Separable Roles for Exonuclease I in Meiotic DNA Double-Strand Break Repair

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Abstract

Exo1 is a member of the Rad2 protein family and possesses both 5'-3' exonuclease and 5' flap endonuclease activities. In addition to performing a variety of functions during mitotic growth, Exo1 is also important for the production of crossovers during meiosis. However, its precise molecular role has remained ambiguous and several models have been proposed to account for the crossover deficit observed in its absence. Here, we present physical evidence that the nuclease activity of Exo1 is essential for normal 5'-3' resection at the Spo11-dependent *HIS4* hotspot in otherwise wild-type cells. This same activity was also required for normal levels of gene conversion at the locus. However, gene conversions were frequently observed at a distance beyond that at which resection was readily detectable arguing that it is not the extent of the initial DNA end resection that limits heteroduplex formation. In addition to these nuclease-dependent functions, we found that an *exo1-D173A* mutant defective in nuclease activity is able to maintain crossing-over at wild-type levels in a number of genetic intervals, suggesting that Exo1 also plays a nuclease-independent role in crossover promotion.

Keywords: *EXO1*, meiosis, recombination, resection, double-strand break.

1. Introduction

During meiosis, recombination is initiated by the formation of DNA double-strand breaks (DSBs) catalysed by the Spo11 protein [1]. Once DSB formation has been achieved, Spo11 must be removed from the DNA ends in order for repair to commence. This removal takes place via endonucleolytic cleavage of the DNA to release Spo11-oligonucleotide complexes [2]. Further DNA end resection then follows resulting in longer 3' overhanging strands [3, 4]. These strands are bound by Rad51 and Dmc1 to form the recombinogenic nucleoprotein filaments required to catalyse strand-invasion [5].

DSBs may be repaired to yield either crossovers or non-crossovers and it is thought that the decision as to which repair pathway is utilised is made at or around the point of strand invasion. The stabilisation of a nascent D-loop to form a single-end invasion (SEI) is the first molecularly measurable event that defines the major crossover pathway [6]. Subsequently, double Holliday junctions (dHJs) are produced [7, 8], resolution of which by an as yet unidentified factor(s) yields a crossover. A number of proteins are required for this process [9] including the Msh4/Msh5 heterodimer [10, 11], which is believed to encircle and stabilise D-loop and Holliday junction structures [12] and the Mlh1/Mlh3 heterodimer. Mlh1/Mlh3 are thought to function after Msh4/Msh5 and may be required (either directly or indirectly) for Holliday junction resolution [13-17]. A minority of crossovers also form independently of the Msh4/Msh5 pathway, many of which are dependent upon the structure-specific endonuclease complex Mus81/Mms4 [18-20]. While physically detectable intermediates specific to the non-crossover pathway have not been identified, it is thought likely to proceed via synthesis-dependent strand-annealing [21, 22]. The initiating events in this pathway are similar to those in the crossover pathway; however, a detectable SEI intermediate is not produced. Instead, following DNA synthesis, the invading strand is displaced and anneals to the complementary sequence uncovered by resection on the opposite side of the DSB. DNA synthesis then acts to fill in the remaining gaps, followed by ligation of the DNA ends.

Exo1 (a member of the Rad2/XPG family of nucleases) possesses both 5'-3' exonuclease and 5' flap endonuclease activities [23] and has been shown to be

involved in a wide variety of cellular processes during mitotic growth. In yeasts [24-26], *Drosophila* [27] and mice [28], expression of Exo1 is strongly up-regulated in cells undergoing meiosis suggesting an important conserved role for Exo1 during the recombination process. To date, the most comprehensive analyses concerning the meiotic role of Exo1 have been carried out in the yeast *Saccharomyces cerevisiae*. In this organism, crossing-over was shown to be reduced 1.5-2 fold in *exo1Δ* strains compared to wild type [25, 29, 30]. Consequently, increased levels of meiosis I non-disjunction occurred resulting in the formation of aneuploid spores. Compared to any single mutant, no further reduction in crossing-over was observed in *exo1Δ msh4Δ* or *exo1Δ msh5Δ* double mutants suggesting that Exo1 acts to promote inter-homologue crossing-over via the Msh4/Msh5 pathway [29, 30].

These studies also indicated that deletion of *EXO1* confers allele and/or locus specific effects on gene conversion [29, 30]. While Tsubouchi *et al.* [25] were unable to detect any reduction in gene conversion at *ARG4* or the artificial *HIS4:LEU2* hotspot using recombination assays between heteroalleles, Khazanehdari & Borts [30] reported a significant two-fold reduction in gene conversion at all four alleles of *HIS4* studied. They also noted an approximately two-fold reduction in conversion at two alleles of *MET13* and at one of the three *LEU2* alleles tested. Conversely, Kirkpatrick *et al.* [29] reported no effect on the gene conversion of markers at *HIS4* but did observe a significant reduction at *ARG4*. As gene conversion requires the incorporation of allelic markers into heteroduplex DNA (hDNA) [31], Exo1 may therefore be required for the formation or extension of hDNA tracts. In view of these results and the biochemical function of Exo1 as a nuclease, it has been suggested that Exo1 functions in the resection of meiotic DSBs prior to strand invasion [25, 30]. This has been demonstrated recently at mitotic DSBs [32, 33].

To date, experiments designed to demonstrate a direct role for Exo1 in meiotic resection have relied upon deletion of *DMC1* to aid the visualisation of resection intermediates on Southern blots [25, 34]. In the absence of *DMC1*, breaks are unable to undergo strand invasion and hyper-resected DSBs accumulate [35]. While these studies demonstrated that Exo1 is partially responsible for the hyper-resection observed, it remains unclear as to what extent this reflects normal patterns of

resection. Additionally, there is uncertainty as to how Exo1 acts to promote crossing-over. It has been proposed that decreased resection following DSB formation may reduce the stability of initial strand invasion attempts, resulting in fewer SEIs being formed [30]. However, observations from mice indicate that when Exo1 is absent, meiotic defects only become apparent at a later stage (after the crossover function of Msh4/Msh5)[36, 37]. This argues that a reduction in resection may not account for the crossover deficit observed and that Exo1 may have an alternative critical function during meiosis.

In this work, we aimed to determine whether or not Exo1 is required for normal resection following Spo11-catalysed DSB formation in otherwise wild-type cells. We further sought to test the hypothesis that resection tract length correlates with hDNA formation and ultimately determines the propensity with which a DSB is repaired to yield a crossover. To do this we employed both physical and genetic methods of analysis, focussing upon the natural *HIS4* hotspot, where Exo1 has previously been implicated in DSB processing [30].

2. Materials and Methods

2.1 *Yeast strains and media*

All strains are derivatives of the *Saccharomyces cerevisiae* SK1 isolate [38]. The alleles used have been described previously [19, 39-43] and were introduced by transformation or crossing to existing strains. The *EXO1* ORF was replaced with the *kanMX4* cassette conferring resistance to geneticin as described by Wach *et al.* [44] using the plasmid template pFA6-kanMX4. The *exo1-D173A* mutation was introduced via a PCR-based allele replacement method [45] using plasmid template pEAM71 [46]. All alleles were confirmed by PCR, restriction digest and linkage analysis where appropriate. The *exo1-D173A* mutation was confirmed by DNA sequencing. Full strain genotypes are listed in Tables 1 and 2. The media used have been described previously [47].

2.2 *Liquid sporulation*

For tetrad analysis, haploid strains were mixed and allowed to mate for 4 hours on YPD at 30°C before being replicated to minimal medium (supplemented with 240 µl

of 0.5% (w/v) adenine hemisulphate per plate) to select for diploids and grown overnight at 30°C. For the physical analysis and random spore experiments, pure diploid colonies were used. Sporulation conditions were the same for all experiments. Cultures were grown to saturation in YPD, diluted 1:400 into SPS (supplemented with 40mg/ml adenine hemisulphate) and grown to a cell density of 5.5×10^7 cells/ml. Cells were harvested by centrifugation, washed once in 2% (w/v) potassium acetate (pH 7) and finally resuspended in 2% (w/v) potassium acetate (pH 7) in 1.5 x the volume of SPS used. These cultures were transferred to a 2.8 L baffled flask and incubated with vigorous shaking at 23°C. 23°C was chosen as the sporulation temperature as gene conversion and DSBs at the *HIS4* hotspot have been shown to be temperature-dependent, occurring at a higher frequency at lower temperatures [47, 48]. For DNA analysis, aliquots were removed (25 ml up to 5 hours and 15 ml thereafter) and 10% (w/v) sodium azide added to a final concentration of 0.1% (w/v) before harvesting by centrifugation and storing of the cell pellets at -80°C. For tetrad and random spore analysis, cell samples were taken after 24 hours sporulation and processed immediately.

2.3 Genetic analysis

Tetrad dissection and analysis was carried out as described previously [49]. Crossing-over and gene conversion (6:2, 2:6 segregation) was assessed in tetrads with four viable spores. Tetrads exhibiting three or more non-Mendelian segregation events (any segregation pattern other than 4:4) were scored as false tetrads and excluded from further analysis. Map distances in centiMorgans (cM) were calculated as described by Perkins *et al.* [50]. Random spores were prepared according to the protocol outlined by Jessop *et al.* [51] and appropriate dilutions were plated out onto two types of media: synthetic complete and synthetic complete lacking histidine, both of which were supplemented with 60 µg/ml canavanine and 10 µg/ml cyclohexamide. The double drug selection ensured that only haploid meiotic products were analysed. Spores were incubated at 30°C for 3 days before individual colonies were patched onto YPD. Following growth overnight at 30°C, the patches were replicated to YPD supplemented with either 300 µg/ml hygromycin B or 100 µg/ml nourseothricin. After a further overnight incubation at 30°C, patches were scored for their ability to grow on these plates. Statistical comparisons were made using either the G-test of

homogeneity or the *t*-test as appropriate and the Dunn-Sidak correction for multiple testing was applied [52]. This guards against type 1 errors but increases the probability of type 2 errors.

2.4 Loss of restriction site analysis by qPCR

In order to measure resection intermediates without having to rely upon the use of the *dmc1Δ* mutation or an artificial hotspot, we modified an assay developed by Hodgson *et al.* [53] that measures single-stranded DNA (ssDNA). This assay uses restriction enzyme digestion to cleave all double-stranded DNA (dsDNA) at a specific target site, followed by quantitative PCR (qPCR) using a pair of oligonucleotide primers designed to anneal each side of the enzyme cut site. Any DNA present at the target site in single-stranded form (such as that produced by 5'-3' resection) becomes refractory to enzyme cleavage and is therefore able to be amplified in the subsequent PCR step. A second qPCR reaction is directed to the rDNA array. No target sites for any of the restriction enzymes used are present within the rDNA region chosen, enabling it to serve as an internal measure of the amount of DNA per reaction. The oligonucleotide sequences used for these experiments are given in Table 3.

DNA was extracted from cells using a modified version of the CTAB method [54] in which hexamine cobalt chloride was omitted from all solutions and the DNA resuspended in 100-150 µl of 1x TE. For each time point, approximately 750 ng of genomic DNA was digested with 25 units of enzyme for 30 mins at 37°C in a total volume of 50 µl. Following digestion, 20 µl of the digest was diluted into 80 µl of ice-cold water, 2 µl of which was added to each qPCR reaction to give approximately 6ng of template DNA per reaction. The qPCR reactions were carried out in a total volume of 10 µl using 1x SensiMix™ SYBR (Bioline) and 400 nM forward and reverse oligonucleotides. At each time point, qPCR was directed to both the target site of interest and the rDNA region. Thermocycling was carried out using a LightCycler® 480 Real-Time PCR System (Roche). PCR reactions were initiated by heat activation of the *Taq* polymerase at 95°C for 10 mins, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate and “no-template” controls for each oligonucleotide pair used were included in every run. Amplicons were between 100 and 150 base pairs in length and primer specificity was

validated by melt-curve analysis. Standard curves were included on every plate and were constructed from a five-point 1 in 10 serial dilution of DNA taken from the 0 hour time point of the time course being analysed (starting from approximately 25 ng template DNA per reaction). Crossing-point (Cp) values were calculated using the automated Cp-calling algorithm provided by the LightCycler® Analysis Software (based on the second derivative maximum method). In general, the mean Cp value of the three replicates was used for subsequent analysis. However, if a single reaction differed from its duplicates by more than one Cp value, it was discarded and the mean Cp value of the remaining two reactions used instead.

Mean Cp values for each triplicate sample were imported into Microsoft Excel and standard curves constructed for both the target site primer pair and rDNA primer pair by plotting the mean Cp values obtained against the $\log^{10}$ of the dilution factor used. A line of best fit was drawn and the slope (m) and y intercept (b) of the line calculated. For each experimental sample, a value for the amount of template DNA present at the start of the PCR (x) at both the target and rDNA loci were obtained by reference to the appropriate standard curve using the formula:

$$x = 10^{\frac{MeanCp - b}{m}}$$

The relative proportions of ssDNA and dsDNA in the sample were then calculated using the equations:

$$\%ssDNA_{MEASURED} = \frac{x_{TARGET}}{x_{rDNA}} \times 100$$

$$\%dsDNA_{MEASURED} = 100 - \left[\frac{x_{TARGET}}{x_{rDNA}} \times 100 \right]$$

In order to compare between different target sites and time courses, it was necessary to normalise the data from each experiment to take account of the efficiency with which digestion of dsDNA had occurred. This was done by reference to the amount of ssDNA measured in the 0 hour time point of that time course (where 0 hour is assumed to represent 100% dsDNA) using the equation:

$$\%ssDNA = \%ssDNA_{MEASUREDINSAMPLE} - \left[\frac{\%dsDNA_{MEASUREDINSAMPLE}}{\%dsDNA_{MEASUREDAT0hr}} \times \%ssDNA_{MEASUREDAT0hr} \right]$$

The ssDNA values calculated provided a conservative estimate of the overall proportion of chromatids resected as the resected strand was unable to serve as a template for PCR in the first amplification cycle.

2.5 Southern blot analysis

Approximately 3 µg of genomic DNA was digested with 25 units of *Bgl*II at 37°C for 3.5 hours in a total volume of 80 µl. The DNA was separated on a 0.7% agarose gel before transfer to a nylon membrane in 20x SSC and probing with an α-³²P radio-labelled *HIS4* fragment (+523 to +1,547 base-pairs, relative to the *HIS4* open reading frame). Hybridisation and washing was carried out as described in Allers and Lichten [55]. Bands were quantified using a Typhoon Phosphorimager (GE Healthcare) and CellQuant software. The percentage of DSBs present in each time point was calculated using the formula:

$$\%DSBs = \left[\frac{DSB\ signal}{DSB + parental\ signal} \right] \times 100$$

2.6 Cumulative curves

In order to convert the non-cumulative DSB frequencies into cumulative curves, the background DSB signal measured at 0 hours was subtracted from each time point. The DSB lifespan in each time course was then calculated as described by Padmore *et al.* [56] by dividing the area under the non-cumulative curve by the amount of DSB measured in *rad50S* strains. Subsequently, the areas between each adjacent pair of time points under the noncumulative curve were divided by the lifespan of the DSB and the resulting values describing the proportion of total DSBs present at each time point summed to produce a cumulative curve. Cumulative curves for ssDNA were calculated in the same way. For all time courses, the lifespan of each resection intermediate was assumed to be equivalent to the lifespan of the DSB in the same time course.

3. Results

3.1 *Resection at the natural HIS4 hotspot requires the nuclease activity of Exo1*

DSBs at the natural *HIS4* hotspot are spread over an approximately 200 bp region which centres approximately 300 bp upstream of the *HIS4* start codon [42, 57] (Supplemental Figure 1A). In a *rad50S* strain where unresected DSBs accumulate [43], approximately 13% of all molecules receive a DSB at *HIS4* when assessed by Southern blotting and this is unaffected by the status of *EXO1* (Methods 2.5 and Supplemental Figure 1A). There are no other DSBs within 1.3 kb upstream or 5.9 kb downstream of the *HIS4* ORF (Supplemental Figure 1B). We first characterised resection in a wild-type strain by measuring the amount of ssDNA produced at the *NheI*, *ClaI*, *AatII* and *BglIII* restriction sites (situated approximately 0.5 kb, 0.8 kb, 1.25 kb and 2 kb from the *HIS4* hotspot respectively) at various time points throughout meiosis (Figure 1A). This was done using a qPCR-based assay that specifically amplifies ssDNA (Methods 2.4). Attempts to measure ssDNA closer to the break site were unsuccessful, perhaps due to DSBs occurring within the amplicon or degradation of the 3' end. DSB formation at the *HIS4* locus was also measured from the same time courses using quantitative Southern blot analysis and the results from these experiments are presented in Figure 1B. In contrast to previous reports (based on degree of DSB “smearing”) in which DSBs were purported to exhibit a constant degree of resection at all time points [35, 58], we observed a slight delay in resection reaching the sites used in this study. Thus, a greater proportion of the DSBs persisting at later time points were subject to detectable resection compared to those in earlier time points. This suggests that the processivity of resection in these experiments is slower than has been observed previously. There are a number of potential explanations for this including differences in the assays used to estimate resection and the temperature of sporulation. Additionally, if the DNA/chromatin context affects resection [53], it may be the case that the natural *HIS4* locus is less amenable to processive resection than the loci used in previous studies.

In order to estimate the overall proportion of DSBs resected as far as each target site over the entire time course, cumulative curves for both DSBs and ssDNA were constructed (Figures 1C, Methods 2.6). From this we demonstrate that there is a very sharp resection gradient in wild-type cells, with the vast majority of resection terminating before reaching the *AatII* site (955 bp from *HIS4*, approximately 1.25 kb

from the DSB). As can be seen in Figure 1D, when ssDNA was measured in *exo1Δ* cells there was considerably less resection than in the wild type (2.3x less at 500 bp from the DSB and 5x less at the site approximately 800 bp away). This difference is entirely due to loss of the nuclease activity of *EXO1* as DSBs in a strain containing an *EXO1* allele that remains structurally intact but is defective in nucleolytic activity, *exo1-D173A* [23, 46], also underwent very little resection (Figure 1B).

3.2. Resection tract length does not strictly correlate with frequency of gene conversion

It has previously been proposed that *EXO1* is responsible for the 5'-3' resection of meiotic DSBs and that a gradient of resection could contribute to the formation of gene conversion gradients [4, 30, 31]. Therefore, we assessed the effect that deleting *EXO1* had on the *HIS4* gene conversion gradient by tetrad dissection of strains containing alleles both proximal and distal to the DSB. Deletion of *EXO1* significantly reduced the levels of gene conversion measured at both alleles (Figure 2; $p < 0.0256$, G-test). While this was similar to the effect that *EXO1* deletion had upon the resection gradient, there did not appear to be a direct correlation between resection and conversion. This was most clearly demonstrated in the wild-type strain in which over one third of the gene conversion tracts that were measured 96 bp from the start codon (approximately 400 bp from the DSB) also extended as far as 1,688 bp away (approximately 2 kb from the DSB; Figure 2). However, despite significant quantities of resection being observed 500 bp from the DSB, virtually no ssDNA was detected at the site of the DSB distal allele even though considerable amounts of hDNA must have formed there (Figures 1 and 2). This argues that more hDNA is produced during meiosis than can be accounted for by detectable resection of the DSB. Accordingly, the slopes of the resection gradients in the region assessed were approximately 3-fold greater than the slopes of the conversion gradients in both wild-type and *exo1Δ* strains (Figures 1D and 2).

In order to rule out the possibility that the requirement for Exo1 in gene conversion at *HIS4* was a locus-dependent effect, we also tested the effect of *exo1Δ* at 7 other alleles. There was a significant reduction in gene conversion at two of these loci (Table 4) when compared to wild type. At the five other loci, gene conversion was

also reduced but the frequencies measured were individually too low to obtain statistically significant results. However, the probability of observing the rates of gene conversion at all five loci changing in the same direction in *exo1Δ* cells (*ie.* less than wild-type) if *exo1Δ* had no effect is $p=0.031$ (binomial exact probability). If we enlarge the group to include the four other loci, then the probability of all 9 exhibiting lower conversion levels in *exo1Δ* is $p<0.002$. Thus in the strains used here, deletion of *EXO1* significantly reduces gene conversion. Furthermore, the influence of Exo1 on heteroduplex tract length appears to be dependent upon the nucleolytic activity of the enzyme as gene conversion at the *his4-xho* and *leu2-r* alleles was also significantly reduced when measured in *exo1-D173A*. However, there was no significant change in the frequency of gene conversion at the 7 remaining alleles in this strain, perhaps due to the numbers of tetrads assessed.

3.3 Crossovers are maintained when the nuclease activity of Exo1 is absent

We next sought to determine if the reduced hDNA tracts and reduced resection observed in the absence of the nuclease activity of Exo1 correlated with reduced crossing-over. To do this, we analysed recombination in a range of genetic intervals on three chromosomes (Figure 3A) in wild type, *exo1Δ* and *exo1-D173A* tetrads. Consistent with previous studies, deletion of *EXO1* resulted in a statistically significant 1.5-2 fold reduction in crossing-over in all intervals examined (Figure 3C, Supplemental Table 1). This reduction in crossing-over was accompanied by a significant decrease in spore viability (Figure 3B, Supplemental Table 2) from 95.8% to 60.4% and a significant alteration in the distribution of viable spore tetrad classes. The *exo1Δ* spore viability measured in this work was significantly lower than that reported previously in the SK1 background by Tsubouchi *et al.* [25]. This can be accounted for by the difference in the temperature of sporulation used in each study as increasing temperature in *exo1Δ* strains appears to improve homologue disjunction at meiosis I (Supplemental Table 2). An effect of temperature upon spore viability has been reported previously in *msh4Δ* [59] and *pch2Δ* [60] mutants.

Surprisingly, crossing-over in *exo1-D173A* tetrads was significantly higher than in *exo1Δ* cells in all but the *HIS4-LEU2* and *CEN8-ARG4* intervals. The only interval with significantly reduced crossing-over in *exo1-D173A* cells compared to wild type

was *LEU2-MAT* (Figure 3B, Supplemental Table 1). As there is little crossover deficit in *exo1-D173A* cells, the crossover defect observed in the *exo1Δ* strain is unlikely to be due to a defect in exonuclease activity. Instead, Exo1 may also play a structural role in promoting crossing-over. Consistent with the near wild-type levels of crossing-over in *exo1-D173A* meiosis, spore viability was significantly improved compared to the *exo1Δ* strain and there were fewer meiosis I non-disjunctions (Figures 3B, 3D; Supplemental Table 3). However, the pattern of spore viability was different from wild type with more tetrads containing a single dead spore (Figure 3B, Supplemental Table 2). A potential explanation for this effect is discussed in 4.4 and illustrated in Figure 5.

3.4 The relationship between hDNA tract length and crossover formation

In order to further investigate the apparent discordance between hDNA tract length and crossing-over, we sought to measure the association of crossing-over with gene conversion at the *HIS4* hotspot. Ideally, one would use tetrad analysis to identify gene conversions at alleles placed varying distances from the DSB and determine the frequency with which they are associated with crossing-over. However, due to the relatively low frequency of gene conversion at the distal allele used (*his4-bgl*) such an approach was not feasible. In order to obtain sufficient gene conversions to accurately assess crossover association, we instead designed a random spore assay based on *his4* heteroalleles and selected His⁺ prototrophic recombinants. We constructed two heteroallelic diploids: *his4-xho/his4-cla* and *his4-cla/his4-bgl* (Figure 4). In systems such as this one, the majority of His⁺ prototrophs derive from recombination events in which the hDNA tract or the mismatch repair tract terminates between the two alleles (Supplemental Figure 2). Thus, His⁺ prototrophs arising from the *his4-xho/his4-cla* heteroalleles require hDNA to extend past *his4-xho* but terminate prior to reaching the *his4-cla* allele and are therefore ‘short’. In contrast, prototrophs from a *his4-cla/his4-bgl* diploid come from ‘long’ events terminating after *his4-cla* but before *his4-bgl*.

We selected 600 His⁺ prototrophs from wild-type, *exo1Δ* and *exo1-D173A* strains containing these two pairs of heteroalleles. The association of each event with crossing-over was determined by the arrangement of the flanking markers. Like all

such assays (for example, that of Martini *et al.* [39]), one of the two possible recombinants and one of the two potential parental configurations are favoured due to the placement of the heteroalleles and the nature of the selection (Supplemental Figure 2). For example, since the parental strand undergoing DSB formation is the recipient of information, His⁺ events in the *his4-cla/his4-bgl* cross preferentially arise when the *his4-cla* strand is broken. This leads to the selective recovery of His⁺ Nat^R-Hyg^R non-crossover recombinants and His⁺ Nat^R crossover recombinants. Similarly in the *his4-xho/his4-cla* cross, prototrophs arise predominantly when the *his4-xho* strand is broken resulting in His⁺ non-crossovers that are drug sensitive and crossovers that are Nat^S Hyg^R (Table 5, Supplemental Figure 2).

In addition to the gene conversion events, the configuration of flanking markers was also analysed in 600 spores without first selecting for histidine prototrophy in order to assess the overall rate of crossing-over in the interval. No statistically significant differences (G-test, $p > 0.0258$) between strains were detected using this approach although the difference between the wild-type and the *exo1Δ* strain was $p = 0.03$. However, the pattern of crossing-over measured (Table 6) mirrored that observed by tetrad analysis in the *HIS4-LEU2* interval and the adjacent *LEU2-MAT* interval where *exo1-D173A* cells exhibited a crossover frequency intermediate between the wild-type and *exo1Δ* strains. This suggests that for recombination initiating at *HIS4*, both the nuclease-dependent and independent functions of Exo1 are required for crossing-over to proceed as normal. We predicted that if *exo1-D173A* cells were able to maintain crossing-over despite reduced hDNA formation, an increase in the association of gene conversions with crossing-over would be evident. Consistent with this, crossing-over in *exo1-D173A* spores was significantly increased compared to wild-type when both ‘short’ and ‘long’ hDNA tracts were selected for (Table 5). Furthermore, a significant reduction in crossover association was observed in *exo1Δ* cells compared to *exo1-D173A* when ‘short’ hDNAs had been produced. This suggests that the non-catalytic function of Exo1 is important for ensuring that shorter hDNA tracts undergo crossing-over. When longer hDNA tracts were selected for, no such reduction in association was observed, arguing that intermediates containing longer hDNA tracts do not require Exo1 to be resolved into crossovers.

4. Discussion

4.1 *DSB end resection is reduced when EXO1 is absent*

In *exo1Δ*, the extent to which DSBs are resected is reduced. Furthermore, the data are consistent with all DSBs being resected less, rather than a subset of DSBs being selectively processed by Exo1. A more severe defect was observed when ssDNA was measured approximately 800bp from the DSB compared to 500 bp, suggesting that the residual resection occurring in the absence of Exo1 is less processive. This is consistent with the data of Hodgson *et al.* [53] based on their analysis of resection at a break induced by the *VMA1*-derived endonuclease (VDE). The identity of the protein(s) responsible for this remaining resection is unclear. A recent study demonstrated a role for Sgs1 and Dna2 in producing the hyper-resected intermediates that form during meiosis in *dmc1Δ* mutants [34]. This is reminiscent of the dual resection pathways mediated by Exo1 and the 5' single-stranded exonuclease Dna2 (in conjunction with the helicase Sgs1 to first unwind the DNA) at DSBs occurring during mitotic growth [32, 33]. However, if these same enzymes are involved in catalysing normal meiotic resection, they are noticeably less able to compensate for Exo1 during meiosis than in other phases of the cell cycle. Indeed, a recent study of meiotic resection by Zakharyevich *et al.* [61] demonstrated that the absence of Sgs1 does not have any significant effect upon meiotic resection tract lengths. Therefore, the most likely candidates are the structure-specific endonuclease Mre11 and/or Sae2 (both of which are required for the initial endonucleolytic cleavage event that releases Spo11 following DSB formation [62-68]). In support of this idea, strains containing mutations in *SAE2* and *MRE11* have been shown to exhibit resection defects at a VDE break [53, 68, 69]

4.2 *DNA synthesis and Exo1 may collaborate in the creation of heteroduplex DNA*

While Exo1 was shown to contribute to both gene conversion and resection at *HIS4* in this study, there did not appear to be a one-to-one correlation. Specifically, gene conversion tracts extended much further than resection tracts, implying that DNA synthesis commonly takes place beyond the point reached by resection before the initial strand invasion event. Although this finding contradicts a previous study at the *ARG4* locus in which resection and gene conversion gradients were shown to be

similar [4], it is in accord with the lengths of meiotic recombination-related DNA synthesis observed by Terasawa *et al.* [70]. By monitoring the incorporation of thymidine analogues during meiosis, synthesis tracts associated with a crossover were measured at 1.5 kb-1.9 kb, lengths far in excess of those estimated to occur during resection [4, 35, 58; this study]. We cannot rule out the possibility that rapid turnover of longer resection tracts prevents these intermediates from being detected. However, the apparent disparity between the amount of DNA resected and DNA synthesised supports previous observations implying that it is the extent of heteroduplex made by the combination of DNA synthesis and second-end capture that determines the length of hDNA formed during meiosis, rather than the length of ssDNA in the first SEI [6, 51, 71, 72] (Figure 5).

Nonetheless, Exo1 evidently influences hDNA tract length in some manner as its absence results in significantly reduced amounts of gene conversion. If extensive synthesis past the initial resection point occurs, this may result in the formation of DNA flaps following capture by the resected strand on the opposite side of the DSB. We suggest that Exo1 acts either to remove these flaps or to catalyse a second phase of resection as DNA synthesis proceeds (Figure 5). Such a nucleolytic activity may not be detectable using our assay because it would occur within the context of dsDNA. This 5' end removal would allow the DNA synthesised using the homologous chromosome as a template to be incorporated into heteroduplex DNA on the chromatid that received the DSB, thereby enabling conversion tracts to extend beyond the initial resection (Figure 5). A similar role for Exo1 was previously postulated by Abdullah *et al.*[73] and such a function fits well with the hDNA extension model proposed by Maloisel *et al.*[72]. When Exo1 is absent, some limited secondary resection may be catalysed by an alternative nuclease. This alternative 5' processing mechanism may also account for the observation that *exo1-D173A* tetrads exhibited a lower level of non-Mendelian segregation at the *his4-xho* allele than was observed in *exo1Δ* cells, as substrate binding by the nucleolytically defective protein could reduce the ability of any substitute nuclease to access the DNA end.

It remains to be determined whether this second phase of resection usually occurs coordinately as DNA synthesis proceeds or later, following the displacement of the

synthesised DNA and partial strand annealing (Figure 5). If synthesis and resection are generally coordinated, it may be that synthesis itself is reduced in the absence of Exo1. Alternatively, the same amount of nascent DNA may be produced but upon capture of the second end, a 3' flap could form [73]. Removal of this 3' flap rather than assimilation of the DNA into the heteroduplex would thus result in a reduced hDNA tract. Similar 3' flapped substrates have been shown to be an ideal substrate for the Mus81/Mms4 complex [74] and indeed a minor role for Mms4 in the removal of such flaps has been proposed previously to account for the increased gene conversion observed in *mms4Δ* strains [18, 19]. Physical analysis has also implicated Mus81/Mms4 in resolving aberrant recombination intermediates during meiosis [75, 76].

4.3 A structural role for Exo1 in crossover promotion

The maintenance of crossing-over in *exo1-D173A* meiosis compared to *exo1Δ*, despite equivalent defects in resection and hDNA formation, argues that relatively short resection tracts remain largely proficient for strand invasion at most loci. Similar observations have been made previously at mitotic DSBs [32, 33]. Furthermore, it can be argued that dHJ formation is also largely unaffected by the reduction in nucleolytic processing. In light of this and the finding that the non-catalytic function of Exo1 appears to be most important when short hDNA tracts have occurred, we suggest that Exo1 is required to stabilise either the strand invasion structure or the dHJ intermediate, allowing it to be resolved as a crossover (Figure 5). A late role for Exo1 fits well with observations from *Exo1^{-/-}* mice in which synapsis occurs normally but chiasmata are not maintained [36, 37]. The finding that crossing-over is only maintained at levels intermediate between wild-type and *exo1Δ* strains in some intervals suggests a locus-specific requirement for the nucleolytic activity of Exo1 in some regions of the genome. The basis for this is unclear; perhaps at some loci, more extensive resection is essential to guarantee stable strand invasion. Alternatively, these sites may be more prone to unwinding of crossover-designated intermediates than others, an activity which could be exacerbated by the shorter hDNA tracts seen in *exo1-D173A* cells.

Exo1 has previously been suggested to act in a nuclease-independent manner during mitotic DNA mismatch repair (MMR). This function requires Exo1 to physically interact with Mlh1 [77]. A five amino acid motif R/S-S-K-(Y/F)-F known as the Mlh1 interacting protein (MIP) box in Exo1 mediates this interaction by binding a conserved S2 motif in Mlh1 [77, 78]. The physical presence of Exo1 was proposed to be important for the stabilisation of the Mlh1 and Pms1 heterodimer, thus supporting the formation of a larger multi-protein complex involved in MMR [79]. It is therefore tempting to speculate that Exo1 may behave similarly during meiosis by supporting the formation/function of the Mlh1/Mlh3 complex and a recent physical analysis of crossing-over at the *HIS4LEU2* hotspot by Zakharyevich *et al.* [61] supports such a conclusion. However, in the Y55 strain background, a MIP box mutant *exo1-S445A F447A F448A* that is defective in Mlh1 binding was found to exhibit a wild-type crossover phenotype in six genetic intervals when assessed genetically. This resulted in a wild-type spore viability (V.E. Cotton & R.H. Borts, data not shown), arguing that this same interaction motif may not be essential for the non-catalytic function of Exo1 during crossover promotion.

4.4 The nature of the spore death in the separation-of-function allele

In the *exo1-D173A* strain, an increased frequency of three-viable spore tetrads was observed compared to both wild-type and *exo1Δ* (Figure 3). We hypothesise that these events could arise if the lengths of resection that occur are sufficient to enable strand invasion but insufficient to allow strand capture. If strand capture is unsuccessful, a half crossover and a single broken chromatid could result leading to the death of the spore inheriting the broken chromatid (Figure 5). Similar situations have been suggested to occur in the absence of the strand annealing activity of Rad52 [80] and during heteroduplex rejection of the second-end capture during homeologous recombination [81]. Given that such an event need only occur at a single DSB to cause the death of one spore within a tetrad and each cell is estimated to undergo approximately 150 DSBs per meiosis [82], only a very low frequency of these aberrant events would be required to produce the viability pattern observed in *exo1-D173A* meiosis. The differential ability of a break to undergo strand invasion and strand capture could be explained either by differing amounts of resection either side of the DSB or if the length of resection required for strand capture is greater than that

necessary for strand invasion. Broken chromatids may similarly arise in *exo1Δ* cells but the high levels of meiosis I non-disjunction that occur may mask this phenotype.

4.5 Conclusions

In conclusion, Exo1 appears to perform at least three roles during meiotic DSB repair. Firstly, it functions in the 5' to 3' end resection that takes place following DSB formation. Secondly, it is required for the additional removal of DNA after strand invasion which allows newly synthesised DNA to be incorporated into hDNA thus leading to gene conversion and finally, it plays a nuclease-independent role in the stabilisation of crossover-designated intermediates. In contrast to earlier models proposed to explain the crossover deficit observed in *exo1Δ* strains, it appears to be this nuclease-independent function that is the most important in terms of ensuring crossing-over at the majority of loci.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure 1 - Resection analysis at *HIS4*.

(A) Schematic diagram indicating the approximate locations of the resection assay target sites used with respect to the *HIS4* hotspot. PCR primer pairs were designed to anneal either side of these sites. Amplification was only possible from these primers when the restriction site indicated was present in single-stranded form and thus protected from restriction endonuclease cleavage. Each pair was used in conjunction with a second pair of primers at the rDNA locus that served to measure the total amount of DNA within the reaction. (B) Non-cumulative curves describing the formation of DSBs at *HIS4* and the production of ssDNA at approximately 0.5 kb, 0.8 kb, 1.25 kb and 2 kb from the *HIS4* hotspot in wild-type (RKD51), *exo1*Δ (RKD52) and *exo1-D173A* (RKD55) meiotic time course experiments. Each data point plotted is the average of two independent time courses. Bars indicate the range observed. (C) The non-cumulative data for the wild-type and *exo1*Δ time courses presented were converted to cumulative curves as described in Methods 2.6. (D) The cumulative amount of ssDNA produced by 12 hours was then divided by the total amount of DSBs formed in order to estimate the overall proportion of DSBs resected as far as each target site during the 12 hour period monitored. The proportions observed are plotted with respect to the *HIS4* start codon. For each genotype, the data presented is the mean of two independent time courses. Bars indicate the range observed.

Figure 2 - The effect of *EXO1* deletion on the *HIS4* gene conversion gradient

Following tetrad dissection, gene conversion was measured at two alleles situated 96 bp and 1,688 bp from the *HIS4* start codon (approximately 0.4 kb and 2 kb from the *HIS4* hotspot). Gene conversions result from the presence of mismatches within heteroduplex DNA (hDNA) formed during recombination. In fungi, the frequency of gene conversion is known to decrease with increasing distance from the DSB and this is termed a conversion gradient [31]. In the absence of *EXO1*, gene conversion is significantly reduced at both alleles. The wild-type data was derived from crosses RK358 x RK392 and RK385 x RK392. The corresponding *exo1*Δ strains were RK359 x RK394 and RK387 x RK394.

Figure 3 - Genetic analysis in wild-type, *exo1*Δ and *exo1-D173A* strains

(A) Schematic diagram showing the approximate chromosomal locations of markers used in genetic analysis (drawings not to scale). (B) Patterns of spore viability obtained in wild-type (RK358 x RK392), *exo1Δ* (RK359 x RK394) and *exo1-D173A* (RK399 x RK425) indicating the proportions of viable spores per tetrad. The total number of tetrads dissected for each strain is indicated and the overall viability given in parentheses. (C) Measurements of map distances in six intervals were calculated using the formula of Perkins *et al.* [49]. Comparisons between recombination classes were made by G-test and statistically significant differences ($p < 0.0256$) are indicated by an asterisk. (D) Rates of chromosome III meiosis I non-disjunction were assessed by monitoring the proportion of non-mating spores produced. Statistical comparisons were made as above and significant differences are indicated by asterisks.

Figure 4 - Schematic diagram of *HIS4* marker configurations used in random spore analysis

In order to assess recombination associated with the *HIS4* hotspot, two diploids (A and B) were used containing different pairs of *his4* hetero-alleles. The configurations of markers on the two parental chromosomes (P1 and P2) in these diploids are illustrated. The positions of the *his4-xho*, *his4-cla* and *his4-bgl* alleles are given relative to the *HIS4* start codon and the orientations of the flanking nourseothricin (*NAT*) and hygromycin B (*HPH*) drug-resistance cassettes are shown. The *NAT* and *HPH* cassettes are located 3,806 bp downstream and 5,130 bp upstream of the *HIS4* start codon respectively. The *HIS4* hotspot breakpoint is located approximately 300 bp upstream of the start codon and is indicated by the arrows. The strains used were RKD62 to RKD79.

Figure 5 – Model demonstrating multiple roles for Exo1 during meiotic recombination.

(A) Following DSB formation, Exo1 acts to resect the DNA ends in a 5' to 3' direction. When Exo1 nuclease activity is absent, limited resection may be catalysed by Mre11 and/or Sae2. The overhanging 3' strands can then begin to interact with the homologous chromosome and a D-loop is produced upon invasion. If a break is to be repaired as a non-crossover, DNA synthesis and strand displacement will follow. (B) Along the crossover pathway, the D-loop requires stabilisation by proteins such as

Msh4/Msh5 (shown in orange). If Exo1 also performs an early stabilising function, its absence may lead to displacement of the SEI, collapsing the D-loop. A non-crossover can be formed by SDSA. (C) With a stable SEI, extensive DNA synthesis then takes place, which may or may not be coordinated with processive resection. (D) The synthesised end is eventually displaced and attempts to anneal to the strand uncovered by resection on the opposite side of the break. (E) If there is insufficient resection for this annealing to occur, a half crossover and a single broken chromatid could result. (F) Upon successful annealing, if DNA synthesis and resection do not occur at the same rate, DNA flaps could be formed from displacement of the unresected region. (G) These flaps normally require Exo1 for removal, allowing dHJ to form and undergo subsequent resolution as crossovers. (H) When Exo1 nuclease activity is absent, 3' flaps could be produced that may serve as substrates for Mus81/Mms4 (indicated by green triangle). This would then permit ligation and completion of the dHJ. (I) If instead of/in addition to an early stabilising role, the nuclease-independent function of Exo1 (shown in purple) acts to stabilise the dHJ structure, crossovers would form as normal in *exo1-D173A*. (J) Without this stabilising activity in *exo1Δ*, crossovers would not be produced.

Table 1 – Haploid yeast strains

Strain	Genotype ^a
RK358	<i>his4-xho MATα ade1-1 lys5-p cyh2-z CEN8:URA3 arg4-nsp</i>
RK359	<i>his4-xho MATα ade1-1 lys5-p cyh2-z CEN8:URA3 arg4-nsp exo1Δ::KanMX4</i>
RK385	<i>his4-bgl MATα ade1-1 lys5-p cyh2-z CEN8:URA3 arg4-nsp</i>
RK387	<i>his4-bgl MATα ade1-1 lys5-p cyh2-z CEN8:URA3 arg4-nsp exo1Δ::KanMX4</i>
RK392	<i>leu2-r MATα ade1-1 met13-b trp5-s</i>
RK394	<i>leu2-r MATα ade1-1 met13-b trp5-s exo1Δ::KanMX4</i>
RK399	<i>his4-xho MATα ade1-1 lys5-p cyh2-z CEN8:URA3 arg4-nsp exo1-D173A</i>
RK425	<i>leu2-r MATα ade1-1 met13-b trp5-s exo1-D173A</i>

^aAll strains are in the SK1 background and are also *ho::LYS2 lys2 ura3*

Table 2 – Diploid yeast strains

Strain	Genotype^a									
RKD51	<i>his4-xho</i>	<i>LEU2</i>	<i>MATα</i>	<i>ade1-1</i>	<i>lys5-p</i>	<i>MET13</i>	<i>cyh2-z</i>	<i>TRP5</i>	<i>CEN8:URA3</i>	<i>arg4-nsp</i>
	<i>HIS4</i>	<i>leu2-r</i>	<i>MATa</i>	<i>ade1-1</i>	<i>LYS5</i>	<i>met13-b</i>	<i>CYH2</i>	<i>trp5-s</i>	<i>CEN8</i>	<i>ARG4</i>
RKD52	<i>his4-xho</i>	<i>LEU2</i>	<i>MATα</i>	<i>ade1-1</i>	<i>lys5-p</i>	<i>MET13</i>	<i>cyh2-z</i>	<i>TRP5</i>	<i>CEN8:URA3</i>	<i>arg4-nsp</i>
	<i>HIS4</i>	<i>leu2-r</i>	<i>MATa</i>	<i>ade1-1</i>	<i>LYS5</i>	<i>met13-b</i>	<i>CYH2</i>	<i>trp5-s</i>	<i>CEN8</i>	<i>ARG4</i>
										<i>exo1Δ::KanMX4</i>
RKD55	<i>his4-xho</i>	<i>LEU2</i>	<i>MATα</i>	<i>ade1-1</i>	<i>lys5-p</i>	<i>MET13</i>	<i>cyh2-z</i>	<i>TRP5</i>	<i>CEN8:URA3</i>	<i>arg4-nsp</i>
	<i>HIS4</i>	<i>leu2-r</i>	<i>MATa</i>	<i>ade1-1</i>	<i>LYS5</i>	<i>met13-b</i>	<i>CYH2</i>	<i>trp5-s</i>	<i>CEN8</i>	<i>ARG4</i>
										<i>exo1-D173A</i>
RKD80	<i>his4-xho</i>	<i>LEU2</i>	<i>MATα</i>	<i>ade1-1</i>	<i>lys5-p</i>	<i>MET13</i>	<i>cyh2-z</i>	<i>TRP5</i>	<i>arg4-</i>	<i>rad50S::URA3</i>
	<i>HIS4</i>	<i>leu2-r</i>	<i>MATa</i>	<i>ade1-1</i>	<i>LYS5</i>	<i>met13-b</i>	<i>CYH2</i>	<i>trp5-s</i>	<i>ARG4</i>	<i>rad50S::URA3</i>
RKD81	<i>his4-xho</i>	<i>LEU2</i>	<i>MATα</i>	<i>ade1-1</i>	<i>lys5-p</i>	<i>MET13</i>	<i>cyh2-z</i>	<i>TRP5</i>	<i>arg4-</i>	<i>rad50S::URA3</i>
	<i>HIS4</i>	<i>leu2-r</i>	<i>MATa</i>	<i>ade1-1</i>	<i>LYS5</i>	<i>met13-b</i>	<i>CYH2</i>	<i>trp5-s</i>	<i>ARG4</i>	<i>rad50S::URA3</i>
										<i>exo1Δ::KanMX4</i>
RKD62,63,64	<i>RRP7</i>	<i>his4-xho</i>		<i>FUS1</i>	<i>MATα</i>	<i>ade1-1</i>	<i>CAN1</i>	<i>cyh2-z</i>		
	<i>RRP7-NatMX4</i>	<i>his4-cla</i>		<i>FUS1-HphMX4</i>	<i>MATa</i>	<i>ade1-1</i>	<i>can1</i>	<i>CYH2</i>		
RKD65,66,67	<i>RRP7</i>	<i>his4-xho</i>		<i>FUS1</i>	<i>MATα</i>	<i>ade1-1</i>	<i>CAN1</i>	<i>cyh2-z</i>	<i>exo1Δ::KanMX4</i>	
	<i>RRP7-NatMX4</i>	<i>his4-cla</i>		<i>FUS1-HphMX4</i>	<i>MATa</i>	<i>ade1-1</i>	<i>can1</i>	<i>CYH2</i>	<i>exo1Δ::KanMX4</i>	
RKD68,69,70	<i>RRP7</i>	<i>his4-xho</i>		<i>FUS1</i>	<i>MATα</i>	<i>ade1-1</i>	<i>CAN1</i>	<i>cyh2-z</i>	<i>exo1-D173A</i>	
	<i>RRP7-NatMX4</i>	<i>his4-cla</i>		<i>FUS1-HphMX4</i>	<i>MATa</i>	<i>ade1-1</i>	<i>can1</i>	<i>CYH2</i>	<i>exo1-D173A</i>	
RKD71, 72,73	<i>RRP7</i>	<i>his4-bgl</i>		<i>FUS1</i>	<i>MATα</i>	<i>ade1-1</i>	<i>CAN1</i>	<i>cyh2-z</i>		
	<i>RRP7-NatMX4</i>	<i>his4-cla</i>		<i>FUS1-HphMX4</i>	<i>MATa</i>	<i>ade1-1</i>	<i>can1</i>	<i>CYH2</i>		
RKD74, 75,76	<i>RRP7</i>	<i>his4-bgl</i>		<i>FUS1</i>	<i>MATα</i>	<i>ade1-1</i>	<i>CAN1</i>	<i>cyh2-z</i>	<i>exo1Δ::KanMX4</i>	
	<i>RRP7-NatMX4</i>	<i>his4-cla</i>		<i>FUS1-HphMX4</i>	<i>MATa</i>	<i>ade1-1</i>	<i>can1</i>	<i>CYH2</i>	<i>exo1Δ::KanMX4</i>	
RKD77, 78,79	<i>RRP7</i>	<i>his4-bgl</i>		<i>FUS1</i>	<i>MATα</i>	<i>ade1-1</i>	<i>CAN1</i>	<i>cyh2-z</i>	<i>exo1-D173A</i>	
	<i>RRP7-NatMX4</i>	<i>his4-cla</i>		<i>FUS1-HphMX4</i>	<i>MATa</i>	<i>ade1-1</i>	<i>can1</i>	<i>CYH2</i>	<i>exo1-D173A</i>	

^a All strains are in the SK1 strain background and are also *ho::LYS2 lys2 ura3*. Where multiple strain numbers refer to the same genotype, each originates from an independently isolated diploid colony.

Table 3 - Oligonucleotide sequences used for quantitative PCR analysis

Description	Sequence (5' to 3')
5' at rDNA locus	ATCAGCTTGCGTTGATTACG
3' at rDNA locus	GTTGCCCCCTTCTCTAAGC
5' of <i>Nhe</i> I site (238bp from <i>HIS4</i>)	ATTGGTGGCTTTGTCCTTGC
3' of <i>Nhe</i> I site (238bp from <i>HIS4</i>)	CAAGTGTTCTGGCTGTTTTAGC
5' of <i>Cla</i> I site (532bp from <i>HIS4</i>)	GAGCGTTGTCTAGGGTTGGT
3' of <i>Cla</i> I site (532bp from <i>HIS4</i>)	TCACCCTTGATCCAGATTTCA
5' of <i>Aat</i> II site (955bp from <i>HIS4</i>)	GGCTGCCGATTTGTTCTACT
3' of <i>Aat</i> II site (955bp from <i>HIS4</i>)	TGGCTTAGCATCACCTTTCC
5' of <i>Bgl</i> III site (1688bp from <i>HIS4</i>)	CGCTTCCAAGATTGTTCTAGC
3' of <i>Bgl</i> III site (1688bp from <i>HIS4</i>)	ACATACATTTTGGCGGCAGT

Table 4 – The effect of Exo1 on non-Mendelian segregation

Genotype		Allele								
		<i>his4-xho</i>	<i>his4-bgl</i>	<i>leu2-r</i>	<i>MAT</i>	<i>lys5-p</i>	<i>met13-b</i>	<i>cyh2-z</i>	<i>trp5-s</i>	<i>arg4-nsp</i>
Wild-type	%NMS ^a	29.2 [†]	11.2 [†]	3.6 [†]	0.9	1.4	1.8	0.7	1.3	6.1 [†]
	(NMS/total tetrads)	(275/941)	(38/338)	(34/941)	(8/941)	(13/941)	(17/941)	(7/941)	(12/941)	(58/941)
<i>exo1Δ</i>	%NMS ^a	23.6 [*]	2.5 [*]	1.1 [*]	0.2	0.9	1.0	0.5	0.3	2.9 [*]
	(NMS/total tetrads)	(209/885)	(3/118)	(10/885)	(2/885)	(8/885)	(9/885)	(4/885)	(3/885)	(26/885)
<i>exo1-D173A</i>	%NMS ^a	15.4 ^{**†}	<i>not tested</i>	1.3 [*]	0	1.9	2.5	0.3	1.6	5.6
	(NMS/total tetrads)	(49/319)		(4/319)	(0/319)	(6/319)	(8/319)	(1/319)	(5/319)	(18/319)

^a Comparisons were made using the G-test of homogeneity and statistically significant (p<0.0258) differences are indicated as follows:

^{*} significantly different from wild-type, [†] significantly different from *exo1Δ*.

Table 5 - Association of prototroph formation with crossing-over at *HIS4*

		Configuration of flanking markers					
Heteroalleles	Genotype	Parental		Recombinant		Total ^a	% Crossover associated ^{b,c}
		<i>Nat^R Hph^R</i>	<i>Nat^S Hph^S</i>	<i>Nat^R Hph^S</i>	<i>Nat^S Hph^R</i>		
<i>his4-xho/</i>	Wild-type	34	308	32	226	600	43
<i>his4-cla</i>	<i>exo1Δ</i>	43	320	34	203	600	39.5
	<i>exo1-D173A</i>	17	245	22	316	600	56.3 ^{*†}
<i>his4-cla/</i>	Wild-type	123	24	447	5	599	75.5 [†]
<i>his4-bgl</i>	<i>exo1Δ</i>	78	15	503	4	600	84.5 [*]
	<i>exo1-D173A</i>	83	13	501	3	600	84 [*]

^a For each genotype, data from three independent experiments were pooled.

^b The proportions of parental and recombinant spores were compared using the G-test of homogeneity and statistically significant values (p<0.0169, G-test of homogeneity) are indicated as follows: * significantly different from wild-type, [†] significantly different from *exo1Δ*.

^c All genotypes displayed a significant increase in crossover association when the *his4-cla/his4-bgl* heteroalleles were used compared to the *his4-cla/his4-bgl* heteroalleles.

Table 6 - Crossing-over between flanking markers at the *HIS4* hotspot

Genotype	Configuration of <i>NAT/HPH</i> markers		Recombination frequency ^{a,b}
	Parental	Recombinant	
Wild-type	549	51	8.5
<i>exo1Δ</i>	568	32	5.3
<i>exo1-D173A</i>	558	42	7

^a For each genotype data from 6 independent cultures were pooled.

^b Recombination frequencies were calculated as: (number of recombinants/total number of colonies) x 100.

Figure 1

Figure 2R

Figure 3 **(B)** **(C)** **(D)**

Figure 4
(A)

Figure 5

Supplemental Table 1 – Map Distances

	Wild-type	<i>exo1Δ</i>	<i>exo1-D173A</i>
<i>HIS4-LEU2</i>			
PD ^a	449	540	206
NPD	1	1	1
TT	183	126	59
cM	14.9 [†]	9.9 [*]	12.2
<i>LEU2-MAT</i>			
PD	442	622	172
NPD	32	7	3
TT	425	244	140
cM	34.3 [†]	16.4 [*]	25.1 ^{*†}
<i>CEN8-ARG4</i>			
PD	696	739	248
NPD	2	1	1
TT	185	119	52
cM	11.2 [†]	7.3 [*]	9.6
<i>LYS5-MET13</i>			
PD	555	680	169
NPD	8	2	5
TT	349	186	131
cM	21.8 ^c	11.4 [*]	26.4 [†]
<i>MET13-CYH2</i>			
PD	738	774	257
NPD	0	0	2
TT	179	99	51
cM	9.8 [†]	5.7 [*]	10.2 [†]
<i>CYH2-TRP5</i>			
PD	331	539	114
NPD	35	11	10
TT	557	328	189
cM	41.5 [†]	22.4 [*]	39.8 [†]

^a PD, NPD and TT represent parental ditypes, nonparental ditypes and tetratypes respectively. cM values were calculated as described in Methods 2.3. Comparisons were made using the G-test of homogeneity and statistically significant ($p < 0.0258$) differences are indicated as follows: ^{*} significantly different from wild-type, [†] significantly different from *exo1Δ*.

Supplemental Table 2 - Spore viability at 23°C and 33°C

Genotype	(°C)		Viable spores per tetrad class					Total	%
			4	3	2	1	0	tetrads	viability ^a
Wild-type	23	<i>n</i>	941	54	41	2	8	1046	95.8 [†]
		(%)	(89.9)	(5.2)	(3.9)	(0.2)	(0.8)		
Wild-type	33	<i>n</i>	309	21	2	0	2	334	97.5 ^{*†}
		(%)	(92.2)	(6.3)	(0.6)	(0)	(0.6)		
<i>exo1Δ</i>	23	<i>n</i>	885	231	472	136	476	2200	60.4 [*]
		(%)	(40.2)	(10.5)	(21.5)	(6.2)	(21.6)		
<i>exo1Δ</i>	33	<i>n</i>	220	33	45	10	23	331	81.5 ^{*†}
		(%)	(66.3)	(9.9)	(13.6)	(3.0)	(6.9)		
<i>exo1-D173A</i>	23	<i>n</i>	319	198	199	66	49	831	70.2 ^{*†}
		(%)	(38.3)	(23.8)	(23.9)	(7.9)	(5.9)		

^a Overall viabilities were calculated as (4 x no. of four spore tetrads + 3 x no. of three-spore tetrads + 2 x no. of two-spore tetrads + no. of one-spore tetrads) / (4 x total no. of tetrads) x 100. Comparisons were made using the G-test of homogeneity and statistically significant (p<0.0127) differences are indicated as follows: * significantly different from wild-type at 23°C, [†] significantly different from *exo1Δ* at 23°C.

Supplemental Table 3 - Rates of chromosome III non-disjunction at meiosis I

Genotype	Number of non-maters	Total tetrads	% Non-disjunction^a
Wild-type	1	1046	0.1 [†]
<i>exo1Δ</i>	61	2200	2.8 [*]
<i>exo1-D173A</i>	8	832	1.0 ^{*†}

^a Non-disjunction was calculated as: (number of non-maters/total number of tetrads) x100. Comparisons were made using the G-test of homogeneity and statistically significant (p<0.0258) differences are indicated as follows: ^{*} significantly different from wild-type, [†] significantly different from *exo1Δ*.

Supplemental Figure 1 – DSB formation at *HIS4* in *rad50S* strains

(A) DSBs at *HIS4* were assessed in *rad50S* strains in three independent wild-type, *exo1Δ* and *exo1-D173A* cultures. DNA was digested with *Bgl*II in order to estimate the total amount of breaks formed and the position of the break with respect to the *HIS4* start codon. The mean amount of DSBs produced in wild-type cells (RKD80) was 12.8%, 14.7% in an *exo1Δ* strain (RKD81) and 13.2% in *exo1-D173A* (RKD82). These values were not significantly different from one another ($p > 0.7$, *t*-test). (B) DNA from a *rad50S* culture was digested with *Eco*RV to look for the presence of DSBs downstream of *HIS4*. *Bst*EII digested lambda DNA was used as a molecular weight standard and the size of each band is indicated. The black boxes represent the approximate location of the probe used for DSB analysis.

Supplemental Figure 2 – An example illustrating prototroph formation between heteroalleles

In this example, a *his4-cla*/*his4-bgl* heteroallelic cross is presented. Cassettes conferring resistance to nourseothricin and hygromycin B are inserted on the *his4-cla* chromosome. (A) In such crosses, prototrophs most commonly arise when DSBs occur on the chromosome containing the mutant allele situated closest to the break site (in this case, *his4-cla*). (B) Following strand invasion, DNA synthesis then takes place using the *his4-bgl* strand as a template. If this synthesis terminates between the *his4-cla* and *his4-bgl* sites, a *HIS4* strand is produced. (C) If the invading strand is then displaced to form a non-crossover, a His^+ spore may result depending upon the direction of mismatch repair. This spore will be both Nat^R and Hyg^R (D) Alternatively, if the break is to be repaired as a crossover, a dHJ intermediate will be produced. (E) Resolution of this dHJ in one orientation (indicated by green triangles) will yield a His^+ spore (F). (G) Resolution in the alternative orientation may also produce a His^+ spore (H), depending upon the directionality of mismatch repair. In both cases, the His^+ spore will be $\text{Nat}^R \text{Hyg}^S$. Boxes indicate potential sources of His^+ spores.