Apparent Epigenetic Meiotic Double-Strand-Break Disparity in *Saccharomyces cerevisiae*: A Meta-Analysis

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ABSTRACT Previously published, and some unpublished, tetrad data from budding yeast (*Saccharomyces cerevisiae*) are analyzed for disparity in gene conversion, in which one allele is more often favored than the other (conversion disparity). One such disparity, characteristic of a bias in the frequencies of meiotic double-strand DNA breaks at the hotspot near the *His4* locus, is found in diploids that undergo meiosis soon after their formation, but not in diploids that have been cloned and frozen. Altered meiotic DNA breakability associated with altered metabolism-related chromatin states has been previously reported. However, the above observations imply that such differing parental chromatin states can persist through at least one chromosome replication, and probably more, in a common environment. This conclusion may have implications for interpreting changes in allele frequencies in populations.

KEYWORDS *HIS4*; *ARG4*; double-strand breaks; mismatch repair; genetic recombination

Definitions

Gene conversion: Deviation from normal, 4:4 meiotic segregation, variable in position and involving only a small fraction of a chromosome in any given act. In budding yeast, conversion is characteristically seen either as a 6:2 or 2:6 segregation (full conversion, FC) or as a 5:3 or 3:5 segregation (half conversion, HC), with the number of copies of the dominant, usually wild-type, allele noted first.

Conversion disparity: A significant difference in the frequencies of 6:2 *vs.* 2:6 and/or in 5:3 *vs.* 3:5 tetrads.

His4: Generic term for locus of the wild-type (*HIS4*) allele or the recessive mutant (*his4*) allele.

Arg4: Generic term for locus of the wild-type (*ARG4*) allele or the recessive mutant (*arg4*) allele.

Epigenetic: In this paper, epigenetic refers to a transmissible change in a phenotype of a gene whose nucleotide sequence remains unchanged.

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THE primary metric of evolution is a change in the relative frequencies of a gene and its allele. The relative decline of an allele (see Vitalis *et al.* 2014, for example) is classically understood to indicate that this allele causes diminished reproductive success of the organism. As explained below, however, the same data could indicate that the allele is handicapped at being transmitted through meiosis.

Relevant Features of Meiotic Double-Strand-Break Repair

Meiosis in the yeast *Saccharomyces cerevisiae*, as in human males (Odenthal-Hesse *et al.* 2014), may be viewed in terms of the repair of programmed double-strand breaks (DSBs) occurring at DSB hotspots (Szostak *et al.* 1983). As shown in Figure 1, the repair process involves the loss of a stretch of nucleotides from the broken chromosome, often to be replaced with information from the intact homolog. If the lost nucleotide sequence includes a genetic marker, the repair product (tetrad of haploid cells) may occasionally fail to display normal segregation for the marker, with the allele contributed by the broken parent being underrepresented (gene conversion). If the two parental hotspots are equally subject to DSBs, as is typically true, such gene conversion *per se* will not cause an overall change in allele frequencies in the population; among half conversions (HCs: see *Definitions*), the frequency of

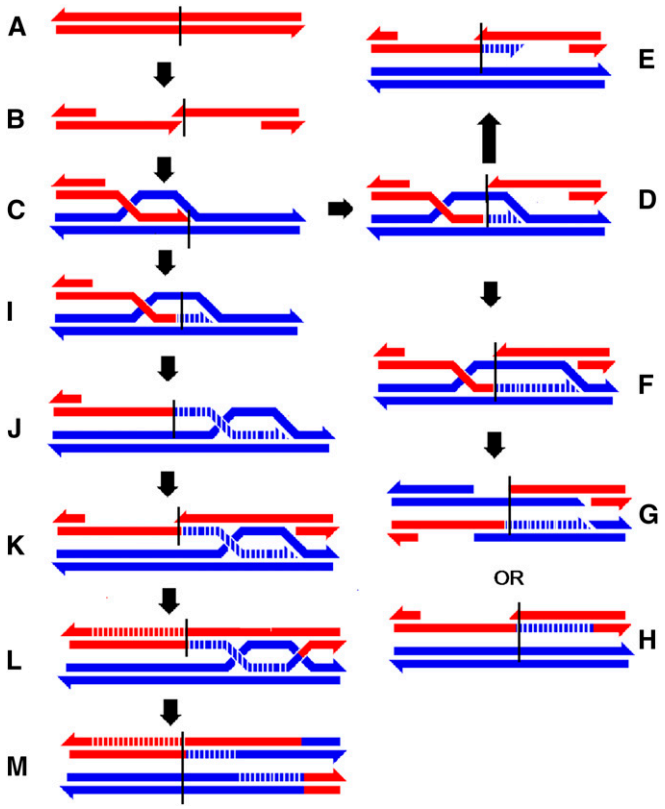

Figure 1 Two pathways for double-strand-break repair in WT yeast (Stahl and Foss 2010). The mitotic pathway (Kohl and Sekelsky 2013): An initiating DSB (A) is followed by resection of 5′ ends (B) and invasion of an intact homolog by one of the 3′-ended overhanging strands so created, resulting in a D-loop (C) and blocking further resection of that strand. The vertical bars mark the level of the initiating break. Extension of the invading strand enlarges the D-loop until enlargement is stopped, perhaps by annealing with the other single strand (D). This pathway gives noncrossovers (E and H), by unwinding of the intermediate, or noninterfering crossovers (G) by cutting of the junctions. In E, G, and H, DNA synthesis will close any gaps. The meiotic pathway (Kohl and Sekelsky 2013) (I–M), which generates interfering crossovers, branches from the mitotic pathway in a manner that blocks the MMR activity of Msh2 (Stahl and Foss 2010) and stabilizes some intermediates at C, creating the relatively long-lived single-end invasion. Eventual extension of the invading strand is accompanied by movement, rather than by enlargement, of the D-loop, similar to the movement of a transcription bubble. Lagging strand synthesis on intermediate J may be required (see Wang *et al.* 2003). Near the DSB, segments of the bivalent with three strands of one color indicate a potential HC in favor of an allele from the blue parent that is located there. In the mitotic pathway, a mismatch in that region can become an FC by MMR. In the meiotic pathway, such a mismatch can be repaired (independently of Msh2) to give either an FC or a normal 4:4 segregation, depending on which strands are the first to be cut when the double-Holliday junction is resolved.

5:3 tetrads will statistically equal the 3:5 tetrad frequency and, among full conversions (FCs: see *Definitions*), the 6:2 and 2:6 tetrads will also be equal. If, however, one hotspot is consistently more subject to DSBs than is its allelic hotspot (DSB disparity), the 5:3 and 3:5 tetrad frequencies will be statistically unequal, as will the 6:2 and 2:6 tetrad frequencies. In the absence of any other source of conversion disparity, we expect these two inequalities to favor the same allele and to be of the same magnitude.

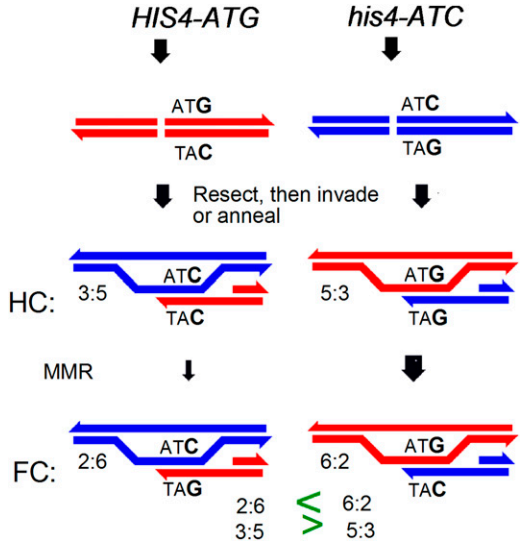

Figure 2 Mismatch repair (naïve expectation). For the marker *his4-ATC*, located close to a DSB hotspot, repair involves intermediate structures with C/C or G/G mismatches, depending on both the location of the cut relative to the marker and on which of the two parents was cut. Repair of such mismatches generates FC 2:6 or 6:2 tetrads, while repair failure may lead to 3:5 or 5:3 tetrads. Since G/G mismatches are repaired to FCs more often than are C/C mismatches (Detloff *et al.* 1991), the *a priori* (naïve) expectation for a marker to the right of the DSB, as drawn, is that 6:2 tetrads will be more frequent than 2:6s while 5:3s will be less frequent than 3:5s.

During an effort to reconcile a maze of contradictory conversion papers, we came to the conclusion that, depending on the protocol employed, DSB disparity can be manifested even when the two allelic hotspots at the *His4* locus of yeast are presumed to be genetically identical. The protocols differed (1) in the number of generations through which the diplophase was propagated prior to sporulation and (2) in whether or not the diplophase was stored in the freezer prior to sporulation. Neither of these differences in protocol can be expected to have altered the nucleotide sequences at the hotspots. Thus, the discrepancy in hotspot properties is likely to reflect alterations in chromatin structure imposed by the differing conditions under which the two haploid parents were propagated prior to their union. To a degree, and depending on conditions, these differences in chromatin structure are retained, for at least one round, and probably more, of DNA duplication, after union of the mating cells. In *Discussion*, the possible significance of such epigenetic DSB disparity will be briefly indicated. Our primary task in this meta-analysis is to present the evidence for the existence of epigenetic changes that are expressed meiotically as disparity in gene conversion.

Materials and Methods

Some of the data discussed here are from the Ph.D. thesis (Rehan 2012) and notebooks of M.B.M.R. The strains and methods employed in that work are described here.

Table 1 Conversion disparity due to MMR disparity for *his4-ATC*

Strain	HC		FC		Total
	5:3	3:5	6:2	2:6	
PD84	56	57	113	33	677
JS102	22	21	46	18	256
Sum	**78**	**78**	**159**	**51**	**933**

Data and sum are from Detloff *et al.* (1991). Sporulation was of established clones of diploids stored in the freezer (P. Detloff, personal communication). The haploid components of the two diploid strains are derived from *HIS4* strains AS4 and AS13 (Stapleton and Petes 1991). To control for possible background effects, two crosses were done. In PD84, the *HIS4* gene of the AS4 parent has been replaced by *his4-ATC*; in JS102, the *HIS4* gene of the A13 parent has been replaced by *his4-ATC*.

Table 2 MMR disparity with palindromic insertion markers

his4-lop				*his4-B2*			
HC		FC		HC		FC	
5:3	3:5	6:2	2:6	5:3	3:5	6:2	2:6
36	32	23	6	49	41	36	20
0.7*		0.003*		0.45*		0.045*	
0.004**				0.04**			

Data from Nag *et al.* (1989). Crosses involve sporulation of A4 × A13-based diploids stored in the deep freeze. The marker *his4-lop* is at the *SalI* site in the first quarter of the *His4* coding sequence, while *his4-B2* is 50 bp upstream from the first codon, putting both markers near the DSB hotspot. * *P*, χ^2 probability that the members of the two HC or FC classes would differ to the observed extent (or more) by chance alone. ** *P*, Fisher's exact probability that 3:5/5:3 would differ from 6:2/2:6 to the observed extent, or more, by chance alone.

Yeast strains

Yeast strains used in the previously unpublished work (Table 4) are derivatives of Y55. Full strain genotypes and details of construction are in Supplemental Material, File S1.

Yeast media

Media are fashioned after those of Cotton *et al.* (2009). See File S1 for details.

Mating and sporulation

Haploid strains were mixed and allowed to mate on a solid YPD medium at 30° overnight prior to sporulation. Mated cells were then replicated to sporulation media, either complete potassium acetate (KAC) or minimal KAC. Plates were then incubated at 23° for 3–5 days until tetrads were formed.

Genetic analysis

Tetrad dissection and analysis were carried out as described previously (Abdullah and Borts 2001) and in File S1.

To the extent they are available to the authors, reagents and strains will be made available.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Studies of conversion disparity due to DSB disparity can be complicated by a second type of conversion disparity, *viz.*, the differential efficiencies of repair of the two kinds of mismatches [mismatch repair (MMR) disparity] that are formed during DSB repair. For historical reasons, the best available data sets for our studies manifest conversion disparities that are composed of these two disparities. In order to understand these complex data, we first look at data that demonstrate MMR disparity by itself. These data (Detloff *et al.* 1991) provide a statistically solid and historically logical foundation for our analysis.

MMR disparity only

The *his4-ATC* marker, located near the *His4* DSB hotspot (Fan and Petes 1996), is the focus of our analysis. This base-pair transversion in the first codon of *His4* is subject to MMR disparity because the two kinds of mismatches resulting from DSB repair (Figure 2) are differentially subject to MMR. When the *his4-ATC* parent is cut, the resulting mismatch, G/G, is well repairable. When the *HIS4* parent is cut, however, the resulting mismatch is C/C, which is poorly repairable by the Msh2-dependent MMR diagrammed in Figure 2 (Stahl and Foss 2010). In budding yeast, Msh2-dependent repair of a mismatch near a DSB generates a 2:6 FC or a 6:2 FC tetrad, while failure to repair may lead to a 3:5 HC or a 5:3 HC tetrad. Since G/G mismatches are repaired, to FCs, more often than are C/C mismatches (Lichten *et al.* 1990; Detloff *et al.* 1991), the *a priori* expectation (Figure 2) is that 6:2 tetrads will be more frequent than 2:6, while 5:3 will be less frequent than 3:5. However, data of Detloff *et al.* (1991) (Table 1), collected from diploids formed between AS4 and AS13 strains (Stapleton and Petes 1991), fail to meet this expectation. Although FC tetrads in favor of *HIS4* (6:2) outnumber those in favor of *his4* (2:6), as expected, HC tetrads manifest no disparity at all. Judging from the statistical equality of the HC classes, we may presume that the two types of mismatches were formed in equal numbers by DSB repair. While many of the G/G mismatches were being repaired to give 6:2 FCs, the poorly repairable C/C mismatches were disappearing at the same rate, although most of those mismatches failed to become 2:6s. A proposal for the molecular basis of this striking feature of MMR at *his4-ATC* is in *Discussion*.

The *his4-ATC* marker is not unique in generating data in which the FCs differ while the HCs do not. Nag *et al.* (1989) collected conversion data for *his4* palindromic insertions using the strain background and methods of Detloff *et al.* (1991), including storage of the diploids in the freezer. These data are telling in three respects: (1) For a given cross, the two FC classes (6:2 and 2:6) are significantly different from each other. (2) The two HC classes, though equally or more abundant than the FCs, are not significantly different from each other, and (3) the data are significantly different from the naïve expectation (Figure 2) that 5:3/3:5 = 2:6/6:2. These three conditions are met for the palindromic inserts *his4-lop* and *his4-B2* (Table 2), in agreement with the *his4-ATC* data.

Table 3 Conversions at *his4-ATC* (zero growth), A4 × A13 background

	HC		FC		Tetrads
	5:3	3:5	6:2	2:6	
Wild type	6	14	13	2	102
msh2	11	20	6	6	126

Data are from Alani *et al.* (1994).

Table 4 Conversion at *his4-ATC* (zero growth), Y55 background

HC		FC	
5:3	3:5	6:2	2:6
(19	43)*	422	585

Conversions are summed from 17 crosses in Rehan (2012), wherein the data are presented as HCs and FCs, without indication of the separate values for the two HC and the two FC classes. Data for the individual crosses and a demonstration of homogeneity that justifies the calculation of the *P*-value are in File S1, Table B. Total tetrads minus 90 (8:0 + 0:8) tetrads (somatic crossovers) and nine (7:1 + 1:7) tetrads were 5191. * $P = 0.004$.

Before we examine the data indicative of environmentally imposed DSB conversion disparity at *his4-ATC*, we ask what the expectations are for such a combination of MMR and DSB disparities. Since DSBs are initiating events, any DSB disparity will affect the FCs and HCs equally. We take it as axiomatic that MMR disparity will be governed by disparity like that seen by Detloff *et al.* (1991) for G/G and C/C mismatches. This disparity leads to an excess of 6:2 tetrads over 2:6 tetrads and has no effect on the HCs. The combination of the two disparities will have different effects depending on which of the two DSB hot spots is the more active. If the hotspot *cis* to *his4-ATC* is cut more often than that of *cis* to *HIS4*, the 6:2/2:6 value will be increased beyond that due to the MMR disparity. On the other hand, if the *HIS4* hotspot is the one that is cut more often, the MMR and DSB disparities will act on the FCs in opposite directions, tending to cancel each other. Regardless of which hotspot has the greater break frequency, the effect on the HCs will be to introduce conversion disparity where there was none, and to reveal, at a glance, the direction and magnitude of the DSB disparity.

Other crosses using Detloff's strains

The data of Detloff *et al.* (1991) look solid, but conversion data for *his4-ATC* collected subsequently differ from Detloff's. Alani *et al.* (1994) examined conversion at *his4-ATC* using Detloff's strains. However, instead of inducing meiosis in an established diploid culture recovered from the freezer, as Detloff *et al.* (1991) had done, these investigators induced meiosis in populations of diploid cells soon after their formation according to a then novel technique called "zero growth" (Reenan and Kolodner 1992), in which the diplophase may, in fact, involve a few generations of growth. The sparse data of Alani *et al.* (1994) (Table 3) differed from Detloff *et al.* (1991) by being in agreement with the *a priori*, naïve expectation of opposite disparities in the HCs and FCs.

Crosses in a different background (Y55)

Whereas the zero-growth wild-type (WT) data in Table 3 were only suggestive of HC disparity, abundant zero-growth data (Table 4), collected (but not previously published) by M.B.M.R. in the laboratory of R.H.B., clearly manifest HC disparity (5:3 < 3:5).

The excess of 3:5 over 5:3 tetrads in Table 4 (as in Table 3) identifies the hotspot *cis* to the *HIS4* allele as the one that is receiving the greater share of DSBs. The disparity in the FCs in Table 4 is in the same direction, favoring the *his4-ATC* allele. The evident difference in the magnitudes of the two disparities is in accord with the expectation that, while the DSB disparity favors the *his4-ATC* allele (as shown by the HC disparity), the MMR disparity reduces that effect for the FCs by favoring the *HIS4* allele, as in Table 1.

The conclusion that the observed HC disparity (Table 4) is the result of DSB disparity is confirmed by crosses in which known requirements for MMR were eliminated. In Detloff's strain, induced to undergo meiosis with the zero-growth protocol, deletion of the MMR gene *MSH2* resulted in 11 5:3s and 20 3:5s (Table 3) of 126 total tetrads (Alani *et al.* 1994). The direction and magnitude of the disparity in the HCs were both unchanged by this loss of MMR, as expected from the observation (Detloff *et al.* 1991) that MMR disparity does not cause disparity of HCs for the *his4-ATC* marker. (The combined wild-type and *msh2* HC disparities reveal significant disparity in the HCs in Alani's data (Table 3) (17 5:3 and 34 3:5; $P = 0.025$). Similarly, in the R.H.B. lab, Hoffmann *et al.* (2005) used the zero-growth protocol to collect conversion data for *his4-ATC* in two MMR-defective derivatives of the Y55 strains used in Table 4. In both mutants (*msh2* and *mlh1*), the disparity in the HCs in favor of *his4-ATC* is significantly demonstrated (Table 5) and is essentially equal in extent in the two MMR-defective genotypes.

Insofar as MMR and DSB disparities are the only appreciable sources of conversion disparity, we may conclude that the disparity in the HCs seen in these MMR-deficient zero-growth crosses represents DSB disparity. By our hypothesis, the conversion disparity of the HCs at *his4-ATC* depends only on DSB disparity and, consequently, should be the same for the MMR proficient and deficient crosses. However, Hoffmann *et al.* (2005) ascribe significance to their failure to see, in the WT cross, the HC disparity that is evident in their MMR-defective crosses. This disagreement in interpretation requires that we quantitatively demonstrate the adequacy of our hypothesis for these data. We do so in *Appendix*, wherein we address the failure of Hoffmann *et al.* (2005) (Table 5), to see significant disparity in either the HCs or FCs in their MMR-proficient cross.

The HC data for the collection of zero-growth crosses (Table 6) are compatible with the null hypothesis that the disparities observed are independent of both the background of the strains involved and their MMR status.

Table 5 Conversions at *his4-ATC* (zero growth), Y55 background

	HC		FC		Tetrads
	5:3	3:5	6:2	2:6	
Wild type	14	15	96	111	1731
msh2	(17	36)*	15	18	545
mlh1	(35	65)**	5	7	585

Data are from Hoffmann *et al.* (2005). * $P = 0.013$ and ** $P = 0.004$.

Table 6 reproducibility of HC disparity in the zero-growth protocol

Source	5:3	3:5
Table 3 wild type	6	14
Table 3 *msh2*	11	20
Table 4 wild type	19	43
Table 5 wild type	14	15
Table 5 *msh2*	17	36
Table 5 *mlh1*	35	65

The data are compatible ($P = 0.67$) with the null hypothesis that they were drawn from the same universe.

Discussion

Unwinding and MMR

The lack of disparity between the two classes of HCs in the data of Detloff *et al.* (1991) (Table 1) provides evidence that the G/G and C/C mismatches were created equally. How is it that they remain equal when they are differentially subject to MMR? In other words, how is it that the relatively unrepairable C/C mismatches seem to "disappear" as often as the G/G mismatches are repaired to give 6:2 tetrads? Following Detloff *et al.* (1991), we propose that the way to get rid of a C/C mismatch without repairing it is to unwind it, with the likely result that it gives rise to a 4:4 tetrad (*e.g.*, as in Figure 1E, on the left side of the DSB site).

To account for the unwinding of the C/C mismatches occurring *pari-passu* with the MMR of G/G, we suggest that Msh2p, after binding equally well to C/C or G/G, activates both a helicase and an endonuclease. When the mismatch is G/G, the endonuclease often makes a nick in the invading strand on the side of the mismatch opposite the invading terminus, while for a C/C mismatch, it does so less often (Wang *et al.* 2003; Qiu *et al.* 2012). The observed equality of the two HC classes is then accounted for by assuming that helicase unwinding, which begins at the invading 3′ end, stops at the MMR-dependent nick. Polymerase then copies the intact strand, completing the MMR. In the absence of a nick to stop it, the helicase unwinds the entire heteroduplex (heteroduplex rejection).

Why was Detloff et al. (1991) ignored?

Detloff's observed FC disparity appears not to have been taken seriously by Hoffmann *et al.* (2005), who did not reference the work, perhaps because of undefined concerns regarding cryptic mismatches in Detloff's strains (P. Detloff, personal communication).

We have explained the appearance of disparity in the HCs of most of the crosses done subsequently to Detloff *et al.* (1991) as being due to DSB disparity arising from the use of the zero-growth protocol. However, data presented pre-Detloff by Lichten *et al.* (1990) are not so easily explained. Lichten *et al.* (1990) offered a set of numbers compatible with the naïvely expected conversion disparity of HCs (Table 7). They arrived at these numbers by summing two sets of data on conversion at a G-to-C transversion (*arg4-nsp*) close to the *Arg4* DSB site. However, only one of the two data sets in the sum manifests the expected FC disparity, while only the other set significantly manifests the naïvely expected HC disparity (Table 7).

Thus, while the conversion disparities in the summed numbers reported by Lichten *et al.* (1990) conform to the naïve expectation for disparate MMR, they cannot be taken seriously. On the other hand, the differences between the MGD409 and the ORD002 data sets have an obvious explanation within the framework of the thesis developed here. For both the FCs and the HCs, the ORD002 data conform with the Detloff data for *his4-ATC*, while the MGD409 data conform with the zero-growth data for *his4-ATC* (*i.e.*, less disparity in the FCs than in the HCs; *e.g.*, Table 4). However, the zero-growth protocol was not introduced until 1992. Consequently, we were tempted to conclude that the MGD409 data look like zero-growth data because this diploid, like the diploids of a zero-growth cross, was not frozen before it was sporulated. Instead, a diploid colony was isolated and then maintained as a patch on a nutrient agar Petri plate. This custom, common now as it was then, allows an estimated minimum of 30–35 generations of diploid growth. Our surmise that MGD409 was maintained on a plate, rather than being frozen, has been confirmed by the recollection of the responsible author (N. Schultes, personal communication). Our appeal to all the authors of Lichten *et al.* (1990) for information regarding ORD002 has so far failed.

Interpretation and significance of the protocol-dependent DSB differences

Abdullah and Borts (2001) demonstrated that a change in the metabolic state of a diploid cell can influence the frequency of gene conversion. Presumably it does so by introducing a change in chromatin structure and, hence, in susceptibility of the hotspot to meiotic DSBs (*e.g.*, Merker *et al.* 2008). The meta-analysis of *His4* data conducted herein provides evidence that epigenetic differences between allelic DSB hotspots, imposed during growth of the parental haploid cultures, can be retained in zygotes resulting from union of those haploids. The *Arg4* data argue that (1) the epigenetic distinction between the homologs that determines their relative DSB rates is maintained for many generations and that (2) some aspect of freezing (or thawing) the diploid removes that distinction.

Of course, the conclusions and surmises of this paper are testable by the execution of properly controlled crosses, studies that we are unable to undertake ourselves. Such studies are needed to clear up the published discrepancies exposed here as well as to prevent the occurrence of further confusions in the yeast meiosis literature. It might also stimulate analyses of the possible importance of epigenetic DSB disparity in

Table 7 Meiotic segregation of *arg4-nsp*

	HC		FC		
Strain	5:3	3:5	6:2	2:6	4:4
MGD409	(4	16)*	49	40	914
ORD002	2	5	(67	23)**	792
Sum	**6**	**21**	**116**	**63**	**1706**

Data, including sum, are from Lichten *et al.* (1990). The FC data for the two strains are statistically incompatible ($P = 0.01$). * $P = 0.014$ and ** $P < 0.0001$.

genomic studies such as those of allele frequencies in populations (Lamb 1998) or of the fate of newly introduced alleles in finite populations (Nagylaki 1983).

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Appendix

Analysis of Conversion at *his4-ATC* in Zero-Growth Crosses

Franklin W. Stahl

In contrast to the data in Table 4, the MMR-proficient cross of Hoffmann *et al.* (2005), using strains of the same background (Y55) and employing the same, zero-growth protocol, failed to demonstrate disparity in either the HCs or the FCs. Our analysis will show that this failure is statistically insignificant and that all features of those data of Hoffmann *et al.* (2005) are compatible with the following concepts (Stahl and Foss 2010): (1) DSB repair in yeast proceeds by two pathways (Figure 1); (2) these pathways differ with respect to MMR; (3) MMR in the meiotic pathway (Kohl and Sekelsky 2013, referred to as the disjunction pathway in Stahl and Foss 2010) is dependent on Mlh1, but not Msh2, and always occurs in the MMR-proficient cross, leading half the time to FC and half the time to 4:4 (restoration); (4) MMR in the mitotic pathway (Kohl and Sekelsky 2013, referred to as the pairing pathway in Stahl and Foss 2010) depends on both Mlh1 and Msh2 and sometimes fails in the MMR-proficient cross.

As elaborated below, the test will consist of calculating a value for the number of tetrads expected in each of the 12 possible conversion categories of Table 5, followed by testing the calculated values for compatibility with observed values. The calculated values will reflect the concepts of Stahl and Foss (2010), reviewed above. These concepts allow us to identify and evaluate, for each pathway, a minimal set of parameters that determine conversion at *his4-ATC*. Whether a DSB repair event will, in fact, result in an FC, an HC, or in 4:4 segregation of the *his4* marker depends on the probabilities that the event suffers a double-strand gap (resulting in an FC) or forms a heteroduplex that either does or does not undergo MMR or simply unwinds (Figure 1E). The parameters for these contingencies are defined in Table A1.

The parameters listed in Table A1 are those whose values dictate the frequencies of each of the four classes of tetrads in the three crosses of Hoffmann *et al.* (2005). A test of the adequacy of our hypothesis requires estimation of those parameters within the framework of the DSB-repair model of Stahl and Foss (2010). That model asserts that MMR in the meiotic pathway occurs only at resolution of the double-Holliday structure by junction cutting and that, in this pathway, all mismatches in the *MLH1* crosses enjoy MMR on that occasion. In *MLH1* crosses, meiotic pathway mismatches are equally likely to be repaired to FC as to 4:4 (Figure 1, L and M), depending on the disposition of the cuts that resolve the double Holliday structure (Stahl and Foss 2010). In the *mlh1* cross, all mismatches are recovered as HCs. Thus, since events in the meiotic pathway are immune to MMR disparity, as in human males (Odenthal-Hesse *et al.* 2014), the only adjustable parameters relevant to the frequencies of tetrad types deriving from that pathway are D, B, and g.

Estimating B, the breakage index: Since neither MMR-deficient cross is subject to MMR disparity, B can be estimated directly from the raw data of each cross (Table 5) as $B = (6{:}2 + 5{:}3)/(\text{FC} + \text{HC})$. The estimates for *msh2* and *mlh1* are 0.372 ± 0.10 and 0.357 ± 0.09, respectively, with an average value of 0.365, which we use for our calculations.

If conversion disparity in the MMR-deficient crosses is, in fact, due only to DSB disparity, then the same value for B, 0.365, should be applicable to both the fractions of 6:2 and 2:6 tetrads within the FC tetrads and the fractions of 5:3 and 3:5 tetrads within the HC tetrads. The statistical tests in Table A2 give large P-values, indicating compatibility with that expectation.

Normalizing data: Since the population sizes of the three crosses of Hoffmann *et al.* (2005) differ, calculations that draw upon data from different crosses require that the observed numbers be normalized to the same population size (Table A3).

Estimating additional parameters from MMR-deficient crosses (Table A4): We return to the *mlh1* and *msh2* mutant crosses (both of which lack MMR disparity) to extract values for D, P, g, and v.

Estimating remaining parameters from MMR-proficient cross (Table A5): The D and g values from Table A4 permit calculation of the number of FC tetrads from the meiotic pathway, which is not subject to MMR disparity.

Next, we subtract these estimated numbers of 6:2 and 2:6 meiotic pathway FCs from the observed numbers of 6:2 and 2:6 tetrads, revealing the number of FCs from the WT mitotic pathway. Whereas the total FCs favored 2:6s, the calculated mitotic pathway FCs favor 6:2s, indicative of MMR disparity (39.6 to 36.7) (Table A5). When the FCs due to gapping are removed from the mitotic pathway FCs, the MMR-disparity value of $m/n = 2.2$ (Table A5) provides a fit of calculated-to-observed values for the FCs (Table A6). (Unless m/n is strain specific, this ratio is probably an underestimate, judging from the FC disparity in Table 1, where the 6:2-to-2:6 ratio is $\sim$3 and is itself an underestimate of m/n depending on the fraction of events in the meiotic pathway. However, since this fraction is apt to be small in the crosses of Table 1, as argued in *Strain-specific differences*, below, the two estimates of m/n are in reasonable agreement.)

Since the fit to the HCs is statistically satisfactory, the entire WT data set of Hoffmann *et al.* (2005) is consistent with the two-pathway rules of Stahl and Foss (2010) as further specified by the demonstration (Detloff *et al.* 1991) that MMR disparity imposes no conversion disparity on HCs at *his4-ATC*. The analysis results in estimates of $B < 0.5$ and $m > n$, supporting the view

(Stahl and Foss 2010) that the failure of Hoffmann *et al.* (2005) (Table 5) to find conversion disparity in their WT cross is a result of opposing MMR and DSB disparities in the FCs combined with a shortage of data for the HCs.

Calculations of the standard errors of the parameter values is unnecessary, as well as difficult (but they are certainly large). Our calculation serves as a demonstration that a complex case of conversion disparity can be successfully modeled within the conventional framework for meiotic DSB repair in budding yeast (Stahl and Foss 2010), and obviates the need to invoke, as did Hoffmann *et al.* (2005), an unknown short-patch mismatch-repair activity functioning only in the absence of Msh2 or Mlh1.

Without further assumption, the nature of the relationship of MMR to unwinding is not revealed by these data. The interesting possibility would be that the unwinding that is responsible for the failure of MMR disparity to be manifested as disparity in HCs is a unique class, executed by the MMR system itself. The excess in the estimated value of u (0.915) over that of v (0.51) (Tables A5 and A6) permits such a test, yielding maximal values of $m = 1.1$ (essentially equal to the theoretical maximum for a probability) and $n = 0.5$. A value of unity for m is the one expected for the simple proposal that the unwinding responsible for the failure of MMR disparity to induce conversion disparity in HCs (Detloff *et al.* 1991) is, in fact, unwinding that occurs only as an action of the MMR system itself (Table A5), as proposed in *Discussion*. To make this attractive possibility more than a suggestion would require larger data sets.

Cross-specific differences: The two AS4 × AS13 data sets differ from the two Y55 sets in two respects. The HC/(HC + FC) ratios in the AS4 × AS13 crosses of Table 1 (156/366 = 0.43) and Table 3 (20/35 = 0.57) are greater than those in the Y55 crosses of Table 4 (62/1069 = 0 .06) and Table 5 (29/236 = 0.12), indicating a higher fraction of mitotic pathway events in the former crosses and suggesting that the high conversion frequencies characteristic of AS4 × AS13 crosses are due to a high rate of predominantly mitotic pathway events. This difference may be intrinsic to the strains or dependent on the differing conditions (*e.g.*, temperature) under which the sporulations are conducted.

Table A1 Parameters needed to specify the 12 tetrad classes in Table 5

Parameter	Description
B	Fraction of DSBs at the *His4* hotspot that occur on the *his4-ATC* chromosome (breakage index); applicable to both DSB-repair pathways (Figure 1B).
D	Number of meiotic pathway events that involve the *his4-ATC* site in a mismatch.
P	Number of mitotic pathway events that involve the *his4-ATC* site in a mismatch.
g	Probability of FC by double-strand gapping; assumed applicable to both DSB-repair pathways (Figure 1B).
v	Probability, in mitotic pathway only, of unwinding a mismatch in the MMR-deficient crosses in a manner that restores 4:4 segregation (*e.g.*, Figure 1E).
u	Probability, in mitotic pathway only, of unwinding a mismatch in the MMR-proficient cross; results in either an FC or a restoration, depending on the reparability of the mismatch (*e.g.*, Figure 1E).
m	Probability of MMR of G/G, giving a 6:2 tetrad; contingent on DNA unwinding in the mitotic pathway.
n	Probability of MMR of C/C, giving a 2:6 tetrad; contingent on DNA unwinding in the mitotic pathway.

Table A2 Conversions at *his4-ATC* for MMR-deficient crosses

	HC			FC		Tetrads
	5:3	3:5		6:2	2:6	
msh2 observed	17	36		15	18	545
msh2 calculated	19.3	33.6		12.1	20.9	
			$P = 0.69$			
mlh1 observed	35	65		5	7	585
mlh1 calculated	36.6	63.4		4.4	7.6	
			$P = 0.88$			

Data observed from Table 5. Calculated values for each cross are derived by applying the breakage index, $B = 0.365$, to the sum of the FCs and to the sum of the HCs, respectively. The *P*-values (χ^2, d.f. = 2) compare the data with the calculated values rounded to the nearest whole numbers.

Table A3 Conversions per 1000 tetrads at *his4-ATC*

Genotype	Conversion type					
	5:3	3:5	HC	6:2	2:6	FC
WT	8.1	8.7	**16.8**	55.5	64.1	**119.6**
msh2	31.2	66.1	**97.3**	27.5	33.0	**60.5**
mlh1	59.8	111.1	**170.9**	8.5	12.0	**20.5**

Data from Table 5 normalized to tetrads per 1000.

Table A4 Estimating parameter values from MMR-deficient crosses

	Expectation	Observed per 1000	Meiotic pathway	Mitotic pathway
FC in *mlh1*	$g(P + D)$	20.5	6.4	17.3
HC in *mlh1*	$(1 - g)(1 - v)P + (1 - g)D$	170.9	73.6	97.3
FC in *msh2*	$g(P + D) + (1 - g)D/2$	60.5	43.2	17.3
HC in *msh2*	$(1 - g)(1 - v)P$	97.3	0.0	97.3

From these four equations and the observed numbers/1000 tetrads (Table A3), the values: $g = 0.08$; $P = 216$; $D = 80$; $v = 0.51$ were extracted by solving simultaneous equations. The values for the two pathways are separately indicated. The steps in extraction of the parameters assured that the sums of the estimated contributions from the two pathways would equal the observed value for all but the smallest class (FC in *mlh1*).

Table A5 Expected tetrad frequencies (per 1000 tetrads) for the MMR-proficient cross of Table 5

	Meiotic pathway	Mitotic pathway	Observed per 1000	Meiotic pathway	Mitotic pathway	Calculated total
6:2	$B[gD + (1 - g)D/2]$	$BP[g + (1 - g)um]$	55.5	15.8	39.6	**55.5**
2:6	$(1 - B)[gD + (1 - g)D/2]$	$(1 - B)P[g + (1 - g)un]$	64.1	27.4	36.7	**64.1**
5:3	0	$BP(1 - g)(1 - u)$	8.1	0	6.1	**6.1**
3:5	0	$(1 - B)P(1 - g)(1 - u))$	8.7	0	10.6	**10.6**

Since HC ratios are unperturbed by MMR disparity (Detloff *et al.* 1991), the ratio 5:3/3:5 is $B/(1 - B)$, giving the expectations 5:3 = 6.1 and 3:5 = 10.6. The numbers of 6:2 and 2:6 tetrads contributed by the meiotic pathway were calculated using $B = 0.365$ and $g = 0.08$. $P = 216$, $D = 80$ from Table A4. These were subtracted from the total observed values to get the mitotic pathway values. From the ratio of mitotic pathway FC numbers, the ratio $m/n = 2.2$ can be obtained, independently of u, and, thus, independently of any assumption about whether all acts of unwinding render a mismatch eligible for repair. Evaluating u from the sum 5:3 + 3:5 gives $u = 0.915$.

Table A6 Conversion at *his4-ATC* in MMR-proficient strain

	HC		FC	
	5:3	3:5	6:2	2:6
Observed	14	15	96	111
Expected	10.6	18.3	96	111

Expected values per 1000 tetrads were calculated as shown in Table A5 and then increased 1.73-fold to compare with observed values (Table 5). Compatibility of HC observed with expected was conducted with a goodness of fit χ^2 test with expectations of 0.367 and 0.633 for the 5:3s and 3:5s, respectively ($P = 0.27$; d.f. = 1).

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Apparent Epigenetic Meiotic Double-Strand-Break Disparity in *Saccharomyces cerevisiae*: A Meta-Analysis

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File S1. Supplemental Materials and Methods

Stahl et al. Apparent Epigenetic Meiotic Double-Strand-Break Disparity in *Saccharomyces cerevisiae*: A Meta-analysis

Materials and Methods

Yeast Strains: Yeast strains (Table A) used in previously unpublished work (Tables 4 and B) are derivatives of Y55 (http://wiki.yeastgenome.org/index.php/Commonly_used_strains#Y55). Deletion strains were created by replacing the entire open reading frame of the relevant gene with a *KANMX4* cassette using PCR-based gene disruption (Wach *et al*. 1994). To generate a meiotically-repressed allele of *BAS1,* the *pClb2-BAS1* strain was made by replacing the native ATG start codon of *BAS1* with the *CLB2* promoter using *pFA6a-pCLB2-HA$_3$-KANMX6* plasmid (Lee and Amon 2003) as the PCR template. Single mutants were verified by PCR. Double or triple mutants were generated by crosses. When the mutant genes were located on different chromosomes, the strains were further confirmed by CHEF gel and southern blot analysis. The *pClb2-BAS1* mutation was verified by DNA sequencing.

Yeast media: Rich growth medium (YPD) consisted of 1% (w/v) yeast extract, 2% (w/v) Peptone, 2% (w/v) dextrose, supplemented with 0.005% (w/v) adenine hemisulphate solution in 0.05 M HCl. Synthetic minimal medium contained 0.68% (w/v) yeast nitrogen base without amino acids and 2% (w/v) dextrose. Synthetic complete medium was synthetic minimal medium plus adenine hemisulphate, arginine, histidine, leucine, methionine, tryptophan and uracil, each at 31.8 mg/L; phenylalanine at 79.5 mg/L; lysine and tyrosine, each at 47.7 mg/L; threonine at 318.2 mg/L and aspartic acid at 159 mg/L ("nutrient mixture"). This medium was also supplemented with 6.25 ml/L of 1% (w/v) leucine and 3 ml/L of 1% (w/v) lysine. Segregants for the *HIS4* and *his4-ATC* alleles were scored on synthetic complete medium minus histidine. Media for scoring the segregation of *NATMX4* and *HPHMX4* cassettes were prepared as YPD plus 100 µg/ml nourseothricin and 300 µg/ml hygromicin B. Two types of sporulation media were used. Complete KAC medium contained 2% (w/v) potassium acetate, 0.22% (w/v) yeast extract, 0.05% (w/v) dextrose and nutrient mixture (Cotton 2007; Cotton *et al*. 2009). For experiments that involve starvation, minimal KAC medium containing 2% potassium acetate was used, supplemented only with nutrients that cells were unable to synthesise.

Mating and sporulation: Haploid strains were mixed and allowed to mate on a solid YPD medium at 30°C overnight prior to sporulation. Mated cells were then replicated to sporulation media, either complete KAC or minimal KAC. Plates were then incubated at 23°C for 3 to 5 days until tetrads were formed.

Genetic analysis: Tetrad dissection and analysis were carried out as described previously (Abdullah and Borts, 2001). Spore colonies were replicated on various media to study the segregation of markers. Crossing over

and gene conversion were analyzed only in tetrads with four viable spores. Analysis of *HIS4* gene conversion in strains that are auxotrophic for histidine, for example the *his1* deletion or the *ade16 ade17* double deletion (Tibbetts and Appling, 2000), was conducted by crossing the dissected spore colonies to a haploid "tester strain" carrying the *his4-ATC* allele. The tester strain is *ADE16*, *ADE17* and *HIS1* and also contains a functional *HO* gene that permits self-diploidisation. After crossing with the germinating cells of the sporulated tester strain, the mated cells were replicated to a synthetic minimal medium supplemented with appropriate nutrients and grown overnight at 30°C. Only diploids that have a functional copy of *HIS4* were able to grow.

Table A Haploid strains

Name	Key feature	Genotype
Y55 2830	Wild type	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CANS ura3-1*
Y55 3569	Wild type	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MAT***α** *trp5-1 cyh2-1 MET13 lys2-c ura3-1*
Y55 3549	*ade1*Δ	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CANS ura3-1 ade1::KANMX4*
Y55 3562	*ade1*Δ	*RRP7::NATMX4 his4-ATC FUS1 HPHMX4 LEU2 MAT***α** *trp5-1 cyh2-1 MET13 lys2-c ura3-1 ade1::KANMX4*
Y55 3593	*ade16*Δ	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CANS ura3-1 ade16::KANMX4*
Y55 3594	*ade16*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MAT***α** *trp5-1 cyh2-1 MET13 lys2-c ura3-1 ade16::KANMX4*
Y55 3571	*ade17*Δ	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CANS ura3-1 ade17::KANMX4*
Y55 3572	*ade17*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MAT***α** *trp5-1 cyh2-1 MET13 lys2-c ura3-1 ade17::KANMX4*
Y55 3595	*gcn4*Δ	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CANS ura3-1 gcn4::KANMX4*
Y55 3596	*gcn4*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MAT***α** *trp5-1 cyh2-1 MET13 lys2-c ura3-1 gcn4::KANMX4*
Y55 3599	*pClb2-BAS1*	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CA ura3-1 pClb2-HA$_3$-BAS1*
Y55 3600	*pClb2-BAS1*	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MAT***α** *trp5-1 cyh2-1 MET13 lys2-c ura3-1 pClb2-HA$_3$-BAS1*
Y55 3602	*ade16*Δ *ade17*Δ	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CANS ura3-1 ade16::KANMX4 ade17::KANMX4*
Y55 3603	*ade16*Δ *ade17*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MAT***α** *trp5-1 cyh2-1 MET13 lys2-c ura3-1 ade16::KANMX4 ade17::KANMX4*
Y55 3612	*bas1*Δ	*HIS4-Hha*I *leu2-r MAT***a** *TRP5 CYH2 met13-2 lys2-d CANS ura3-1 bas1::KANMX4*
Y55 3613	*bas1*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MAT***α** *trp5-1*

		cyh2-1 MET13 lys2-c ura3-1 bas1::KANMX4
Y55 3622	*pClb2-BAS1 ade16*Δ *ade17*Δ	*HIS4-Hha*I *leu2-r MAT**a** TRP5 CYH2 met13-2 lys2-d CANS ura3-1 pClb2-HA$_3$-BAS1 ade16::KANMX4 ade17::KANMX4*
Y55 3623	*pClb2-BAS1 ade16*Δ *ade17*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MATα trp5-1 cyh2-1 MET13 lys2-c ura3-1 pClb2-HA$_3$-BAS1 ade16::KANMX4 ade17::KANMX4*
Y55 3616	*his1*Δ	*HIS4-Hha*I *leu2-r MAT**a** TRP5 CYH2 met13-2 lys2-d CANS ura3-1 his1::KANMX4*
Y55 3617	*his1*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MATα trp5-1 cyh2-1 MET13 lys2-c ura3-1 his1::KANMX4*
Y55 3618	*his1*Δ *ade1*Δ	*HIS4-Hha*I *leu2-r MAT**a** TRP5 CYH2 met13-2 lys2-d CA ura3-1 his1::KANMX4 ade1::KANMX4*
Y55 3619	*his1*Δ *ade1*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MATα trp5-1 cyh2-1 MET13 lys2-cura3-1 his1::KANMX4 ade1::KANMX4*
Y55 3626	*gcn4*Δ *ade1*Δ	*HIS4-Hha*I *leu2-r MAT**a** TRP5 CYH2 met13-2 lys2-d CANS ura3-1 gcn4::KANMX4 ade1::KANMX4*
Y55 3627	*gcn4*Δ *ade1*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MATα trp5-1 cyh2-1 MET13 lys2-c ura3-1 gcn4::KANMX4 ade1::KANMX4*
Y55 3629	*set2*Δ	*HIS4-Hha*I *leu2-r MAT**a** TRP5 CYH2 met13-2 lys2-d CANS ura3-1 set2::KANMX4*
Y55 3630	*set2*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MATα trp5-1 cyh2-1 MET13 lys2-c ura3-1 set2::KANMX4*
Y55 3631	*set2*Δ *bas1*Δ	*HIS4-Hha*I *leu2-r MAT**a** TRP5 CYH2 met13-2 lys2-d CANS ura3-1 set2::KANMX4 bas1::KANMX4*
Y55 3632	*set2*Δ *bas1*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MATα trp5-1 cyh2-1 MET13 lys2-c ura3-1 set2::KANMX4 bas1::KANMX4*
Y55 3644	*bas1*Δ *ade1*Δ	*HIS4-Hha*I *leu2-r MAT**a** TRP5 CYH2 met13-2 lys2-d CANS ura3-1 bas1::KANMX4 ade1::KANMX4*
Y55 3645	*bas1*Δ *ade1*Δ	*RRP7::NATMX4 his4-ATC FUS1::HPHMX4 LEU2 MATα trp5-1 cyh2-1 MET13 lys2-c ura3-1 bas1::KANMX4 ade1::KANMX4*

Table B Conversion disparity in RHB-lab crosses (see Table 4)

Type	Tetrad type								Tetrads	Parents
	6:2	2:6	8:0	0:8	5:3	3:5	7:1	1:7		
Wild	22	24	0	1	2	1	0	0	388	2830 x 3569
*ade1*Δ	11	17	0	0	0	0	0	0	252	3549 x 3562
ade16,17	73	99	17	18	1	7	4	2	417	3602 x 3603
ade16,17	73	115	16	11	6	8	1	2	492[a]	"
Wild	10	22	1	0	1	3			235	2830 x 3569

*gcn4*Δ	19	25	1	0	0	0			467	3595 x 3596
*bas1*Δ	2	4	0	0	0	0			331	3612 x 3613
*ade1*Δ	56	46	5	2	0	0			260	3549 x 3562
*bas1 ade1*Δ	1	6	0	0	0	1			232	3644 x3645
*gcn4 ade1*Δ	19	23	1	1	0	1			275	3626 x 3627
*gcn4 ade1*Δ	28	46	3	2	0	1			245	"
*his1 ade1*Δ	7	14	0	2	1	1			287	3618 x 3619
*his1 ade1*Δ	23	20	0	0	1	3			208	"
set2	22	35	0	2	1	4			224	3629 x 3630
set2	46	64	3	4	5	12			324	"
set2 bas1	7	14	0	0	1	0			339	3631 x 3632
set2 bas1	3	11	0	0	0	1			314	"
Sum	**422**	**585**	**47**	**43**	**19**	**43**	**5**	**4**		
	***p* < 0.0001**				***p* = 0.0035**					

[a] Includes one "aberrant 2:6" tetrad (*His/his, His/his, his/his; his/his*).

Of 17 crosses (Table B) conducted in a study of conversion rates (as a function of genotype, sporulation and growth media), 15 show overall disparity in favor of *his4-ATC* ($p = 0.0036$), providing evidence that, with the "zero-growth" protocol, the *HIS4* DSB site is cut more often than is the *his4-ATC* site. Of the 15 crosses showing such disparity, five have $p < 0.05$ by Chi-square. No crosses show significant disparity in favor of *HIS4*. The two conversion classes separately show a similar bias. For the FCs, 15 of the 17 favor *his4-ATC*. For the HCs, 11 of 13 favor *his4-ATC* ($p = 0.01$). These raw data were presented in Rehan (2012) only as aggregated frequencies of conversion ("NMS").

The validity of the *p* value (0.052) in Table 4 for comparing the disparities of the FCs and HCs is qualified by the small sample sizes of HCs in the 13 crosses that have HCs. Justification for the calculation is provided by two considerations: (1) The mean 5:3/(HC) value calculated from the pooled data (weighted mean = 0.31) and the mean of the individual crosses (0.29) are similar. This similarity suggests that the 13 HC data sets that contribute to the 5:3/(HC) values are drawn from a single universe. (2) The variability among the FC values (6:2 *vs*. 2:6) does not differ significantly from the expectation for samples drawn from a single universe ($p = 0.25$). The uniformity of the FC data further reduces concerns regarding undetected variability in the smaller data set of HCs. This view is consonant with our conclusion that the zero-growth feature of the sporulation protocol, through its introduction of DSB disparity, is the sole determinant of HC disparity in zero-growth crosses from the RHB laboratory.

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