DNA sequence differences are determinants of meiotic recombination outcome

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Meiotic recombination is essential for producing healthy gametes, and also generates genetic diversity. DNA double-strand break (DSB) formation is the initiating step of meiotic recombination, producing, among other outcomes, crossovers between homologous chromosomes (homologs), which provide physical links to guide accurate chromosome segregation. The parameters influencing DSB position and repair are thus crucial determinants of reproductive success and genetic diversity. Using Schizosaccharomyces pombe, we show that the distance between sequence polymorphisms across homologs has a strong impact on meiotic recombination rate. The closer the sequence polymorphisms are to each other across the homologs the fewer recombination events were observed. In the immediate vicinity of DSBs, sequence polymorphisms affect the frequency of intragenic recombination events (gene conversions). Additionally, and unexpectedly, the crossover rate of flanking markers tens of kilobases away from the sequence polymorphisms was affected by their relative position to each other amongst the progeny having undergone intragenic recombination. A major regulator of this distance-dependent effect is the MutSα-MutLα complex consisting of Msh2, Msh6, Mlh1, and Pms1. Additionally, the DNA helicases Rqh1 and Fml1 shape recombination frequency, although the effects seen here are largely independent of the relative position of the sequence polymorphisms.

Correct chromosome segregation during meiosis depends on pairing and physical connection of homologous chromosomes (homologs). Physical connections are established by the repair of programmed DNA double-strand breaks (DSBs) using the homolog rather than the sister chromatid as a template (i.e. interhomolog recombination) and by ensuring that interhomolog recombination intermediates are processed into crossovers (COs). The formation of DSBs by the transensterase Spo11 is thus a key step in initiating recombination during meiosis1. Regions of high-frequency Spo11 recruitment, and thus DSB formation, are called hotspots2. One of the best characterized category of hotspots are cAMP-responsive elements in Schizosaccharomyces pombe, created by point mutations in the ade6 gene that represent binding sites for the Atf1-Pcr1 transcription factor2,3. These include the ade6-M26 hotspot and its derivatives, which are defined by the DNA sequence heptamer 5′-ATGACGT-3′. Although binding of Atf1-Pcr1 and the associated transcription already creates open chromatin at M26-like hotspots3,4, a very high frequency of meiotic recombination requires a conducive chromatin environment in a wider genomic context5,6. This network of parameters determines the overall level of DSB formation at a given genomic locus.

Following break formation, DSB ends are resected to initiate homologous recombination, which during meiosis follows either a Holliday junction/D-loop resolution or a synthesis-dependent strand annealing (SDSA) pathway1,7. As a repair template, either the sister chromatid or the homolog can be used1. Based on this, it has been suggested that the governance of meiotic recombination could be viewed as a two-tiered decision system8. The first decision being template choice (interhomolog vs. intersister recombination), and the second being how the recombination intermediate is resolved - i.e. the CO/non-crossover (NCO) decision. The template choice decision is mainly driven by meiosis-specific factors of the chromosome axis and by the meiotic recombinase Dmc1 supported by its mediators8. In budding yeast there is a basic understanding of how the interhomolog bias is established, although some mechanistic details still remain to be elucidated10. Since homologs are not necessarily identical on a DNA sequence level, a DSB end invading the homolog for repair can generate mismatch-containing

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heteroduplex DNA. Mismatches can be corrected by the mismatch repair system, consisting of the highly conserved MutS and MutL proteins\(^{11}\). Additionally, the MutS-MutL complex can also block strand invasion to avoid recombination between non-homologous sequences\(^{12}\). The CO/NCO-decision happens as the next step; here the decision is taken whether an already established interhomolog recombinational intermediate is processed into a CO or a NCO. Determinants of the CO/NCO-decision are less well studied, but the DNA helicase/translocase FANCM (Fml1 in *S. pombe*) has been shown to limit CO formation in fission yeast and *Arabidopsis*\(^{12,13}\). RecQ-type DNA helicases perform a wide range of regulatory roles in homologous recombination, and one of which probably is the promotion of NCO formation during meiosis in various organisms\(^{14-18}\).

Here, we employ a series of meiotic recombination assays featuring intragenic markers at differently sized intragenic intervals and flanking intergenic markers to identify and characterize intrinsic determinants of template choice and CO/NCO-decision in fission yeast. We show that the relative positions of DNA sequence polymorphisms between homologs have a strong impact on recombination outcome, not only locally in the form of intragenic recombination (gene conversion), but also on the CO frequency between an up- and a downstream marker. The anti-recombinogenic activity of MutS\(-\)/MutL\(-\) factors, and of the DNA helicases Fml1 and Rqh1 modulate recombination outcome differentially when comparing various intragenic intervals.

### Results and Discussion

**Rationale of the meiotic recombination assay.** Our meiotic recombination assay features intragenic markers (point mutations in the *ade6* gene) and flanking intergenic markers (*ura4*\(-\)/aim2 and *his3*\(-\)/aim) (Fig. 1). This assay allows us to monitor various recombination outcomes: (I) intragenic recombination (gene conversion) events producing *Ade*\(^+\) recombinants, (II) crossovers (COs) between the flanking intergenic markers (*ura4*\(-\)/aim2 and *his3*\(-\)/aim), and (III) the ratio of COs vs. non-crossover (NCOs) among intragenic *ade6*\(^+\) recombination events (Fig. 1A). Changes in gene conversion and overall CO frequencies observed in this assay can be explained by an altered frequency of DSB formation at a given *ade6* mutant allele, or a change in repair template usage. The percentages of COs and NCOs among intragenic *ade6*\(^+\) recombination events are the genetic readout for the CO/NCO-decision, representing recombination intermediate processing after successful strand exchange between homologs. The intragenic events are most likely the result of gene conversions with COs or NCOs (non-reciprocal exchange of hereditary information).

The physical distance between point mutations of heteroalleles defines the frequency of intragenic recombination events and their associated CO/NCO ratio. Apart from absolute DSB levels, intragenic recombination frequency is also influenced by the distance between point mutations in a given chromosomal region\(^{19-21}\). Intragenic recombination in our assays (Fig. 1A) has so far been monitored using point mutations within the *ade6* coding sequence, which are at least 1 kb apart\(^{12,22,23}\). We wondered whether the level of COs among intragenic recombination events also changes, when the distance between point mutations was decreased. Therefore, we selected a series of point mutations, which cover almost the complete length of the *ade6* coding sequence (Fig. 1B, Supplementary Table S1). These point mutants include the strong meiotic recombinogenic *ade6*-3083, -3074, and -469 (Fig. 1B, Supplementary Table S1). All hotspots used here mimic a cAMP-response element, which creates a binding site for the Atf1-Pcr1 transcription factor; this in turn generates open chromatin\(^3,5\). It can be safely assumed that a given hotspot will receive the same amount of breakage independent of the *ade6* allele present on the homolog. This means that the differences seen in the combinations of one specific hotspot with various *ade6* alleles will depend on processes downstream of DSB formation. Indeed, the frequency of intragenic recombination positively correlates with the distance between the *ade6* alleles, when the same hotspot is used (Fig. 2A, black and grey lines). Recombination at the *ade6*-M375 allele, which is at a similar position as the strong hotspot alleles *ade6*-3074 & *ade6*-3083, is induced at an overall much lower level (Fig. 2A, green line), but appears to be the acceptor of genetic information when crossed to *ade6*-469 (Fig. 2E), indicating that *ade6*-M375 is somewhat more recombinogenic than *ade6*-469. Intragenic recombination frequency at *ade6*-M375 shows a similar correlation with respect to distance between the DNA polymorphisms as crosses involving hotspots (Fig. 2A). Intragenic intervals of similar size containing the meiotic recombination hotspot alleles, *ade6*-3083, *ade6*-3074, or *ade6*-3049, and a non-hotspot allele produce equivalent intragenic recombination levels (Fig. 2A). Therefore, these hotspot alleles behave similarly in determining intragenic recombination frequency.

Intriguingly, these observations are also largely true for CO frequency among intragenic recombination events: The shorter an intragenic distance between polymorphisms is, the more likely an intragenic recombination event is resolved as a NCO (Fig. 2B). For crosses involving the hotspot alleles *ade6*-3083 or *ade6*-3074 the effect apparently tails off at intragenic distances >600 bp (Fig. 2B). Combining hotspot alleles on both homologs within a cross results in increased overall intragenic recombination rate compared with a hotspot × non-hotspot cross covering a similar intragenic distance between point mutations (Fig. 2C), in line with previous reports\(^21\). However, there is no notable difference in COs among intragenic recombination events (Fig. 2D). This indicates that the frequency of CO among intragenic recombination events is primarily a function of the distance between the *ade6* heteroalleles on the homologs.

The distribution of different NCO/CO classes amongst intragenic recombination events follows a pattern consistent with intragenic NCOs more likely being associated with the hotter allele. This means that the allele more likely to receive a DSB is the recipient of genetic information in the overwhelming majority of cases, which might represent a *bona fide* gene conversion event, e.g. the vast majority of *Ade*\(^+\) NCO events in the *ade6*-3083 × *ade6*-469 cross are Ura\(^+\) His\(^-\), because the *ade6*-3083 allele is linked to the *ura4*\(-\)/aim2 marker (Fig. 2E). If comparable hotspots are combined in a cross the two intragenic NCO classes occur with roughly equal frequency (Fig. 2E; compare cross *ade6*-3083 × *ade6*-3049 to crosses *ade6*-3083 × *ade6*-469 & *ade6*-M375 × *ade6*-3049).
The observed distribution patterns also suggest that, at these long intragenic intervals, a subset of CO events could stem from the processing of one joint molecule, presumably a single Holliday junction or its precursors, positioned between the two ade6 point mutations; in contrast to a gene conversion event being resolved as a CO. This idea makes the following prediction: If CO events among Ade+ recombinants (mostly Ura−His− genotypes) are created by processing of a joint molecule situated between the two ade6 point mutations, then reciprocal Ura+ Ade−His+ recombinants carrying the mutations of both ade6 heteroalleles must exist. To test this, we sequenced the ade6 locus from 32 Ura+ Ade−His+ colonies from an ade6-3083 × ade6-469 cross. Based on the frequency of 0.677% Ura−Ade+His− events among the total viable progeny in such a cross representing 8.375% of recombinants among all Ura−His− colonies (240 Ura−His− colonies among 2,969 total viable progeny, 8.083%), we would expect that 2-3 of the 32 Ura+ Ade−His+ recombinants carrying the mutations of both ade6 heteroalleles must exist. Intragenic COs, if arising at all, are thus potentially only a minor cause in such progeny among gene conversions, which are already relatively rare events. Rather, it is simple gene conversions at single loci, which are primarily generated by mismatch repair or DNA synthesis during DNA repair, that are responsible.

Figure 1. Meiotic recombination assay composed of ade6 heteroalleles flanked by artificially introduced markers ura4+aim2 & his3+aim. (A) Schematic showing the meiotic recombination assay at ade6 (yellow) and its common outcomes. Ade+ recombinants can arise via gene conversion (GC) associated with a crossover (GC-CO) or a non-crossover (GC-NCO). The positions of ade6, and the artificially introduced markers ura4+aim2 (green) and his3+aim (light blue) on chromosome 3 are indicated [in bps]. Positions of point mutations are shown as ▼ and ×. (B) Schematic of the ade6 coding sequence indicating the point mutations and their positions (approximately to scale) used in the recombination assays, hotspots are indicated in red, and non-hotspots in light blue. The distance between the sequence polymorphisms across the homologs is indicated in relation to the given hotspot of each cross [in bp].
MutSα and MutLα are strong negative modulators of recombination frequency specifically at short intragenic intervals. Potential candidates for genetic pathways modulating recombination frequency at intragenic intervals of different lengths are MutS-MutL complexes, which bind to heteroduplex DNA and repair mismatches. Sz. pombe has a streamlined nuclear mismatch repair system consisting of MutSα (Msh2-Msh6), MutSβ (Msh2-Msh3), and a single MutL (MutLα, Mlh1-Pms1); there is also a mitochondrial MutS protein called Msh1. Importantly, the meiotic pro-crossover factors MutSγ (Msh4-Msh5), the meiosis-specific
MutS\textsubscript{\alpha} and MutL\textsubscript{\alpha}, but not MutS\textsubscript{\beta}, are major modulators of the gene conversion (GC) rate, and of the crossover (CO) frequency among GC events. (A,B) Frequency of GC in wild type (WT), msh2, msh3, msh6, mlh1, and pms1 mutants (A) at the intragenic 84bp interval ade6-M216 × ade6-3083: UoA110 × UoA100 (WT, n = 12), UoA478 × UoA476 (msh2-30, n = 6), UoA494 × UoA492 (msh3Δ, n = 6), UoA482 × UoA480 (msh6Δ, n = 6), UoA364 × UoA361 (mlh1Δ, n = 8), UoA407 × UoA405 (pms1-16, n = 5), UoA828 × UoA830 (msh2-30 mlh1Δ, n = 6); (B) at the intragenic 1,320 bp interval ade6-3083 × ade6-469: ALP733 × ALP731 (WT, n = 20), UoA477 × UoA479 (msh2-30, n = 6), UoA493 × UoA495 (msh3Δ, n = 6), UoA481 × UoA483 (msh6Δ, n = 6), UoA362 × UoA371 (mlh1Δ, n = 11), UoA406 × UoA410 (pms1-16, n = 6), UoA827 × UoA829 (msh2-30 mlh1Δ, n = 6). (C,D) Frequency of CO between his3\textsuperscript{\beta-}\textsuperscript{aim} and ura4\textsuperscript{\beta-}\textsuperscript{aim} associated with GC events at ade6 in wild type (WT), msh2, msh3, msh6, mlh1, and pms1 mutants (C) at the intragenic 84 bp interval ade6-M216 × ade6-3083: strains as in (A); (D) at the intragenic 1,320 bp interval ade6-3083 × ade6-469: strains as in (B). n indicates the number of independent crosses. For details of data see Supplementary Table S3.

Figure 3. MutS\textsubscript{\alpha} and MutL\textsubscript{\alpha}, but not MutS\textsubscript{\beta}, are major modulators of the gene conversion (GC) rate, and of the crossover (CO) frequency among GC events. (A,B) Frequency of GC in wild type (WT), msh2, msh3, msh6, mlh1, and pms1 mutants (A) at the intragenic 84bp interval ade6-M216 × ade6-3083: UoA110 × UoA100 (WT, n = 12), UoA478 × UoA476 (msh2-30, n = 6), UoA494 × UoA492 (msh3Δ, n = 6), UoA482 × UoA480 (msh6Δ, n = 6), UoA364 × UoA361 (mlh1Δ, n = 8), UoA407 × UoA405 (pms1-16, n = 5), UoA828 × UoA830 (msh2-30 mlh1Δ, n = 6); (B) at the intragenic 1,320 bp interval ade6-3083 × ade6-469: ALP733 × ALP731 (WT, n = 20), UoA477 × UoA479 (msh2-30, n = 6), UoA493 × UoA495 (msh3Δ, n = 6), UoA481 × UoA483 (msh6Δ, n = 6), UoA362 × UoA371 (mlh1Δ, n = 11), UoA406 × UoA410 (pms1-16, n = 6), UoA827 × UoA829 (msh2-30 mlh1Δ, n = 6). (C,D) Frequency of CO between his3\textsuperscript{\beta-}\textsuperscript{aim} and ura4\textsuperscript{\beta-}\textsuperscript{aim} associated with GC events at ade6 in wild type (WT), msh2, msh3, msh6, mlh1, and pms1 mutants (C) at the intragenic 84 bp interval ade6-M216 × ade6-3083: strains as in (A); (D) at the intragenic 1,320 bp interval ade6-3083 × ade6-469: strains as in (B). n indicates the number of independent crosses. For details of data see Supplementary Table S3.
Interestingly, there is also a substantial shift in CO classes among gene conversion events from mostly Ura^+ His^+ to mainly Ura^− His^+ in mutSo×mutLo mutants at the short intervals ade6-M216 × ade6-3083 and ade6-149 × ade6-3049, but not at the short ade6-51 × ade6-3049 interval (Supplementary Fig. S4A–C). At long intervals (ade6-3083 × ade6-469, ade6-M375 × ade6-469, ade6-M216 × ade6-3049) this shift is not observed (Supplementary Fig. S4D–F). The change in CO classes among gene conversion events at the short intervals (ade6-M216 × ade6-3083 and ade6-149 × ade6-3049) is not a consequence of selective survival or the formation of diploid or disomic spores, because mutSo×mutLo mutants have a spore viability similar to wild type, and the extent of the phenotype is the same in several different mutants (Supplementary Table S3). As with intragenic recombination frequency, the mutSΔ-deletion msh3Δ behaves just like wild type for CO outcome (Fig. 3C,D; p = 0.439 against wild type, two-tailed Mann-Whitney U; Supplementary Figs S4A and S4D).

The observed effects of different parental and recombinant classes amongst progeny having undergone a gene conversion event can be explained by envisioning a DSB 5′ or 3′ of a point mutation leading to a recombination intermediate (D-loop, Holliday junction), which will then be processed immediately at the break site, or ends up somewhat removed from the initial break site by multiple consecutive invasion steps, by branch migration, or both31–33. The genetic makeup of the progeny is, therefore, a compound result of processing distinct recombination intermediates in different ways. The genetic composition of wild-type and mutant progeny resulting from the meiotic recombination assays can be explained as different combinations of scenarios suggested previously34. For example, recombination between ade6-3083 and ade6-M216, which gives rise to mainly Ura^− Ade^− His^+ NCOs and Ura^− Ade^+ His^− COs, may be explained by the model in Fig. 4A. In this model, a bias in favour of Ura^− Ade^− His^+ COs stems from strand exchange/branch migration being constrained to within the region defined by the ade6-3083 − ade6-M216 interval and resolution of the recombination intermediate occurring by D-loop cleavage (Fig. 4A,C). Ura^− Ade^+ His^− COs and additional HJ resolution (Fig. 4A,C).

We also considered whether this alteration of recombination outcome at ade6-M216 × ade6-3083 in mutSo×mutLo mutants, which leads to relatively few Ade^− His^+ Ura^− COs and a big increase in the proportion of Ade^− His^+ Ura^+ COs (Fig. 3, Supplementary Fig. S4A), might have something to do with the complexity of the ade6-3083 allele. This allele consists of multiple substitution mutations and can potentially form a C/C-mismatch in the heteroduplex DNA during strand exchange that is less efficiently repaired during meiosis than other mismatches35. However, as mentioned above, a moderate shift of CO recombinant classes among intragenic events can also be seen at another small interval, ade6-149 × ade6-3049 (Supplementary Fig. S4B). Unlike ade6-3083, ade6-3049 contains only a single nucleotide difference (Supplementary Table S1) and, therefore, the complexity of a given ade6 allele is unlikely to be the critical factor affecting the shift in CO recombinant class. This is complicated by the fact that a third small interval, ade6-51 × ade6-3049, does not show this shift between CO recombinant categories, similar to long intervals (Supplementary Fig. S4C–F). We think that a deficit in heteroduplex rejection and mismatch repair, caused by loss of msh2, could result in strand exchange/branch migration extending beyond the non-hotspot mutation (i.e. ade6-M216 or ade6-149) prior to D-loop cleavage/HJ resolution, with the base-pair mismatches in the recombinant chromosomes remaining unrepaired. Together, these altered features could explain the increase in Ade^− His^+ Ura^− COs at the ade6-M216 × ade6-3083 and ade6-149 × ade6-3049 intervals in mutSo×mutLo mutant crosses (Fig. 4B,C). However, why ade6-51 × ade6-3049 would not show this behavior remains unclear; potentially the positioning of the DSBs in relation to the hotspot mutations could play a role here.

Recombination outcome in a msh2Δ in *Saccharomyces cerevisiae* has also been shown to be more complex than in wild type36,37. Intriguingly, in *S. cerevisiae* the action of Msh2 seems to be restricted to class I COs, which are subjected to CO interference, whereas Mus81-dependent class II COs are unchanged in msh2Δ38. *Sz. pombe* operates only a class II CO pathway via Mus81-processing, completely lacking a class I CO pathway. Nevertheless, the absence of Msh2 in fission yeast has a profound effect on CO frequency, and the way recombination intermediates are processed (Fig. 3, Supplementary Fig. S4).

**Fml1 is a negative modulator of CO frequency among gene conversion events independent of the distance between point mutations.** The DNA helicases, Fml1 and Rqh1, are also prime candidates for modulating recombination frequency at intragenic intervals of different lengths39,40. However, Fml1 apparently does not modulate gene conversion levels, as at all intragenic intervals tested, fml1Δ is similar to wild type (Fig. 5A,B, Supplementary Fig. S5A). In contrast, the RecQ-family DNA helicase Rqh1 is required for wild-type levels of gene conversion12. The deletion of *rqh1* reduces gene conversion frequency to about a third of wild-type percentage at short (ade6-M216 × ade6-3083, ade6-3049 × ade6-469) intervals, and to about a tenth of wild-type frequency at the long ade6-3083 × ade6-469 interval (Fig. 5A,B, Supplementary Fig. S5).

As with long intervals12, fml1Δ results in a ~10 percentage point increase of CO frequency among gene conversion events at short intervals (Fig. 5C,D, Supplementary Fig. S5). The absence of Rqh1 induces moderate increases in CO levels among gene conversion events at the 84 bp ade6-M216 × ade6-3083 and the 1,320 bp ade6-3083 × ade6-469 interval, which are not statistically significant (Fig. 5C,D). However, at the 254 bp ade6-3049 × ade6-469 interval CO frequency among ade6^− events is raised by 17 percentage points in rqh1Δ (p = 3.72 × 10^-8 against wild type, two-tailed Mann-Whitney U) (Supplementary Fig. S5). The ade6-3083 allele contains multiple point mutations and thus represents a more complex situation than the ade6-3049 allele, which only harbors a single point mutation. Fml1 can seemingly drive NCO pathway(s) independently of the complexity of the underlying DNA sequence, because it has the same effect in crosses with complex and single-mutation alleles. In contrast, Rqh1 can apparently fulfill this role only at the simple ade6-3049 allele.

In *Sz. pombe* Fml1 has been shown to specifically limit CO formation during the late CO/NCO-decision12. Fml1 acts as a promotor of NCOs, likely by driving late recombination intermediates into the SDSA pathway, after strand invasion and DNA synthesis has happened. In accordance with this, absence of *fml1* leads to an increase in CO among Ade^+ gene conversion events, but has little effect on intragenic recombination itself (Fig. 5,
Supplementary Fig. S5)12. This role is independent of the size of the intragenic interval, with Fml1 driving 10–12% of NCO recombination in any case.

The deletion of \( rqh1 \) has a very strong meiotic phenotype, leading to reductions in intragenic recombination, CO, and spore viability (Fig. 5, Supplementary Fig. S5). This on its own would indicate an early role in promoting strand exchange and/or DSB resection, but then Rqh1 additionally is capable of promoting NCO formation among \( ade6^+ \) events at some intragenic intervals during later stages of recombination (Fig. 5, Supplementary Fig. S5). Most likely this is due to Rqh1 actually performing the following functions: (I) promotion of interhomolog recombination events, probably in cooperation with Rad55–57 and Rlp1-Rdl1-Sws1, but independently of Sfr1-Swi5,34 potentially also by providing longer resection tracts39; (II) dismantling D-loops, this enables the release of break ends to search for homology elsewhere, starts cycles of multiple consecutive invasion steps, and provides opportunities for Fml1 to drive NCO formation via SDSA; and (III) branch migration of established D-loops and Holliday junctions, thereby promoting heteroduplex DNA formation further away from the break site38.

Overall, these data show that Fml1 has likely no role in modulating gene conversion levels, but drives NCO formation downstream after successful strand invasion and DNA synthesis. Rqh1 promotes intragenic recombination, but also has moderate anti-recombinogenic activity in CO formation among gene conversion events.

In conclusion, factors directly involved in generating CO and NCO recombinants during meiosis have been identified and characterized in recent years12–15,22, and several inroads have been made in understanding how
template choice is regulated and executed during meiotic recombination\cite{10,34}. However, we still only have a basic understanding of how underlying DNA sequence polymorphisms influence meiotic recombination outcomes. This is critically important for understanding recombination event distribution in natural populations, where any two parental genomes will be littered with sequence polymorphisms. Here, we demonstrate that specific DNA sequence differences between the two homologs strongly impact on which outcome is achieved, and that this is largely driven by the action of the MutS–MutL complex. This highlights the importance of the interplay between cis- and trans-factors in shaping the genetic diversity of a given population.

Material and Methods

Bacterial and yeast strains and culture conditions. E. coli strains were grown on LB and SOC media – where appropriate containing 100 μg/ml Ampicillin\cite{40}. Competent cells of E. coli strains NEB10®-beta (New England BioLabs Inc., Ipswich, MA, USA), and XL1-blue (Agilent Technologies, Santa Clara, CA, USA) were transformed following the protocols provided by the manufacturers. S. pombe strains used for this study are listed in Supplementary Table S4. Yeast cells were cultured on yeast extract (YE), and on yeast nitrogen base glutamate (YNB) agar plates containing the required supplements (concentration 250 mg/l on YE, 75 mg/l on YNB). Crosses were performed on malt extract (ME) agar containing supplements at a final concentration of 50 mg/l\cite{41}.

Different ade6 hotspot and non-hotspot sequences (Supplementary Table S1) were introduced by crossing the respective mutant ade6 strain with ade6\textsuperscript{+} strains carrying the ura4\textsuperscript{+} and his3\textsuperscript{+} artificially introduced markers (aim) (UoA95, UoA96, UoA97, UoA98)\cite{22}. The point mutations in the ade6 alleles were verified by Sanger DNA sequencing (Source BioScience, Nottingham, UK) (Supplementary Table S1).

Using an established marker swap protocol\cite{42} the natMX6-marked rqh1Δ-G1 was derived from an existing rqh1Δ::kanMX6 allele\cite{43}, creation of the natMX6-marked pms1-16 insertion mutant allele has been described previously\cite{44}.

Marker cassettes to delete msh3, and msh6, and to partially delete msh2 were constructed by cloning targeting sequences of these genes into pFA6a-kanMX6, pAG25 (natMX4), and pAG32 (hphMX4), respectively, up- and downstream of the dominant drug resistance marker\cite{45,46}. The targeting cassettes were released from plasmids (pALo130, pALo132, pALo134) generated for this purpose by a restriction digest, and transformed into the strains FO652 (msh2 and msh6) and ALP729 (msh3). For specifics of strain and plasmid construction, please refer to Supplementary Materials. Plasmid sequences are available on figshare (https://dx.doi.org/10.6084/m9.figshare.6949274).

Transformation of yeast strains was performed using an established lithium-acetate procedure\cite{47}. All plasmid constructs were verified by DNA sequencing (Source BioScience plc, Nottingham, UK).

All DNA modifying enzymes (high-fidelity DNA polymerase Q5, restriction endonucleases, T4 DNA ligase) were supplied by New England BioLabs. Oligonucleotides were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Genetic and molecular assays. Determination of spore viability by random spore analysis and the meiotic recombination assay have been previously described in detail\cite{22,44}.

Figure 5. The RecQ-family helicase Rqh1, but not the FANCM-type helicase Fml1, is a major modulator of the gene conversion (GC) rate. Rqh1 and Fml1 are major modulators of crossover (CO) frequency among GC events. Frequency of GC in WT, fml1, and rqh1 deletions (A) at the intragenic 84 bp interval ade6-M216 × ade6-3083: UoA110 × UoA100 (WT, n = 12), UoA450 × UoA447 (fml1Δ, n = 9), UoA502 × UoA499 (rqh1Δ, n = 6); (B) at the intragenic 1,320 bp interval ade6-3083 × ade6-469: ALP733 × ALP731 (WT, n = 20), ALP1133 × MCW4718 (fml1Δ, n = 15), ALP781 × ALP780 (rqh1Δ, n = 10). Frequency of CO between his3\textsuperscript{+}-aim and ura4\textsuperscript{+}-aim2 associated with GC events at ade6 in WT, fml1, and rqh1 deletions (C) at the intragenic 84 bp interval ade6-M216 × ade6-3083: strains as in (A); (D) at the intragenic 1,320 bp interval ade6-3083 × ade6-469: strains as in (B). n indicates the number of independent crosses. For details of data see Supplementary Table S3.
To test whether intragenic COs exist, genomic DNA of Ura⁺ Ade⁻ His⁺ progeny from an ade6-3083 × ade6-469 (ALP733 × ALP731) cross was used to PCR-amplify the ade6 locus (oligonucleotides oUA219 5'-AAAGTTGGATTTCCACAATGC-3' and oUA66 5'-GTCATGGTCCCTATGC-3') for Sanger sequencing (Eurofins Scientific, Brussels, Belgium) with oUA219, oUA66, or nested oligonucleotides oUA779 5'-CTCATAAGCTGAGCTC-3' and oUA780 5'-AAGCTTCCTCATAGCACGG-3'.

**Data presentation and statistics.** Raw data is available on figshare (https://doi.org/10.6084/m9.figshare.6949274). Line graphs were produced using Microsoft Excel 2016 (version 16.0.4638.1000, 32-bit). Box-and-whisker plots were created in R (version i386, 3.0.1) (http://www.r-project.org/) using the standard settings of the boxplot() function. The lower and upper ‘hinges’ of the box represent the first and third quartile, and the bar within the box indicates the median (=second quartile). The ‘whiskers’ represent the minimum and maximum of the range, unless they differ more than 1.5-times the interquartile distance from the median. In the latter case, the borders of the 1.5-times interquartile distance around the median are indicated by the ‘whiskers’ and values outside this range (‘outliers’) are shown as open circles. R was also used to compute Kruskal-Wallis test and Tukey’s Honest Significant Differences employing the kruskal.test() and TukeyHSD() functions, respectively. Mann–Whitney U tests were performed as previously described.

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Author contributions
A.L. and M.C.W. conceived this study. A.L., S.D.B., S.J.M., M.N.A. and M.J. conducted the experiments. A.L. drafted the manuscript. All authors read, revised and approved the manuscript.

Competing interests
The authors declare no competing interests.

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SUPPLEMENTARY MATERIALS

DNA sequence differences are determinants of meiotic recombination outcome

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Specifics of yeast strain and plasmid construction

The open reading frame of msh2 (SPBC19G7.01c) overlaps with the open reading frame of cwf14, to avoid potentially affecting Cwf14 expression and function only a small portion of the 5' end of msh2 was deleted. A targeting cassette for msh2 was constructed by cloning an upstream flanking sequence of msh2 (PCR using oligonucleotides oUA47 5'-AATAAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' and oUA48 5'-AATTAGGATCCGATGCTAGTAAATACAC-3' on genomic DNA of ALP1594) digested with PvuII and BamHI into pAG32' linearized with PvuII and BamHI. The resulting plasmid (pALo129) was linearized by digesting with SacI and SpeI and a part of the coding sequence of msh2 (PCR using oligonucleotides oUA49 5'-AATTAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' and oUA50 5'-AATTAACAGCTGCTTTTCGTAGTAAATACAC-3' on genomic DNA of ALP1594) digested with SacI and SpeI was inserted by standard cloning to give pALo130. The transformation cassette was released by a PvuII-SpeI digest and transformed into the standard lab strain FO652. This construct almost completely removes the msh2 coding sequence plus an additional 299 bps upstream of the Start codon. Correct integration was monitored by selection for hygromycin B resistance and verified by PCR; all strains carrying the msh2-30::hphMX4 insertion mutation are derived by crossing from the original transformant (UoA459).

A deletion cassette for msh3 (SPAC8F11.03) was constructed by cloning an upstream flanking sequence of msh3 (PCR using oligonucleotides oUA51 5'-AATTAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' and oUA52 5'-AATTAACAGCTGCTTTTCGTAGTAAATACAC-3' on genomic DNA of ALP1594) digested with PvuII and BamHI into pAG25' linearized with PvuII and BamHI. The resulting plasmid (pALo131) was linearized by digesting with SacI and SpeI and a downstream flanking sequence of msh3 (PCR using oligonucleotides oUA53 5'-AATTAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' and oUA54 5'-AATTAACAGCTGCTTTTCGTAGTAAATACAC-3' on genomic DNA of ALP1594) digested with SacI and SpeI was inserted by standard cloning to give pALo132. The transformation cassette was released by a PvuII-SpeI digest and transformed into the standard lab strain ALP729. This construct almost completely removes the msh3 coding sequence: at the 5' end an additional 37 bps upstream of the Start codon are deleted, and at the 3' end the last 12 bps of the coding sequence are retained. Correct integration was monitored by selection for CLONNAT-resistance and verified by PCR; all strains carrying the msh3A-32::natMX4 deletion are derived by crossing from the original transformant (UoA460).

A deletion cassette for msh6 (SPCC285.16c) was constructed by cloning an upstream flanking sequence of msh6 (PCR using oligonucleotides oUA55 5'-AATTAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' and oUA56 5'-AATTAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' on genomic DNA of ALP1594) digested with PvuII and BamHI into pFA6a-kanMX6' linearized with PvuII and BamHI. The resulting plasmid (pALo133) was linearized by digesting with SacI and SpeI and a downstream flanking sequence of msh6 (PCR using oligonucleotides oUA57 5'-AATTAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' and oUA58 5'-AATTAACAGCTGCTTTTCGTAGAAGGGAGCAGCAG-3' on genomic DNA of ALP1594) digested with SacI and SpeI was inserted by standard cloning to give pALo134. The transformation cassette was released by a PvuII-SpeI digest and transformed into the standard lab strain FO652. This construct almost completely removes the msh6 coding sequence: at the 5' end an additional 4 bps upstream of the Start codon are deleted, and at the 3' end the last 9 bps of the coding sequence are retained. Correct integration was monitored by selection for G418-resistance and verified by PCR; all strains carrying the msh6A-34::kanMX6 deletion are derived by crossing from the original transformant (UoA461).

All plasmid constructs were verified by DNA sequencing (Source BioScience plc, Nottingham, UK). DNA modifying enzymes (high-fidelity DNA polymerase Q5, restriction endonucleases, T4 DNA ligase) were supplied by New England BioLabs. Oligonucleotides were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Plasmid sequences are available online as supporting material (https://dx.doi.org/10.6084/m9.figshare.6949274).
**Supplementary Figure S1.** Intragenic COs between the 3083 and the 469 point mutations in ade6 could not be confirmed. The ade6 locus was sequenced in 32 Ade- Ura+ His+ colonies from an ade6-3083×ade6-469 (ALP733×ALP731) cross, no instances carrying both mutations were recorded. wt (wild type), 3083, and 469 in bold indicate the status of the sequence confirmed by Sanger sequencing at the 5' and 3' ends, respectively. At the 3' end, the presence of 469 was assumed in some cases (not bold, black) based on the colony being Ade- and having a wt sequence at the 5' end.

| colony number | 5' end | 3' end |
|---------------|--------|--------|
| 1             | 3083   | wt     |
| 2             | wt     | 469    |
| 3             | 3083   | wt     |
| 4             | wt     | 469    |
| 5             | wt     | 469    |
| 6             | wt     | 469    |
| 7             | wt     | 469    |
| 8             | 3083   | wt     |
| 9             | wt     | 469    |
| 10            | 3083   | wt     |
| 11            | wt     | 469    |
| 12            | wt     | 469    |
| 13            | 3083   | wt     |
| 14            | wt     | 469    |
| 15            | 3083   | wt     |
| 16            | 3083   | wt     |
| 17            | 3083   | wt     |
| 18            | 3083   | wt     |
| 19            | 3083   | wt     |
| 20            | wt     | 469    |
| 21            | wt     | 469    |
| 22            | 3083   | wt     |
| 23            | wt     | 469    |
| 24            | 3083   | wt     |
| 25            | wt     | 469    |
| 26            | 3083   | wt     |
| 27            | 3083   | wt     |
| 28            | wt     | 469    |
| 29            | 3083   | wt     |
| 30            | 3083   | wt     |
| 31            | wt     | 469    |
| 32            | wt     | 469    |
Supplementary Figure S2. MutLα is a major modulator of gene conversion (GC) rate. Frequency of GC in wild type (WT), and mlh1Δ. (A) at the intragenic 33 bp interval ade6-149×ade6-3049: UoA122×UoA497 (WT, n = 6), UoA368×UoA512 (mlh1Δ, n = 6); (B) at the intragenic 53 bp interval ade6-3049×ade6-51: UoA120×UoA463 (WT, n = 6), UoA366×UoA511 (mlh1Δ, n = 6); (C) at the intragenic 1,335 bp interval ade6-M375×ade6-469: ALP1541×ALP731 (WT, n = 16), UoA510×UoA371 (mlh1Δ, n = 6); (D) at the intragenic 1,168 bp interval ade6-M216×ade6-3049: UoA99×UoA123 (WT, n = 12), UoA368×UoA361 (mlh1Δ, n = 12); n indicates the number of independent crosses. For details of data see Supplementary Table S3.
Supplementary Figure S3. MutLα is a major modulator of crossover (CO) frequency among gene conversion (GC) events. Frequency of CO between his3<sup>+</sup>-aim and ura4<sup>+</sup>-aim2 associated with GC events at ade6 in wild type (WT), and mlh1Δ. (A) at the intragenic 33 bp interval ade6-149×ade6-3049: UoA122×UoA497 (WT, n = 6), UoA368×UoA512 (mlh1Δ, n = 6); (B) at the intragenic 53 bp interval ade6-3049×ade6-51: UoA120×UoA463 (WT, n = 6), UoA366×UoA511 (mlh1Δ, n = 6); (C) at the intragenic 1,335 bp interval ade6-M375×ade6-469: ALP1541×ALP731 (WT, n = 16), UoA510×UoA371 (mlh1Δ, n = 6); (D) at the intragenic 1,168 bp interval ade6-M216×ade6-3049: UoA99×UoA123 (WT, n = 12), UoA368×UoA361 (mlh1Δ, n = 12); n indicates the number of independent crosses. For details of data see Supplementary Table S3.
Supplementary Figure S4. Distribution of non-crossover (NCO: Ura+ His- & Ura- His+) and crossover (CO: Ura+ His+ & Ura- His+) classes among Ade* gene conversion (GC) events in wild type (WT), msh2, msh3, msh6, mlh1, and pms1 mutants (percentages in each class are shown as means ± Std. Dev. (A) at the intragenic 84 bp interval ade6-M216×ade6-3083: UoA110×UoA100 (WT, n = 12), UoA478×UoA476 (msh2-30, n = 6), UoA494×UoA492 (msh3Δ, n = 6), UoA482×UoA480 (msh6Δ, n = 6), UoA364×UoA361 (mlh1Δ, n = 8), UoA407×UoA405 (pms1-16, n = 5), UoA828×UoA830 (msh2-30 mlh1Δ, n = 6); (B) at the intragenic 33 bp interval ade6-149×ade6-3049: UoA122×UoA497 (WT, n = 6), UoA368×UoA512 (mlh1Δ, n = 6); (C) at the intragenic 53 bp interval ade6-3049×ade6-51: UoA120×UoA463 (WT, n = 6), UoA366×UoA511 (mlh1Δ, n = 6); (D) at the intragenic 1,320 bp interval ade6-3083×ade6-469: ALP733×ALP731 (WT, n = 20), UoA477×UoA479 (msh2-30, n = 6), UoA493×UoA495 (msh3Δ, n = 6), UoA481×UoA483 (msh6Δ, n = 6), UoA362×UoA371 (mlh1Δ, n = 11), UoA406×UoA410 (pms1-16, n = 6), UoA827×UoA829 (msh2-30 mlh1Δ, n = 6); (E) at the intragenic 1,335 bp interval ade6-M375×ade6-469: ALP1541×ALP731 (WT, n = 16), UoA510×UoA371 (mlh1Δ, n = 6); (F) at the intragenic 1,168 bp interval ade6-M216×ade6-3049: UoA99×UoA123 (WT, n = 12), UoA368×UoA361 (mlh1Δ, n = 12); n indicates the number of independent crosses. For details of data see Supplementary Table S3.

Supplementary Figure S5. Rqh1 and Fml1 modulating meiotic recombination outcome at the intragenic 254 bp interval ade6-3049×ade6-469: (A) Frequency of gene conversion (GC) in wild type (WT), fml1, and rqh1 mutants, UoA120×ALP731 (WT, n = 31), ALP1716×MCW4718 (fml1Δ, n = 11), MCW6587×ALP731 (rqh1Δ, n = 10); (B) Frequency of crossovers (CO) among GC events at ade6 in wild type (WT), fml1, and rqh1 mutants, crosses as in (A). n indicates the number of independent crosses. For details of data see Supplementary Table S3.
Supplementary Table S1. Sequence and position (counted from the A of the start codon ATG as first position) of ade6 point mutations (indicated in bold)

| allele   | position | DNA sequence                  | reference                      |
|----------|----------|-------------------------------|--------------------------------|
| ade6-M216| G47A     | ggtcaattgATGaaacatgtg         | Szankasi et al., 1988³         |
| ade6-M375| G133T    | aacaaattgCTGagacatgtg         | Szankasi et al., 1988³         |
| ade6-M26 | G136T    | aattgattgATGaaacatgtg         | Szankasi et al., 1988³         |
| ade6-2074| G136T/G142C | aattgattgATGaaacatgtg     | Steiner & Smith, 2005⁴          |
| ade6-3083| A131G/G134T/G136T/G142C | aattgattgATGaaacatgtg     | Steiner & Smith, 2005⁴          |
|          | /G144T/A146G/A148C |                                   |                                |

¹previously reported as T956C³
²previously reported as T956C³
³previously reported as T956C³
⁴previously reported as T956C³
⁵previously reported as T956C³

Supplementary Table S4. Yeast strain list

| Strain | Relevant genotype | Origin |
|--------|-------------------|--------|
| ALP729 | h²³ arg2-D4 his3-D1 leu1-32 ura4-D18 | lab strain³ |
| ALP731 | h⁴⁰° ade6-649 his3⁴⁰°-aim3-D4 his3-D1 ura4-D18 | Lorenz et al., 2010¹⁰ |
| ALP733 | h⁴⁰° ade6-3083 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | Lorenz et al., 2010¹⁰ |
| ALP780 | h⁴⁰° rhp1::kanMX6 ade6-649 his3⁴⁰°-aim3-D4 his3-D1 ura4-D18 | Lorenz et al., 2014¹¹ |
| ALP781 | h⁴⁰° rhp1::kanMX6 ade6-3083 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | Lorenz et al., 2014¹¹ |
| ALP1133| h⁴⁰° fml1::hphMX4 ade6-3083 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | Lorenz et al., 2012²⁰ |
| ALP1541| h⁴⁰° ade6-M375 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | Lorenz et al., 2012²⁰ |
| ALP1594| h⁴⁰° ade6-750 arg3-D4 his3-D1 leu1-32 ura4-D18 | lab strain¹² |
| ALP1716| h⁴⁰° fml1::hphMX4 ura4⁴⁰°-aim2 ade6-3049 his3-D1 leu1-32 ura4-D18 | this study |
| FO652  | h⁴⁰° arg3-D4 his3-D1 leu1-32 ura4-D18 | lab strain¹¹ |
| FO1285 | h⁴⁰° ade6-M26 ura4⁴⁰°-aim2 arg3-D4 his3-D1 leu1-32 ura4-D18 | lab strain |
| MCV4718| h⁴⁰° fml1::hphMX4 ade6-649 his3⁴⁰°-aim3-D4 his3-D1 ura4-D18 | Lorenz et al., 2012²⁰ |
| MCV6587| h⁴⁰° rhp1::kanMX6 ura4⁴⁰°-aim2 ade6-3049 his3-D1 leu1-32 ura4-D18 | this study |
| U0A95  | h⁴⁰° ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A96  | h⁴⁰° ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A97  | h⁴⁰° his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A98  | h⁴⁰° his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A99  | h⁴⁰° ade6-M216 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A100 | h⁴⁰° ade6-M216 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A104 | h⁴⁰° ade6-3074 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A106 | h⁴⁰° ade6-3074 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A110 | h⁴⁰° ade6-3083 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A112 | h⁴⁰° ade6-704 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A115 | h⁴⁰° ade6-704 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A116 | h⁴⁰° ade6-62 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A119 | h⁴⁰° ade6-52 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A120 | h⁴⁰° ade6-3049 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A122 | h⁴⁰° ade6-3049 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A123 | h⁴⁰° ade6-3049 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A361 | h⁴⁰° mih1::kanMX6 ade6-M216 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A362 | h⁴⁰° mih1::kanMX6 ade6-3083 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A364 | h⁴⁰° mih1::kanMX6 ade6-3083 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A366 | h⁴⁰° mih1::kanMX6 ade6-3049 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A368 | h⁴⁰° mih1::kanMX6 ade6-3049 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A371 | h⁴⁰° mih1::kanMX6 his3⁴⁰°-aim6 ade6-469 arg3-D4 his3-D1 ura4-D18 | this study |
| U0A405 | h⁴⁰° pms1::natMX4 ade6-M216 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A406 | h⁴⁰° pms1::natMX4 ura4⁴⁰°-aim2 ade6-3083 his3-D1 leu1-32 ura4-D18 | this study |
| U0A407 | h⁴⁰° pms1::natMX4 ade6-3083 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A410 | h⁴⁰° pms1::natMX4 his3⁴⁰°-aim ade6-469 arg3-D4 his3-D1 ura4-D18 | this study |
| U0A447 | h⁴⁰° fml1::natMX6 ade6-M216 ura4⁴⁰°-aim2 his3-D1 leu1-32 ura4-D18 | this study |
| U0A450 | h⁴⁰° fml1::natMX6 ade6-3083 his3⁴⁰°-aim arg3-D4 his3-D1 ura4-D18 | this study |
| U0A459 | h⁴⁰° msh2-30::hphMX4 arg3-D4 his3-D1 leu1-32 ura4-D18 | this study |
| U0A460 | h⁴⁰° msh2-32::kanMX6 arg3-D4 his3-D1 leu1-32 ura4-D18 | this study |
| U0A461 | h⁴⁰° msh6-34::natMX4 arg3-D4 his3-D1 leu1-32 ura4-D18 | this study |
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