Meiotic Recombination in *Neurospora crassa* Proceeds by Two Pathways with Extensive Holliday Junction Migration

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**Abstract**

Analysis of thousands of Δmsh-2 octads using our fluorescent recombination system indicates that, as in other filamentous fungi, symmetric heteroduplex is common in the *his-3* region of *Neurospora crassa*. Symmetric heteroduplex arises from Holliday junction migration, and we suggest this mechanism explains the high frequency of His* spores in heteroallelic crosses in which recombination is initiated *cis* to the *his-3* allele further from the initiator, cog*. In contrast, when recombination is initiated *cis* to the *his-3* allele closer to cog*, His* spores are mainly a result of synthesis-dependent strand annealing, yielding asymmetric heteroduplex. Loss of Msh-2 function increases measures of allelic recombination in both *his-3* and the fluorescent marker gene, indicating that mismatches in asymmetric heteroduplex, as in *Saccharomyces cerevisiae*, tend to be repaired in the direction of restoration. Furthermore, the presence of substantial numbers of conversion octads in crosses lacking Msh-2 function suggests that the disjunction pathway described in *S. cerevisiae* is also active in Neurospora, adding to evidence for a universal model for meiotic recombination.

**Introduction**

Meiotic recombination is a feature common to all sexually reproducing organisms, generating crossovers (COs), or reciprocal exchanges between chromosomes, which are required for chromosomes to segregate correctly during meiosis [1]. Recombination also ensures that variations within parental sequences come together in novel ways after meiosis, producing new material for natural selection. Rearrangement of sequence variations occurs not only as a result of new combinations of parental chromosomes and of crossing over between variations within each chromosome, but also from gene conversion [2, 3], where the copy number of one sequence increases at the expense of another.

A long-standing model of recombination was developed to explain what was thought to be a single pathway in *Saccharomyces cerevisiae*, in which a programmed double-strand break (DSB) is processed to yield a double Holliday junction that can be resolved in two ways to result in a CO or a non-crossover (NCO) [4, 5]. Current models, however, are derived from
bodies of tetrad data from several organisms, including seminal studies using *Neurospora crassa* [3], *Ascobolus immersus* [6], *Sordaria fimicola* [7] and *S. cerevisiae* [8]. Reconciliation of apparently conflicting data obtained in *S. cerevisiae* has resulted in the conclusion that there are at least two pathways for crossing over during meiosis [9–13]. The “pairing” and “disjunction” pathways [13–15], known as the Class 2 and Class 1 CO pathways respectively [10, 16] also appear to apply to data from Sordaria [7], Drosophila [17–19] and Arabidopsis [20, 21]. It is thought that the Class 2 pathway is a direct descendant of the pathway for mitotic recombination, while the Class 1 pathway is a meiosis-specific modification evolved to regulate COs and ensure chromosome segregation [16].

The Class 1 pathway is dependent on the synaptonemal complex, requires Msh4/Msh5 proteins and generates interfering COs [9]. In *S. cerevisiae*, intermediate Class 1 molecules are short, contain fully ligated double Holliday junctions, and are believed to be resolved to yield only COs [10, 13, 15, 22]. The Class 2 pathway, in contrast, is Msh4/Msh5-independent with longer intermediates that yield predominantly NCOs as a result of synthesis-dependent strand annealing (SDSA) [23]. Invasion of the homolog by the 3’-overhanging end of a DSB [5] is followed by DNA synthesis to yield a transient intermediate that can be resolved by unwinding, as mediated by anti-crossover helicases such as Sgs1 [16]. Such unwinding results in newly synthesized DNA annealing to the initiating chromosome. It is possible for the 3’ end to invade the homolog more than once, leading to alternate patches of hetero- and homoduplex, a mechanism described as template-switching [23–26]. Any Holliday junction intermediates arising from the Class 2 pathway are resolved by protein complexes such as Mus81–Mms4 [27], yielding equal frequencies of NCOs and non-interfering COs [13, 15, 28].

Heteroduplex (hDNA) generated during recombination can be symmetric, present on both homologs adjacent to a Holliday junction, or asymmetric. Asymmetric hDNA is formed when a free 3’ end, resulting from digestion of the 5’ ends on either side of the DSB, invades the homologous chromosome, or when DNA, newly synthesized using the homolog as template, anneals with the initiating chromosome. Symmetric hDNA forms when a Holliday junction migrates further than the region in which DNA synthesis fills the 5’ gap, as the paired strands in each chromatid involved in recombination proceed to unwind and anneal with the homolog.

Evidence of the extent and nature of hDNA formed during recombination is often destroyed by the correction of mismatched DNA bases by mismatch repair (MMR) proteins. In eukaryotes, homologs of *Escherichia coli* MutS (Msh2, 3 and 6 proteins in *S. cerevisiae*) act as heterodimers to recognize mismatches, and the resultant complex then recruits a heterodimer of MutL homologs (*S. cerevisiae* Mlh1-3, Pms1) to repair the mismatch (reviewed in [23, 29, 30]). Thus, a useful strategy for a study of recombination is to disable MMR, leaving hDNA largely uncorrected. Since Msh2 is thought to be involved in recognition of all types of mismatch [29] yet results in little disturbance to meiosis, *MSH2* inactivation has been the usual choice [31]. However, it has been suggested Msh2 is required only in the Class 2 pathway [15], so we must therefore consider the possibility that only hDNA generated by the Class 2 pathway will lack correction in the absence of *MSH2*.

Using *S. cerevisiae* hybrids with genomes that differed at 46,000 or 52,000 positions, analysis of tetrads by 454 sequencing and high density microarrays indicated that about 90 COs and 45 NCOs occur in each meiosis [32, 33]. Even in an SK1/S288C hybrid where 62,000 SNPs distinguish the genomes, an average of 73 COs and 27 NCOs per meiosis was detected [31]. Since, despite the level of heterology, the combined NCO + CO frequency is similar to the estimated DSB frequency in a homologous diploid [34], the SK1/S288C hybrid was used to compare wild-type and *MSH2*-deletion meioses [31]. Lack of Msh2 was found to increase NCOs by a factor of three and to increase COs from 73 to 92 per meiosis. hDNA patterns suggested that not all NCOs occur by simple SDSA but that a substantial proportion (35%) are a result of
Holliday junction dissolution by the combined action of a helicase and a type I topoisomerase [31]. Aberrant 4:4 segregation (Ab 4:4), indicating symmetric hDNA, was rarely seen despite the large number of markers and the absence of MMR [31]. Similarly, in an earlier study using poorly repaired mismatches at HIS4 [35], Ab 4:4 tetrads made up only 4.6% of non-Mendelian segregation (NMS, defined as any deviation from 4:4 segregation).

These data are in stark contrast to tetrad analyses using spore color mutations in S. fimicola in which the frequency of Ab 4:4 segregation is similar to that of gene conversion events, showing 6:2 segregation ([36, 37]; please note that henceforth we describe aberrant segregations as 5:3 or 6:2 regardless of which allele is present in excess). In both S. fimicola and A. immersus [38], Ab 4:4 segregation makes up 20–30% of NMS. However, in our recent study of 52,000 Neurospora ascii, where alleles of a histone H1-GFP fusion gene substituted for spore color [39], we ignored Ab 4:4 ascii, so the frequency of symmetric hDNA is currently unknown in Neurospora.

This GFP-based recombination reporter system [39, 40] has made it feasible to analyse recombination outcomes in thousands of ascii relatively rapidly. Normal Mendelian segregation includes ascii with GFP alleles in separate halves of the ascus or in pairs on either side, indicating that segregation of GFP has occurred at the first or second meiotic division respectively [39]. The latter is evidence that a CO has separated the centromere from the parental GFP allele before the first division of meiosis and so can be used as a measure of CO frequency between the centromere and the site of GFP integration on that chromosome. More rarely, an ascus will display NMS such as gene conversion or post-meiotic segregation (PMS), a result of hDNA formed during recombination, with or without mismatch repair respectively. In a Δmsh-2 homozygote, hDNA generated in the Class 2 (pairing) pathway is expected to remain unpaired. If conversion-type and restoration-type repair are equally likely, the frequency of 5:3 Class 2 pathway ascii in a cross lacking Msh-2 function should be twice the frequency of 6:2 Class 2 ascii when Msh-2 is active. If repair in the Class 1 (disjunction) pathway is msh-2-independent in Neurospora as it is in yeast [15], the frequency of 6:2 Class 1 ascii should be unchanged by lack of Msh-2 function.

We have deleted msh-2 by the split-marker method [41] in several different Neurospora strains, allowing isogenic analysis of the effect of Msh-2 on allelic recombination in his-3. This region was chosen because of the well-known recombination hotspot, cog, that initiates recombination about 3 kb from the 3′ end of the his-3 coding sequence [42, 43]. There are two codominant alleles of the hotspot, cog and cog+, and recombination stimulated by either cog allele is suppressed by the unlinked rec-2+ gene [44]. In the absence of cog+, relief of rec-2+–mediated suppression increases allelic recombination only 4-fold, with no detectable effect on local crossing over. In contrast, a single copy of cog+ results in >40 times the level of allelic recombination and 6-fold more crossing over than seen in the rec-2+ cross, while two copies of cog+ increase allelic recombination >100-fold and crossing over 12-fold compared to the suppressed level [45]. It is therefore clear that recombination is rarely initiated at cog when the cog+ allele is present.

We have analyzed Δmsh-2 octads with markers segregating on three Neurospora chromosomes for comparison with a previous study of wild-type octads [46] and compared allelic recombination frequencies in Δmsh-2 and otherwise isogenic Msh2+ crosses. When combined with our GFP-based octad analyses, our data are supportive of a generally universal mechanism for recombination but suggests there are features yet to be fully described.

**Results**

**The meiotic MMR function of Neurospora msh-2**

For comparison with 148 octads collected from previous analysis of an Msh-2+ cross (T12105 × T12282; Table 1 [46]), we collected 41 octads from an otherwise isogenic msh-2
Table 1. Neurospora strains. All N. crassa strains used in this study are listed below. Strain numbers are those used by the Catcheside laboratory, as many of these cultures are not held by the Fungal Genetics Stock Center.

| Stock no. | Genotype |
|-----------|----------|
| T10998    | A, his-3<sup>3</sup>K874, cog<sup>+</sup>, ad-3; cot-1; rec-2 |
| T11089    | A, his-3<sup>3</sup>K874, cog, ad-3; cot-1; am, rec-2 |
| T11802    | a, lys-4, his-3<sup>11201</sup>, cog; cot-1; am, rec-2 |
| T11805    | a, lys-4, his-3<sup>11201</sup>, cog<sup>+</sup>; cot-1; rec-2 |
| T12298    | A, his-3<sup>3</sup>K874, cog<sup>+</sup>, ad-3; cot-1; rec-2; Δmsh-2 |
| T12299    | a, lys-4, his-3<sup>11201</sup>, cog<sup>+</sup>; cot-1; rec-2; Δmsh-2 |
| T12105    | a, cog<sup>+</sup>; cot-1; rec-2 |
| T12282    | A, lys-4, his-3<sup>3</sup>K874, cog, ad-3; am, rec-2 |
| T12342    | A, lys-4, his-3<sup>3</sup>K874, cog, ad-3; cot-1; am, rec-2; Δmsh-2 |
| T12344    | a, cog<sup>+</sup>; cot-1; rec-2; Δmsh-2 |
| T12498    | A, his-3<sup>3</sup>Δ::pccg-1::hH1::5′GFP, cog; rec-2 |
| T12515    | a, his-3<sup>3</sup>Δ::pccg-1::hH1::3′GFP, cog; am, rec-2 |
| T12520    | rid<sup>o</sup><sub>1</sub>, a, his-3<sup>3</sup>Δ::pccg-1::hH1::5′GFP, cog<sup>+</sup>; rec-2 |
| T12529T12571 | rid<sup>o</sup><sub>1</sub>, a, his-3<sup>3</sup>Δ::pccg-1::hH1::3′GFP, cog; am, rec-2rid<sup>o</sup><sub>1</sub>, a, his-3<sup>3</sup>Δ::pccg-1::hH1::5′GFP, cog<sup>+</sup>; rec-2; Δmsh-2 |
| T12582    | rid<sup>o</sup><sub>1</sub>, A, his-3<sup>3</sup>Δ::pccg-1::hH1::3′GFP, cog; am, rec-2 |
| T12651    | rid<sup>o</sup><sub>1</sub>, A, his-3<sup>3</sup>Δ::pccg-1::hH1::3′GFP, cog; am, rec-2; Δmsh-2 |
| T12705/06/07 | A, his-3<sup>3</sup>K874, cog, ad-3; cot-1; am, rec-2; Δmsh-2 |
| T12708/09/10 | a, lys-4, his-3<sup>11201</sup>, cog; cot-1; am, rec-2; Δmsh-2 |
| T12711    | rid<sup>o</sup><sub>1</sub>, a, his-3<sup>3</sup>Δ::pccg-1::hH1::5′GFP, cog<sup>+</sup>; rec-2; Δmsh-2 |
| T12713    | rid<sup>o</sup><sub>1</sub>, A, his-3<sup>3</sup>Δ::pccg-1::hH1::5′GFP, cog; am, rec-2; Δmsh-2 |

The am allele is K314, lys-4 is STL4, cot-1 (colonial temperature-sensitive mutation) is C102t, and ad-3 is K118.

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deletion cross (T12344 × T12342; Table 1). The crosses used for this analysis were heterozygous at four loci on LGI (mat, lys-4, his-3 and ad-3; Fig 1) and at a single locus on each of LGIV and LGV (cot-1 and am respectively; Fig 1). Of the 148 octads where MMR was wild-type, there were four showing NMS (6+:2M at lys-4, 6+:2M at his-3, 6+:2M and 2+:6M at cot-1 [46]). In contrast, of the 41 octads from the cross lacking Msh-2 function, five displayed NMS, a greater frequency than in the Msh-2<sup>+</sup> cross (p = 0.03). All the detected NMS octads from the Δmsh-2 cross showed post-meiotic segregation (PMS; 5+:3M at his-3, 5+:3M and 3+:5M at cot-1 and 5+:3M and 3+:5M at am), confirming that Neurospora MSH-2 has the meiotic MMR function predicted by similarity to the Msh2 protein of S. cerevisiae. Note that because the number of octads analyzed is small, the detected NMS events may not represent all such events in this cross.

**Meiotic silencing has no detectable effect on msh-2 phenotype**

A null msh-2 mutant generated by repeat-induced point mutation [49] has been found to be recessive with respect to recombination [50]. A deletion mutant could however be dominant due to meiotic silencing, whereby a DNA coding sequence that lacks a partner on the homologous chromosome is prevented from being expressed during meiosis [51, 52].

Meiosis is subtly disturbed in crosses homozygous for Δmsh-2, resulting in deformed and infertile asci (Fig 2D) relative to the wild-type cross (Fig 2A), and a slight delay in sporogenesis. In addition, the frequency of His<sup>+</sup> progeny of a cross heteroallelic for his-3<sup>3</sup>K874 and his-3<sup>3</sup>K1201
mutations [48] is significantly increased in crosses homozygous for \(\Delta msh-2\) relative to otherwise isogenic crosses homozygous for \(msh-2^+\), while crosses heterozygous \(\Delta msh-2^+/msh-2^+\) (Table 2) give His\(^+\) spore frequencies indistinguishable from those of the \(msh-2^+\) homozygote. Regardless of which parent is deleted for \(msh-2\), perithecia from \(\Delta msh-2^+/msh-2^+\) heterozygotes appear normal (Fig 2B and 2C). Thus, there is no detectable silencing of \(msh-2^+\) in either hemizygote, and \(\Delta msh-2\) behaves as classically expected for a recessive mutant.

Loss of Msh-2 function increases allelic recombination—under most circumstances

When both chromosomes carry \(cog^+\) in a cross that is heteroallelic for mutations in \(his-3\) (Fig 3A), the His\(^+\) spore frequency is increased \(~1.5\)-fold by loss of Msh-2 function (Table 2). In crosses heterozygous for \(cog^+\), when the \(his-3\) allele closer to \(cog\) is \(cis\) to \(cog^+\) (Fig 3B), the frequency of His\(^+\) spores is similarly increased by loss of Msh-2 function (Table 2). In contrast, when the \(his-3\) allele closer to \(cog\) is \(trans\) to the only copy of \(cog^+\) (Fig 3C), the frequency of His\(^+\) spores is unchanged by loss of Msh-2 function (Table 2).

In a cross in which one strain has a 5\(^0\)GFP and the other a 3\(^0\)GFP non-fluorescent mutant construct, each inserted between \(his-3\) and \(cog\) (Fig 4), the pattern of fluorescence in an ascus indicates if a recombination event has occurred in the meiosis from which the ascus was derived [39]. We have data (Table 4) from crosses that differ only in that wild-type \(msh-2\) in T12529 and T12520 has been replaced by \(hph\), conferring hygromycin resistance and generating the otherwise isogenic
Δmsh-2 strains T12713 and T12711 respectively (Table 1). These crosses are, as in the 5’GFP × 3’GFP crosses above, heterozygous cog/cog’, with cog’ closer and cis to the 5’GFP mutation.

The frequency of 6+:2M asci is little altered by loss of Msh-2 function (Table 4), although the spectrum of NMS is very different from when Msh-2 is active. The frequency of unrepaired mismatches (5+:3M, 7+:1M, Ab6+:2M, Ab5+:3M) is much increased by loss of Msh-2 function, as is the frequency of unrepaired symmetric hDNA (Ab 4:4). Overall, loss of Msh-2 function increases NMS ~3.5-fold, most of which is equally divided between 5+:3M and Ab 4:4.

**Discussion**

Our analyses have generated data that suggest a number of conclusions. Firstly, the frequency of octads showing PMS is increased 13-fold by loss of Msh-2 function (Table 4), so repair of a

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**Fig 2. The effect of msh-2 deletion on meiosis is recessive.** Whether the msh-2 parent is the male (B) or the female (C), rosettes from heterozygous crosses appear the same as the homozygous wild-type (A). In contrast, homozygous deletion (D) results in abnormal spores and a reduction in fertility, although there are some asci with eight viable spores.

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Table 2. Loss of Msh-2 function increases allelic recombination in his-3 only when cog+ is cis to the his-3 allele closer to it. The His+ frequency is increased by a factor of 1.5 in his-3K874 cog+ × his-3K1201 cog+ crosses (Fig 3A), while remaining unchanged by heterozygosity for Δmsh-2. However, although lack of Msh-2 function similarly increases His+ frequency in his-3K874 cog+ × his-3K1201 cog+ crosses (Fig 3B), there is no effect on His+ frequency in his-3K874 cog × his-3K1201 cog+ crosses (Fig 3C).

| Cross                  | Genotype            | His+  | p value |
|------------------------|---------------------|-------|---------|
| his-3K874 cog+ × his-3K1201 cog+ | Msh-2+              | 946   | 0.370   |
|                        | Msh-2+/Δmsh-2       | 975   | 0.001   |
|                        | Δmsh-2              | 1460  |         |
| his-3K874 cog+ × his-3K1201 cog+ | Msh-2+              | 690   | 0.020   |
|                        | Δmsh-2              | 1228  |         |
| his-3K874 cog × his-3K1201 cog+ | Msh-2+              | 390   | 0.630   |
|                        | Δmsh-2              | 427   |         |

All crosses are between strains carrying his-3K874 and his-3K1201 alleles and are homozygous for rec-2. His+ is the mean frequency of histidine-independent progeny per 10⁵ viable spores. Strains used to obtain these data are T10998, T11089, T11801, T11805, T12298, T12299 and T12705-T12710 (Table 1; S1 Table).

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substantial proportion of hDNA is msh-2-dependent in Neurospora, as it is in yeast. Secondly, both the ~3-fold increase in the total frequency of NMS (Table 4) and the ~1.5-fold increase in recombinant His+ spores in the absence of Msh-2 function (Table 2) indicate MMR at his-3 and GFP is strongly biased in the direction of restoration. The mismatches in his-3 and GFP are all >3 kb from the putative initiation site cog+ and mismatches >1 kb from a DSB are also preferentially restored in yeast [53, 54]. Finally, the frequency of 6:2 segregation of the 5’GFP

![Fig 3. Possible arrangements of cog+, cog and alleles his-3K1201 and his-3K874. The centromere is to the left of the figure and ad-3 is to the right. The figure is not to scale. In A, the cross is homozygous for cog+; in B, cog+ is cis to his-3K874, the mutant site closer to cog; while in C, cog+ is cis to his-3K1201, the mutant site further from cog.](image-url)

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mutation is little changed by loss of Msh-2 function (Table 4), indicating the existence of a DSB repair pathway in which MMR is msh-2-independent, as postulated for yeast [15]. Together, these data are supportive of the two pathway model for meiotic recombination [9–13], adding to the evidence that it may be universal.

On the other hand, additional data make the picture more complex. In a cog/cog+ heterozygote, recombination is known to be overwhelmingly initiated at cog+ [24, 45, 47]. In crosses heterozygous cog/cog+ and heteroallelic for his-3 point mutations, most His+ spores (2/3 to 3/4) result from conversion of the site cis to cog+, irrespective of whether that site is the one closer to or further from cog+ [45]. In the case of his-3TM429, a mutation generated by a reciprocal translocation between LGI and LGVII that separates the 5' and 3' ends of the his-3 coding sequence, the his-3TM429 allele cannot experience gene conversion. In a his-3TM429 heterozygote in which the point mutant is further from cog than TM429, cog+—stimulation of recombination absolutely requires the point mutant to be cis to cog+ [47]. In this case, conversion on the far side of Fig 4. GFP constructs inserted at his-3. Plasmids are based on pMF280 [38], shown at top of figure, in which the arrows indicate the directions of transcription for the his-3 and sgfp coding sequences. A mutation (substitution of T for A at nucleotide 628) was placed in the 3' end of the sgfp sequence in pMF280 to give phis-3GFP3' [38]. pGFP5+cog+ (lower part of figure) was made by joining the left side of pMF280, including the 5'-truncated his-3 sequence, sgfp, hH1 and the cgg-1 promoter, to sequences amplified from a cog+ strain [39] and a mutation (substitution of T for G at nucleotide 26) placed in the 5' end of the sgfp sequence. The constructs were targeted to his-3 by transformation of his-3 mutant strains and selection for growth without histidine. "ini" indicates the putative recombination initiation site within cog+ [24], so recombination is initiated about 6 kb from the GFP5' mutation.

Table 3. Allelic recombination in GFP is increased by loss of Msh-2 function. In each cross (msh-2+ is T12498 × T12515 and Δmsh-2 is T12571 × T12651; Table 1), one chromosome carries a 3'GFP and the other a 5'GFP construct, each inserted at the same position between cog and his-3. PMS is increased 13-fold (p < 0.0001) and frequency of GFP+ spores is increased 1.4-fold (p = 0.003) by loss of Msh-2 function.

| Octad type | msh-2+ asci | Asci % | Δmsh-2 asci | Asci % |
|------------|-------------|--------|-------------|--------|
| No fluorescence | 13444 | 99.66 | 18158 | 99.15 |
| 1+:7M | 8 | 0.06 | 138 | 0.75 |
| 2+:6M | 34 | 0.25 | 15 | 0.08 |
| 3+:5M | 0 | 0 | 4 | 0.02 |
| 4+:4M | 4 | 0.03 | 0 | 0 |
| Ab 2+:6M | 0 | 0 | 1 | 0.005 |
| Total | 13490 | 100 | 18316 | 100 |

C ± SE is the calculated frequency of conversion events in each 100 asci ± the standard error of that frequency. GFP+ ± SE is the calculated frequency of GFP+ spores in each 100 spores ± the standard error of that frequency.

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| 1+:7M | 8 | 0.06 | 138 | 0.75 |
| 2+:6M | 34 | 0.25 | 15 | 0.08 |
| 3+:5M | 0 | 0 | 4 | 0.02 |
| 4+:4M | 4 | 0.03 | 0 | 0 |
| Ab 2+:6M | 0 | 0 | 1 | 0.005 |
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C ± SE is the calculated frequency of conversion events in each 100 asci ± the standard error of that frequency. GFP+ ± SE is the calculated frequency of GFP+ spores in each 100 spores ± the standard error of that frequency.
TM429 from cog is thought to occur by template-switching [23, 24], whereby recombination initiated on one chromosome proceeds via SDSA using as template not only the homolog but also the sister [24]. But if the point mutant is closer to cog than TM429, cog+-stimulation of recombination occurs even if the point mutant is not in cis to cog+ [24, 47].

We have already noted that in his-3 K1201 × his-3 K874 crosses, if the cross is homozygous for cog+ (Fig 3A), or if the only copy of cog+ is cis to his-3 K874, the mutant site closer to the initiator (Fig 3B), loss of Msh-2 function increases recombinant His++ spores about 1.5-fold (Table 2). However, if the only copy of cog+ is trans to his-3 K874 (Fig 3C), loss of Msh-2 function has no effect on recombination frequency (Table 2). In addition, the need for conversion of a site more distant from cog+ without co-conversion of the closer site only reduces the frequency of His++ spores to about 56% of the frequency observed with a single copy of cog+ cis to his-3 K874 (Table 2).

It is not immediately obvious how conversion of the his-3 allele more distant from cog+ occurs at such high frequency, since it is evident that recombination is initiated predominantly at cog+ [24, 45, 47] and the initiating chromosome has been shown to be the usual recipient of information [47, 55]. One mechanism that springs to mind is template-switching [23, 24], the same way that recombination crosses the TM429 translocation breakpoint [24]. However, in a cross between his-3 K1201 and his-3 K874, the His+ frequency is ~0.01% [47], compared to ~1% in a cross between the similarly positioned his-3 K874 [48] and his-3 K1201 (Table 2; [45]). Since the requirement to cross the breakpoint leads to a 100-fold decrease in His++ spores, this suggests that template switching is infrequent and thus likely to make little contribution to the frequency of His++ spores when cog+ is trans to his-3 K874.

Data from crosses heterozygous 5’GFP/GFP+ indicate the frequency of Ab 4:4 is high even in msh-2+ NMS octads in Neurospora (0.25% of octads and 30% of NMS; Table 4), in contrast to studies using S. cerevisiae, in which Ab 4:4 has rarely been detected [31]. Although some may be a result of spindle slippage during meiosis or of spores slipping past each other in an ascus [56], loss of Msh-2 function increases Ab 4:4 frequency to 1.2% of octads (46% of NMS). Thus symmetric hDNA appears to be very common at his-3 in Neurospora, occurring at a

### Table 4. GFP analysis of Δmsh-2 homozygotes reveals a range of recombination outcomes near his-3.

| Octad type | msh-2+ asci | Asci % | Δmsh-2 asci | Asci % |
|------------|-------------|--------|-------------|--------|
| D1         | 10384       | 87.54  | 5530        | 79.06  |
| D2         | 1378        | 11.62  | 1261        | 18.03  |
| 6+:2M      | 60          | 0.50   | 30          | 0.43   |
| 5+:3M      | 5           | 0.04   | 82          | 1.17   |
| 7+:1M      | 0           | 0      | 4           | 0.06   |
| 8+:0M      | 3           | 0.03   | 1           | 0.01   |
| Ab 5+:3M   | 0           | 0      | 2           | 0.03   |
| Ab 6+:2M   | 2           | 0.02   | 2           | 0.03   |
| Ab 4+:4M   | 30          | 0.25   | 83          | 1.18   |
| Total      | 11862       | 6995   |             |        |
| CX ± SE    | 5.9 ± 0.19  | 9.3 ± 0.50 |        |
| C ± SE     | 0.59 ± 0.07 | 1.19 ± 0.13 |        |

D1 is the number of asci showing first division segregation of GFP and D2 the number with second division segregation. CX represents the percentage of crossover events between the centromere and GFP while C indicates the frequency of conversion asci per 100 asci (± the standard errors of each frequency).

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similar level to that demonstrated in other filamentous fungi [36–38]. The frequency of symmetric hDNA suggests another mechanism that may explain the frequency of conversion of sites trans to cog+, that of Holliday junction migration (HJM).

The probability that a His+ spore will be produced by a meiotic event depends on how often recombination is initiated at cog+ [24, 45], the chance that hDNA includes the closer mutant site in his-3 [57] or covers both mutant sites, and how mismatches within his-3 are repaired. In crosses heterozygous for cog+, the sum of these probabilities depends on whether the mutant site closer to cog is on the same chromosome as cog+ or not (Fig 5). When cog+ is cis to his-3K874, SDSA can result in a His+ spore (Fig 5A) but not when cog+ is trans to his-3K874 (Fig 5B). Conversely, whether cog+ is trans or cis to the closer mutant site, HJM is equally likely to produce symmetric hDNA covering only the closer mutant site (his-3K874 in Fig 5), followed by resolution of the junction between the mutant sites to yield a His+ spore (Fig 5). Although symmetric hDNA covering both mutant sites can also result in a His+ spore if the two sites are repaired in opposite directions, this mechanism would result in a decrease in His+ spores in the absence of Msh-2 function.

At this point, synthesis may continue (top section of A and B), generating a migrating D-loop (see [57]). Ligation of ends does not occur at this early stage, so the recombination intermediate can unwind, giving conversion without a crossover (SDSA). Note that DNA synthesis can switch readily between the homolog and the initiating chromatid (or the sister, not shown in this figure), provided that the sequences of the homologs are sufficiently similar for binding of the end to occur [57]. If the SDSA structure (A top section) is unrepaired, the meiosis yields a single His+ spore. Mismatch repair of the his-3K874 mutation to wild-type can give two His+ spores, while repair of the newly synthesized strand in the hDNA will result in no His+ spore.

Alternatively, ligation of ends results in Holliday junctions, one or both of which may migrate towards his-3 (A and B, lower sections). If the left-most junction is resolved between the sites of the his-3K874 and his-3K1201 mutations, this can yield a single His+ spore. Once again, mismatch repair can increase the number of His+ spores to two or reduce it to zero, depending on the direction of repair. Note that when cog+ is cis to his-3K874 (A), both SDSA
and Holliday junction migration can result in His⁺ spores, while if cog⁺ is trans to his-3⁴⁸⁷⁴ (B), His⁺ spores cannot be generated by SDSA.

As described in the two pathway model for recombination in S. cerevisiae [13, 14], mismatches in hDNA from the pairing pathway are usually restored to 4:4 by a MSH2-dependent mechanism [15]. Thus if most of the asymmetric hDNA in Neurospora were a product of the pairing pathway, loss of Msh-2 function would lead to recovery of unrepaired hDNA and 5:3 segregation, which appears to be the case (Tables 3 & 4). The two pathway model [15] also suggests that the 6:2 segregation seen in Δmsh-2 octads is a result of the disjunction pathway, in which MMR is MSH2-independent, and such resolutions would be predicted to be as COs [15]. Our data do not conflict with this hypothesis but suggest that symmetric hDNA is also part of the msh-2-dependent pairing pathway, since loss of Msh-2 function increases the frequency of Ab 4:4 and 5:3 segregation to similar extents (Table 4).

We suggest that SDSA may be responsible for the majority of His⁺ spores when cog⁺ is homozygous, or when cog⁺ is cis to the mutant site closer to it (Table 2). SDSA with template switching is known to be responsible for His⁺ spores in crosses heteroallelic for his-3⁴⁸⁷⁴ when the point mutant is further from cog⁺ than the translocation [24]. Our data suggest that repair of mismatches in asymmetric hDNA shows about a 4-fold bias towards restoration. Perhaps the free end in the unligated intermediate [15] may direct repair to the newly synthesized strand.

We also suggest that junction migration is responsible for the high frequency of His⁺ spores from heteroallelic crosses when cog⁺ is trans to the closer mutant site. Since loss of Msh-2 function does not alter His⁺ spore frequency in this situation (Table 2, Fig 3C), it seems likely that the direction of repair in symmetric hDNA is unbiased, although repair direction may be determined by the direction of cutting of a Holliday junction when the intermediate is resolved, perhaps by Mus81–Mms4 [27].

In general, recombination in Neurospora appears to follow the “rules” of the two pathway model [13, 15], with evidence for both the pairing and the disjunction pathways and suggesting MSH2-independence of the latter pathway in Neurospora as in S. cerevisiae. Our data indicate that MSH2-dependent MMR of mismatches generated by recombination initiated at cog⁺ is biased in the direction of restoration to normal 4:4 segregation, as seen in yeast studies of markers distant from an initiating DSB site [58, 59]. However, this analysis has also revealed the substantial contribution of HJM to gene conversion at his-3 of Neurospora, a completely different picture to that seen in S. cerevisiae, where HJM is rarely detected [31, 35]. Thus, a gradient in HJM may substitute for or add to conversion gradients resulting from changes in the direction of MMR [58], from the extent of hDNA formation during DNA repair synthesis [60] or from MMR-regulated hDNA rejection [59, 61]. Since HJM is likely to have a significant impact on recombination in other filamentous fungi and conceivably in higher eukaryotes, the existence of this feature in Neurospora indicates the need to study recombination in a wide range of model organisms.

Methods
Culture and media
Culture methods were as described previously [62], except that crosses were supplemented with 200μg/ml l-histidine, 500μg/ml l-alanine, 500μg/ml l-arginine, 200μg/ml adenine and 400μg/ml l-lysine as required. Vegetative cultures were supplemented with 200μg/ml l-histidine, 500μg/ml l-arginine, 500μg/ml l-alanine, 400μg/ml adenosine and 400μg/ml l-lysine as required. Recombination assays and crosses on solid media were as described previously [45, ...
46]. Microscopy and data collection were as described previously [39]. Transformation of Neurospora conidia was by electroporation [63].

Generation of DNA constructs for deletion of msh-2

Left and right flanks were amplified from cosmid G4:D9 [64] using primer pairs 

msh2Lfwd (ATCAGTCTCCATCTCATACCC) with msh2Lrev (gtctgtgaatgtatcgcgtACTGAATGTGATGGTGGAC) and msh2Rfwd (tctggtgaattgtatcgcgtACTGAATGTGATGGTGGAC) with msh2Rrev (TTCCCTTTTCCCTTCC). Left and right flank constructs were respectively fused to incomplete overlapping portions (HY and YG) of the hygromycin phosphotransferase (hph) cassette, using fusion PCR [65]. Fusion of the left flank and HY used primer pairs msh2Lfwd with HY (NLC37: GGATGCCTCCGCTCGAAGTA; [41]), while the left flank fusion used primers YG (NLC38: CGTTGCAAGACCTGCCTGAA; [41]) with msh2Rrev.

Construction of strains

Original strains with GFP inserted at his-3 (T12515, T12520 and T12529; Table 1), contain the 3'GFP, 5'GFP and GFP+ constructs respectively (Fig 4), and were made as described previously [39]. The 5' allele is a substitution of T for G at nucleotide 26 of the GFP coding sequence (p.Glu6*), while the 3' allele is a substitution of T for A at nucleotide 628 (p.Lys210*) [39].

Most msh-2 deletion strains were made using the standard split-marker process [41], by transformation with the left and right deletion constructs (described above), and selecting for growth on hygromycin. Strains transformed were T10998, T11805, T12105, T12282, T12520 and T12529, to give T12298, T12299, T12344, T12342, T12711 and T12713 respectively (Table 1). After separation of heterokaryons [66], homokaryotic transformants were confirmed by Southern analysis. Additional msh-2 deletion strains were extracted as hygromycin-resistant progeny of crosses. T12705, T12706 and T12707 are from a cross between T11089 × T12299; T12708, T12709 and T12710 are from T11802 × T12298; T12571 from T12298 × T12520 and T12651 from T12299 × T12582 (Table 1).

Statistical analysis of recombination data

For analysis of octad data using genetic markers, $\chi^2$ or Fisher’s exact tests were used to assess the probability that the distribution of genotypes in the progeny of the mismatch-repair competent and otherwise isogenic msh-2 deletion crosses could differ by chance.

For data obtained from asci segregating GFP alleles, frequency of conversion (C) within GFP was calculated as conversion events per 100 asci, where an ascus that has experienced a single conversion event (6+:2M, for example) is considered as one, while if two events have happened in the same ascus (8+:0M), that ascus is counted as two. So for example, if the total number of asci is 1000, of which there were four 6+:2M asci and three 8+:0M asci, the frequency of conversion is $\frac{(4 + (2 \times 3))}{1000}$ x 100%, or 1%. Aberrant 4+:4M asci (++++M +MMM and +MMM+M+, for example) were included in the total ascus count but were not considered to be conversion asci. As the 5' GFP mutant is cis to cog*, 2+:6M asci are relatively uncommon and because they cannot be differentiated from asci with dead spores, they are routinely not scored [39]. Standard errors (SEs) of proportions were calculated according to the following formula—$SE = \sqrt{\frac{p(1-p)}{n}}$ where $p$ is the proportion of recombinant asci (as a fraction of unity) and $n$ the number of asci scored.

For recombination assay data, two-tailed t-tests were used to compare frequencies of His+ spores, with each frequency transformed ($p \rightarrow \sin^2 p$) prior to comparison [67]. To compare allelic recombination frequencies in crosses between mutant GFP alleles with those in crosses...
between mutant his-3 alleles, the proportion of recombinants per $10^5$ spores was calculated as the number of individual fluorescent spores divided by the total number of spores, multiplied by $10^5$. SEs were calculated as described for asci segregating mutant and wild-type GFP alleles (above), except that $p$ is the proportion of recombinant spores (as a fraction of unity) and $n$ the number of spores scored.

Supporting Information
S1 Table. “A” is the number of colonies on medium lacking histidine (selective plates), while “C” is the number of colonies on fully supplemented medium (viable count). In all crosses except those marked thus /C3, the number of spores plated on A was 200 times that plated on C.

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Author Contributions
Conceived and designed the experiments: PJY FJB. Performed the experiments: PJY FJB. Analyzed the data: PJY DEAC. Contributed reagents/materials/analysis tools: DEAC. Wrote the paper: PJY FJB DEAC. Provided the Neurospora stocks, equipment and facilities necessary for this project to proceed: DEAC.

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