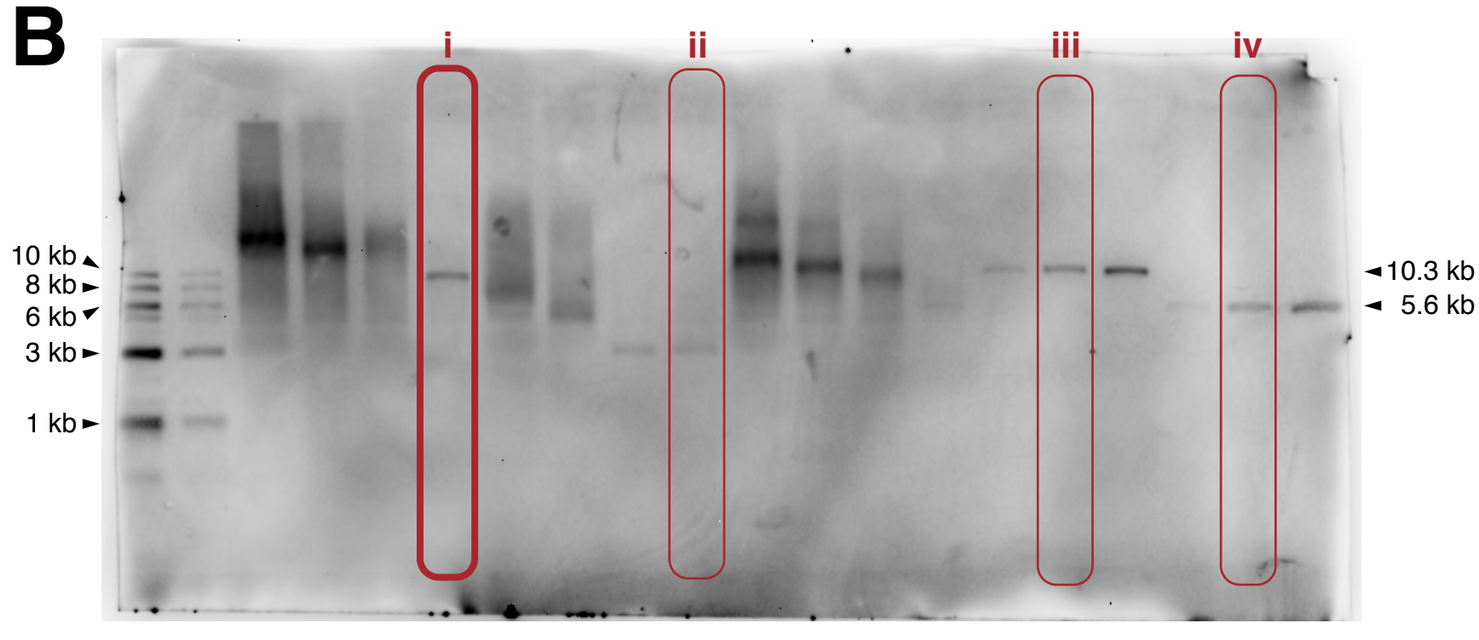
Supplementary file S3. Primers sets used in qPCR measurements of resection.

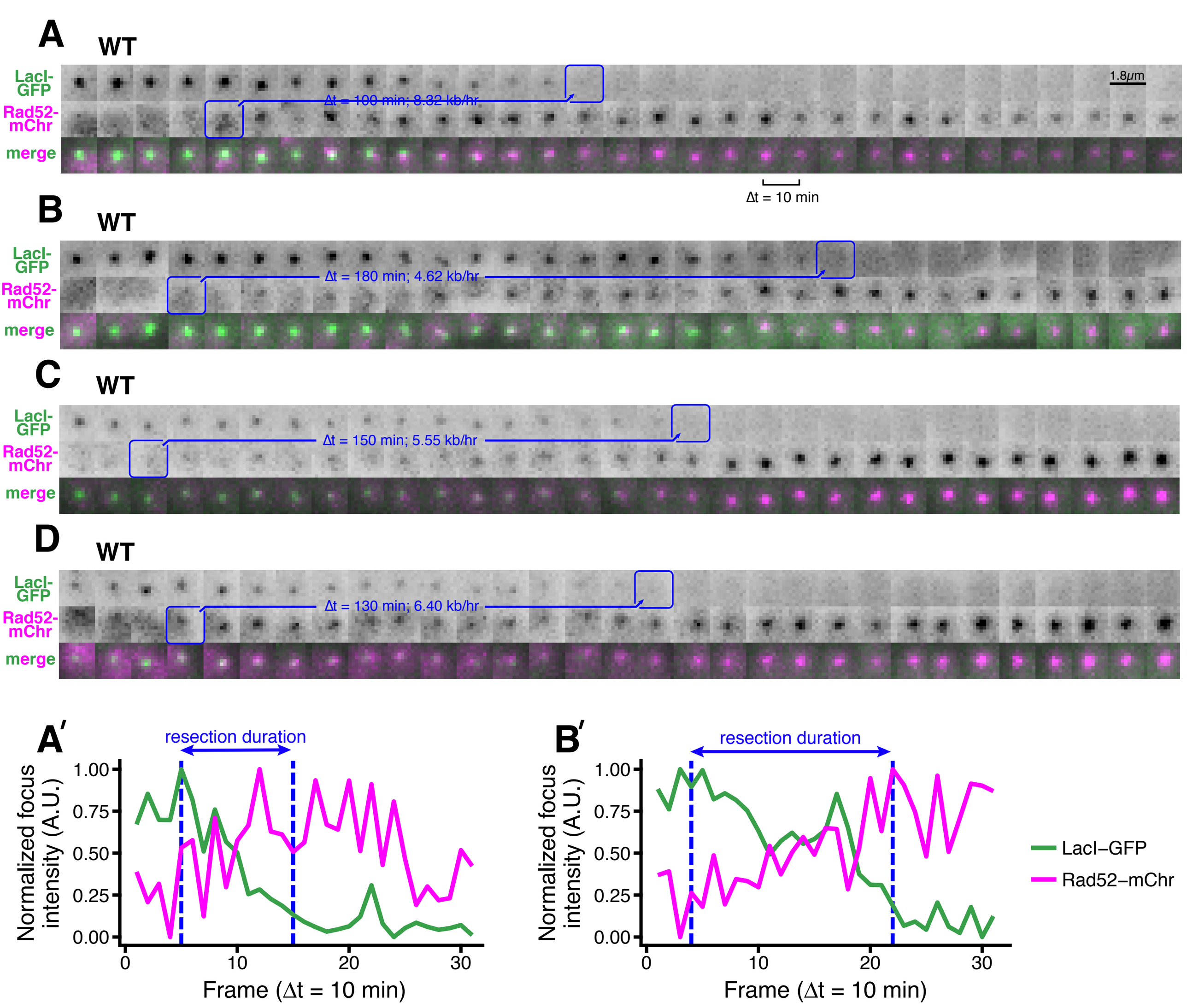
ApoI site position relative to HOcs at Mmf1	Forward primer (5'-3')	Reverse primer (5'-3')
-168v1	ATCACCGATGGAAACAGTGAACTCAT	GCTGATTTGCTAGCAGTCTTCAGCTC
-168v2	GGAAACAGTGAACTCATATCATATCCA	GCAGTCTTCAGCTCAGATAATAAGG
-300	AGAAACTTTTACAAACCTCGCGT	TGAGTTCACTGTTTCCATCGGT
-3023	AGCTTGTAATAATCGATGCCAAAGG	GTTGAGGCTAAACGACCCATT
+13150	GCCAGCTATGACAAAAGGCC	TAGGATCGTAGTTGCCAGCG
-14253	AGCTGGTTGGAAGGCATATCA	CGCAAACAAGGCATCGACTTT
Control primers		
Ncb1v0	AGACGTATTTGAGTGATAGTGCTCGCTGC	CGTCCTTCCGATGTTGCTTTAACGCATACTC
Ncb1v1	GCCGCTGAACACATTATTAAAGC	CGCCACTTCCAAAGCTTCAG
spanning HOcs@Mmf1	GGAGGCCCAGAATACAGTTTC	CGCACGTCAAGACTGTCAAG

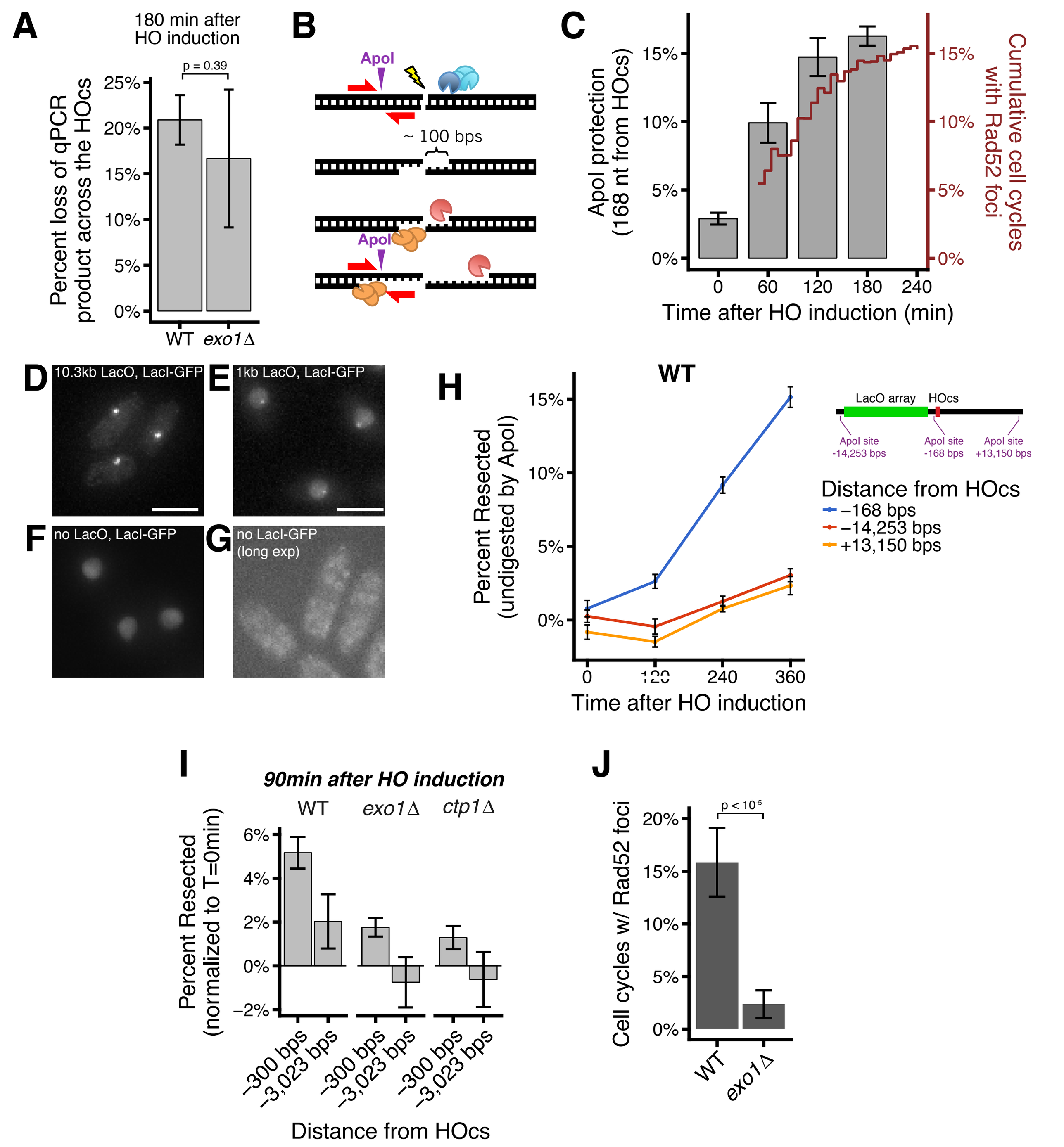
The -168v1 and Ncb1v0 primers were used in panel Figure 1 – figure supplement 3C. For all other plots, the -168v2 and Ncb1v1 primers were used.

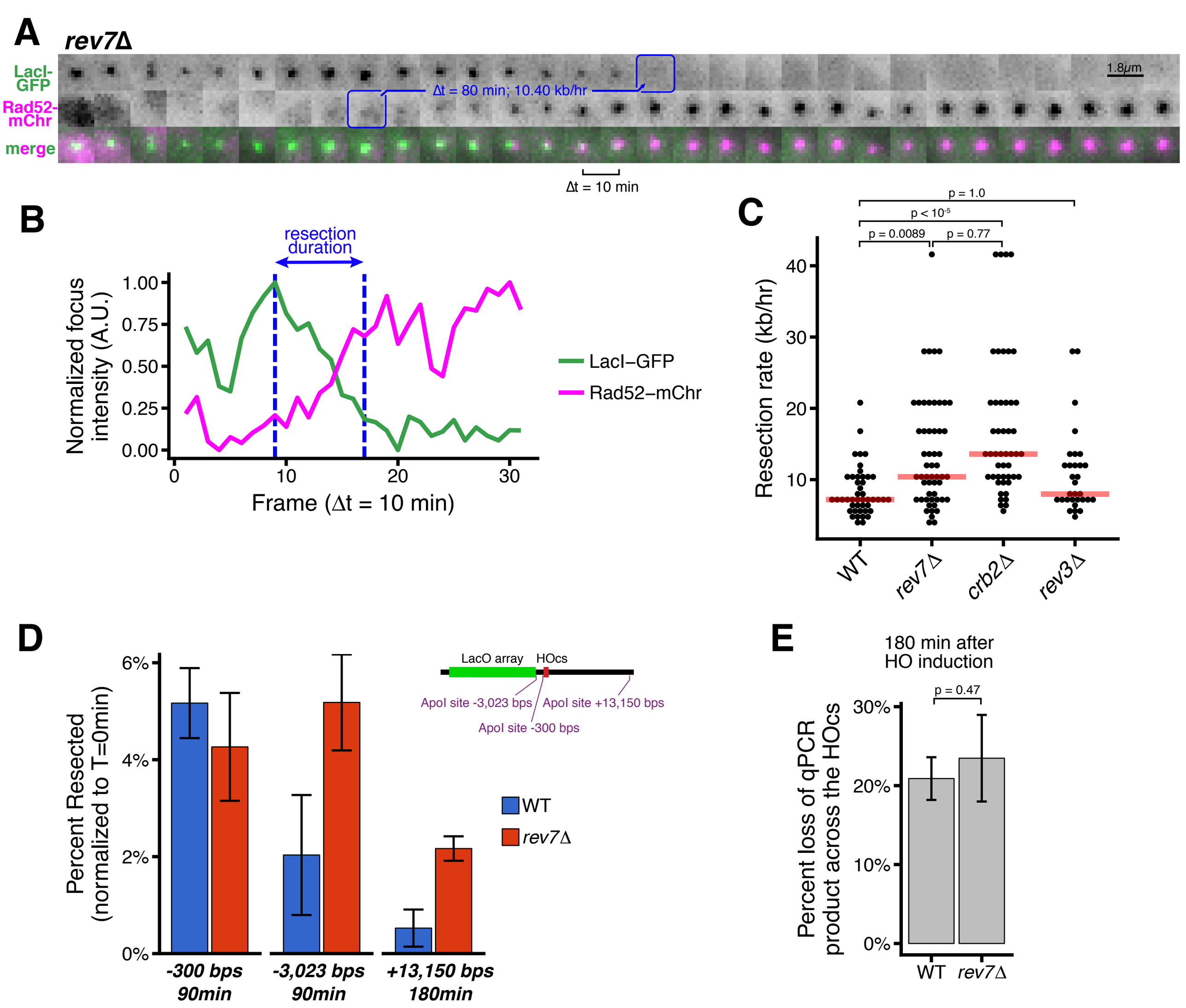

A
10.3 kb
3.6 kb
LacI-GFP
lacO array
inducible DSB
(HO nuclease)
Time
Rad52-mCherry
B
WT
LacI-GFP, Rad52-mChr, TRANS
6μm
M
G1
S
G2; DNA damage checkpoint arrest
daughter #1
LacI-GFP
Rad52-mChr
merge
Δt = 150 min; 5.55 kb/hr
Δt = 140 min; 5.94 kb/hr
daughter #2
Δt = 10 min
C
exo1Δ
Does not resect through the LacO array within 4.5 hours
1.8μm
Δt = 10 min
D
daughter #1
resection duration
M
Normalized focus intensity (A.U.)
Frame (Δt = 10 min)
LacI–GFP
Rad52–mChr
E
daughter #2
resection duration
M
Normalized focus intensity (A.U.)
Frame (Δt = 10 min)
LacI–GFP
Rad52–mChr
F
p < 10-5
Resection rate (kb/hr)
N.D.
WT
exo1Δ
crb2Δ

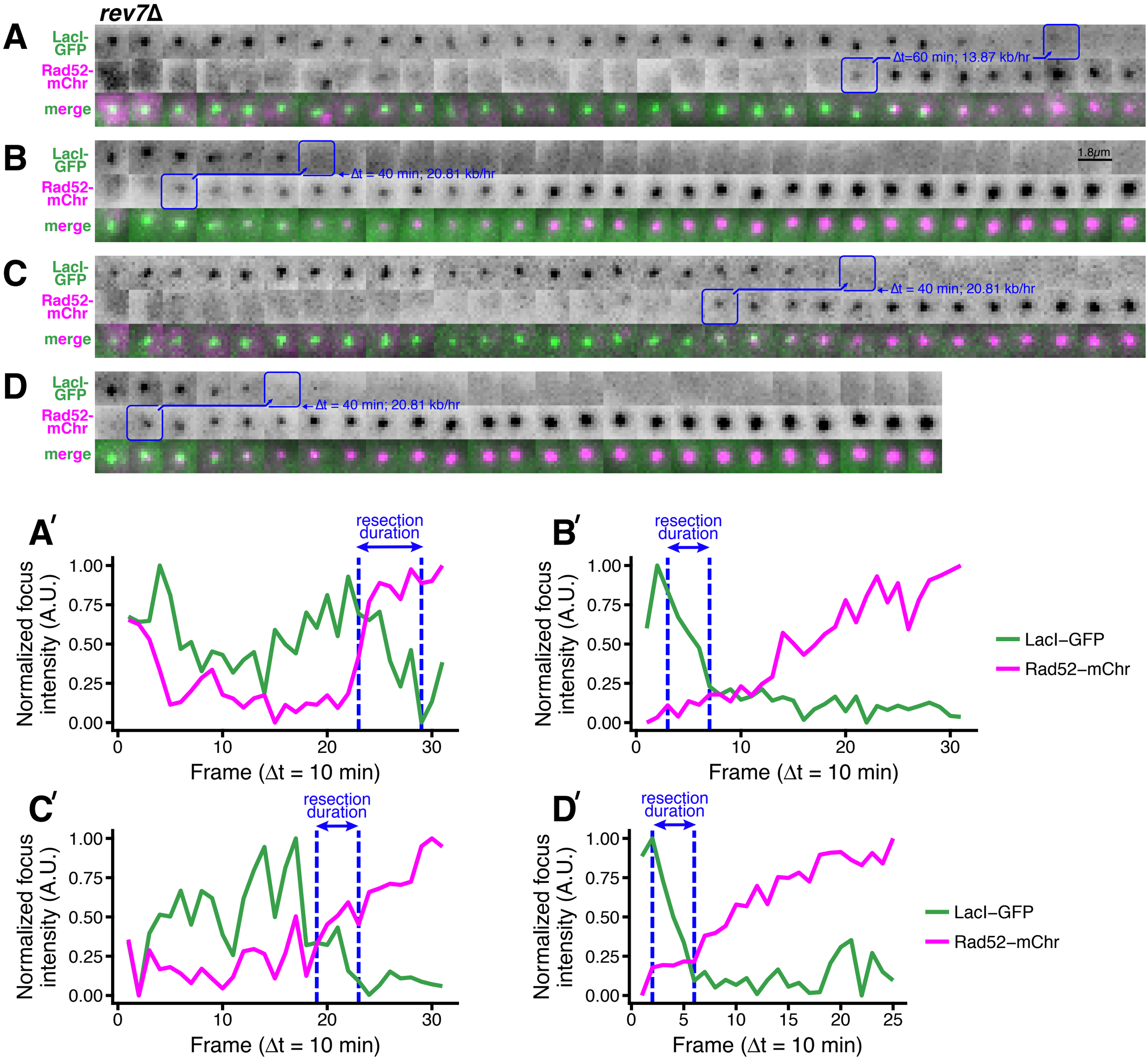

A
Insert: linearized pSR10_ura4_10.3kb
256 LacO repeats (10.3 kb)
HincII
HincII
ampR
ura
[...]
qPCR ApoI site
-168 bps from HOcs
HOcs
hphR
Insert: MX6-based HOcs cassette
qPCR ApoI site
+13,150 from HOcs
Chromosome II
ApoI site; -300 bps
[...]
qPCR ApoI site
-14,253 from HOcs
Chr II: 3442981
syj1
sbh1
mmf1
rpl1693
apl1
Chr II: 3446249
mug178
ade9
qPCR ApoI site
+590 bps from HOcs
3570 bps
[...]
10.6 kb

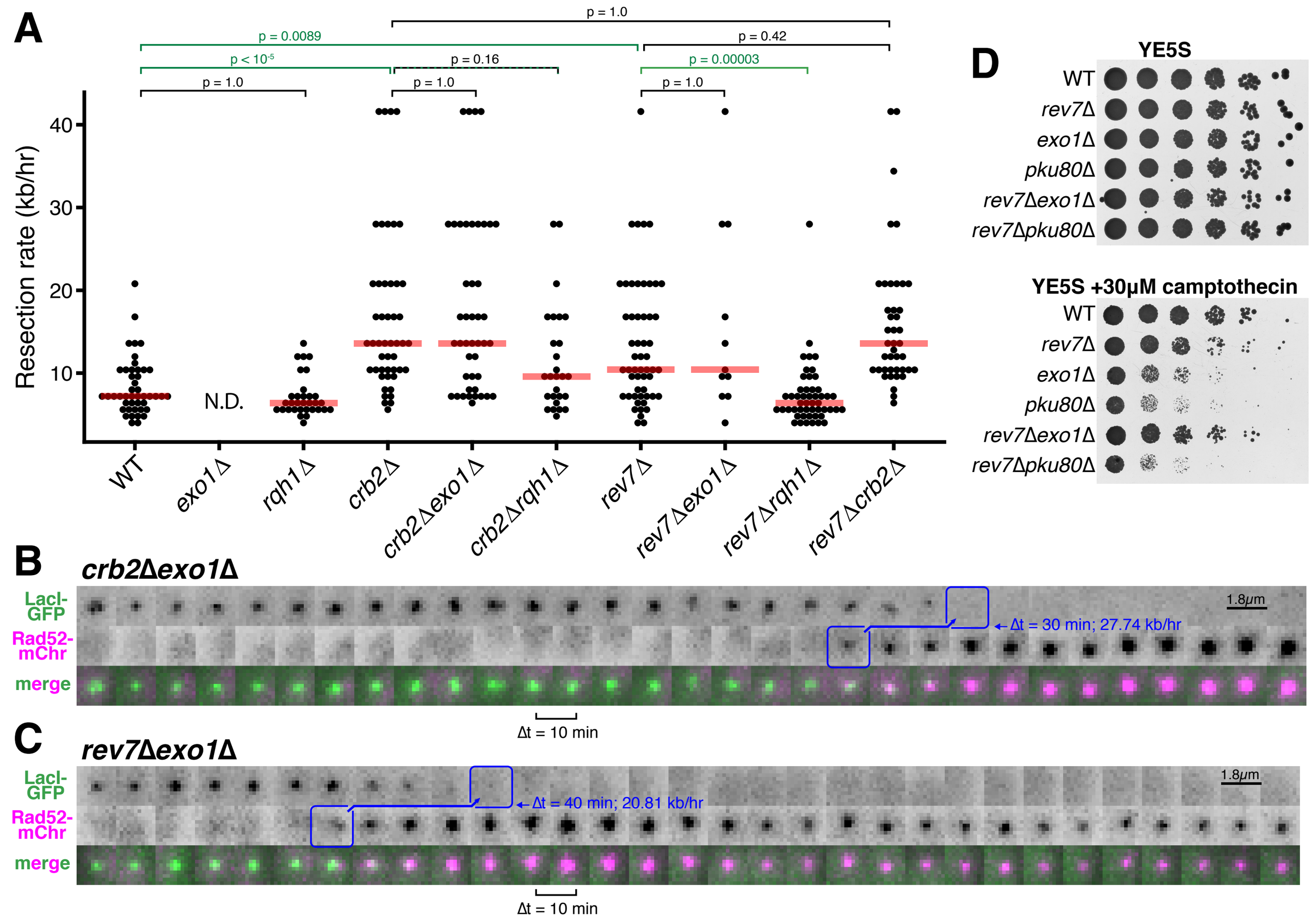

B
i
ii
iii
iv
10 kb
8 kb
6 kb
3 kb
1 kb
10.3 kb
5.6 kb

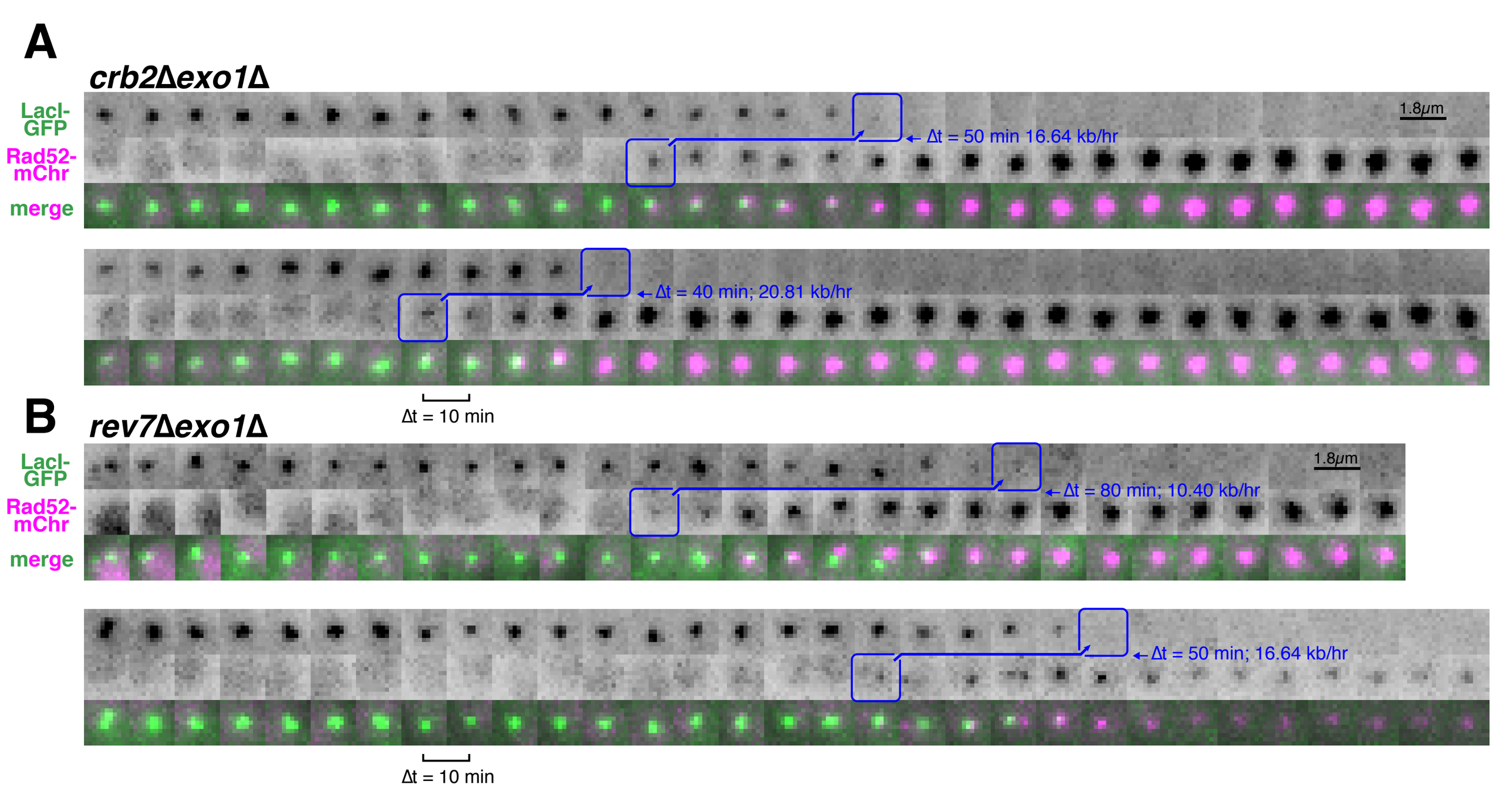

A
WT
LacI-GFP
Rad52-mChr
merge
Δt = 100 min; 8.32 kb/hr
1.8μm
Δt = 10 min
B
WT
LacI-GFP
Rad52-mChr
merge
Δt = 180 min; 4.62 kb/hr
C
WT
LacI-GFP
Rad52-mChr
merge
Δt = 150 min; 5.55 kb/hr
D
WT
LacI-GFP
Rad52-mChr
merge
Δt = 130 min; 6.40 kb/hr
A′
resection duration
Normalized focus intensity (A.U.)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Frame (Δt = 10 min)
B′
resection duration
Normalized focus intensity (A.U.)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Frame (Δt = 10 min)
LacI–GFP
Rad52–mChr

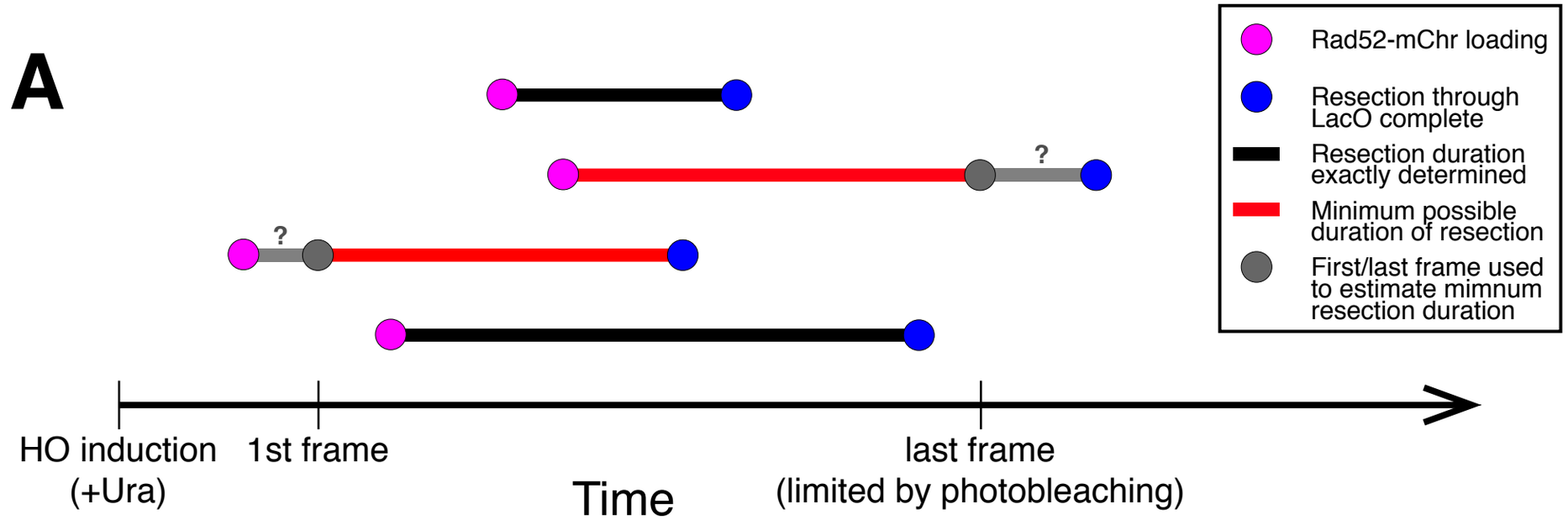

A
180 min after HO induction
p = 0.39
Percent loss of qPCR product across the HOcs
25%
20%
15%
10%
5%
0%
WT
exo1Δ
B
Apol
~ 100 bps
Apol
C
Apol protection (168 nt from HOcs)
Cumulative cell cycles with Rad52 foci
15%
10%
5%
0%
0
60
120
180
240
Time after HO induction (min)
D
10.3kb LacO, LacI-GFP
E
1kb LacO, LacI-GFP
F
no LacO, LacI-GFP
G
no LacI-GFP (long exp)
H
WT
Percent Resected (undigested by Apol)
15%
10%
5%
0%
0
120
240
360
Time after HO induction
LacO array
HOcs
Apol site -14,253 bps
Apol site -168 bps
Apol site +13,150 bps
Distance from HOcs
–168 bps
–14,253 bps
+13,150 bps
I
90min after HO induction
WT
exo1Δ
ctp1Δ
Percent Resected (normalized to T=0min)
6%
4%
2%
0%
–2%
–300 bps
–3,023 bps
Distance from HOcs
J
p < 10-5
Cell cycles w/ Rad52 foci
20%
15%
10%
5%
0%
WT
exo1Δ

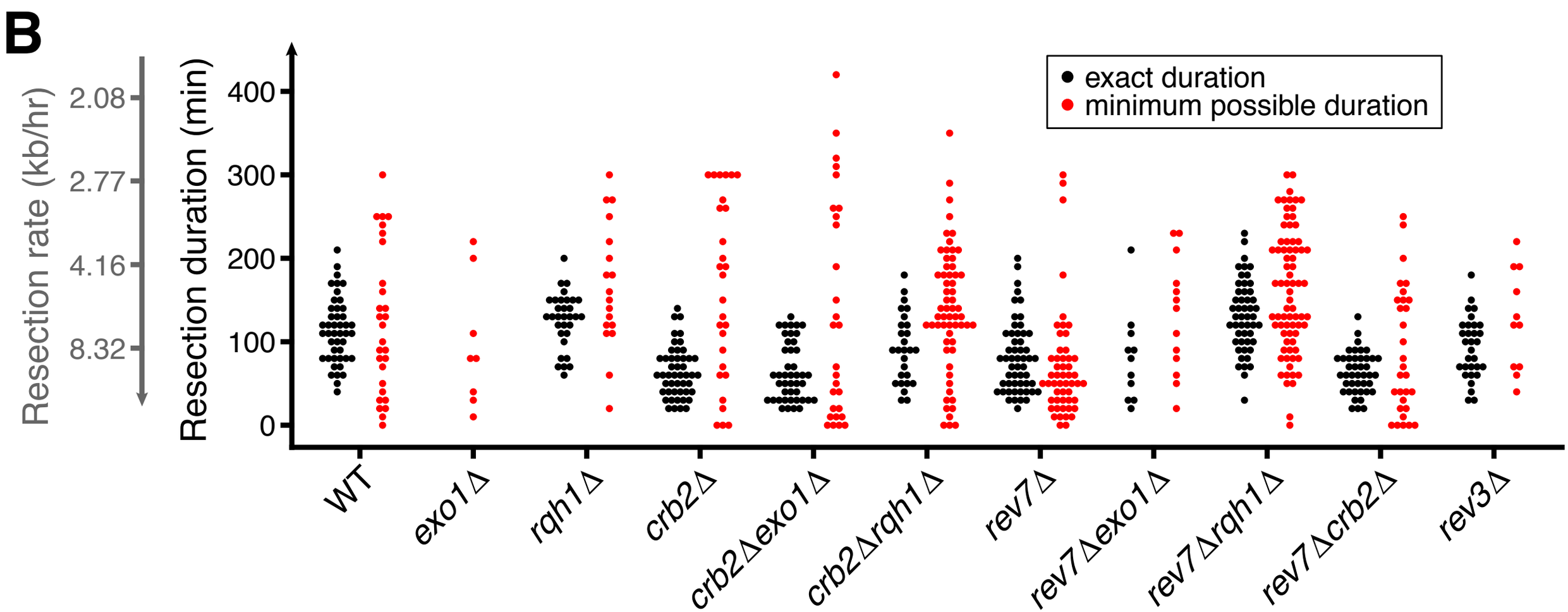

A
rev7Δ
LacI-GFP
Rad52-mChr
merge
Δt = 80 min; 10.40 kb/hr
1.8μm
Δt = 10 min
B
resection duration
Normalized focus intensity (A.U.)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
Frame (Δt = 10 min)
LacI–GFP
Rad52–mChr
C
p = 1.0
p < 10-5
p = 0.0089
p = 0.77
Resection rate (kb/hr)
40
30
20
10
WT
rev7Δ
crb2Δ
rev3Δ
D
Percent Resected (normalized to T=0min)
6%
4%
2%
0%
LacO array
HOcs
ApoI site -3,023 bps
ApoI site +13,150 bps
ApoI site -300 bps
WT
rev7Δ
-300 bps 90min
-3,023 bps 90min
+13,150 bps 180min
E
180 min after HO induction
p = 0.47
Percent loss of qPCR product across the HOcs
30%
20%
10%
0%
WT
rev7Δ


rev7Δ
A
LacI-GFP
Rad52-mChr
merge
Δt=60 min; 13.87 kb/hr
B
Δt = 40 min; 20.81 kb/hr
1.8μm
C
Δt = 40 min; 20.81 kb/hr
D
Δt = 40 min; 20.81 kb/hr
A′
B′
C′
D′
resection duration
Normalized focus intensity (A.U.)
Frame (Δt = 10 min)
LacI–GFP
Rad52–mChr


A
p = 1.0
p = 0.0089
p = 0.42
p < 10-5
p = 0.16
p = 0.00003
p = 1.0
p = 1.0
p = 1.0
Resection rate (kb/hr)
40
30
20
10
N.D.
WT
exo1Δ
rqh1Δ
crb2Δ
crb2Δexo1Δ
crb2Δrqh1Δ
rev7Δ
rev7Δexo1Δ
rev7Δrqh1Δ
rev7Δcrb2Δ
D
YE5S
WT
rev7Δ
exo1Δ
pku80Δ
rev7Δexo1Δ
rev7Δpku80Δ
YE5S +30μM camptothecin
WT
rev7Δ
exo1Δ
pku80Δ
rev7Δexo1Δ
rev7Δpku80Δ
B
crb2Δexo1Δ
LacI-GFP
Rad52-mChr
merge
1.8μm
Δt = 30 min; 27.74 kb/hr
Δt = 10 min
C
rev7Δexo1Δ
LacI-GFP
Rad52-mChr
merge
1.8μm
Δt = 40 min; 20.81 kb/hr
Δt = 10 min


A
crb2Δexo1Δ
LacI-GFP
Rad52-mChr
merge
1.8μm
Δt = 50 min 16.64 kb/hr
Δt = 40 min; 20.81 kb/hr
Δt = 10 min
B
rev7Δexo1Δ
LacI-GFP
Rad52-mChr
merge
1.8μm
Δt = 80 min; 10.40 kb/hr
Δt = 50 min; 16.64 kb/hr
Δt = 10 min


A
Rad52-mChr loading
Resection through LacO complete
Resection duration exactly determined
Minimum possible duration of resection
First/last frame used to estimate mimnum resection duration
?
?
HO induction (+Ura)
1st frame
Time
last frame (limited by photobleaching)


B
Resection rate (kb/hr)
2.08
2.77
4.16
8.32
Resection duration (min)
400
300
200
100
0
exact duration
minimum possible duration
WT
exo1Δ
rqh1Δ
crb2Δ
crb2Δexo1Δ
crb2Δrqh1Δ
rev7Δ
rev7Δexo1Δ
rev7Δrqh1Δ
rev7Δcrb2Δ
rev3Δ