Regulation of Budding Yeast Mating-Type Switching Donor Preference by the FHA Domain of Fkh1

Jin Li^a, Eric Coić^b, Kihoon Lee, Cheng-Sheng Lee, Jung-Ae Kim^c, Qiuxin Wu, James E. Haber^*

Department of Biology and Rosenstiel Center, Brandeis University, Waltham, Massachusetts, United States of America

Abstract

During *Saccharomyces cerevisiae* mating-type switching, an HO endonuclease-induced double-strand break (DSB) at MAT is repaired by recombination with one of two donors, *HMLa* or *HMRa*, located at opposite ends of chromosome III. *MATa* cells preferentially recombine with *HMLa*; this decision depends on the Recombination Enhancer (RE), located about 17 kb to the right of HML. In *MATa* cells, HML is rarely used and RE is bound by the MATa2-Mcm1 corepressor, which prevents the binding of other proteins to RE. In contrast, in *MATa* cells, RE is bound by multiple copies of Fkh1 and a single copy of Swi4/Swi6. We report here that, when RE is replaced with four LexA operators in *MATa* cells, 95% of cells use HMR for repair, but expression of a LexA-Fkh1 fusion protein strongly increases HML usage. A LexA-Fkh1 truncation, containing only Fkh1's phosphothreonine-binding FHA domain, restores HML usage to 90%. A LexA-FHA-R80A mutant lacking phosphothreonine binding fails to increase HML usage. The LexA-FHA fusion protein associates with chromatin in a 10-kb interval surrounding the HO cleavage site at MAT, but only after DSB induction. This association occurs even in a donorless strain lacking HML. We propose that the FHA domain of Fkh1 regulates donor preference by physically interacting with phosphorylated threonine residues created on proteins bound near the DSB, thus positioning the strand invasion step of interchromosomal recombination even for non-MAT sequences. We also find that when RE binds to the region near the DSB at MATa then Mec1 and Tel1 checkpoint protein kinases but partially depends on casine kinase II. RE stimulates the strand invasion step of interchromosomal recombination even for non-MAT sequences.

Introduction

*Saccharomyces* mating-type switching occurs through a DSB-initiated intrachromosomal gene conversion event at MAT, using one of two donors on chromosome III, HML and HMR (Figure 1A) [1–3]. Switching is initiated by expression of the site-specific HO endonuclease that cleaves only one site in the yeast genome, MATa or MATa. The unexpressed mating-type genes in HML and HMR also contain HO cleavage sites, but they are not cut because these regions are heterochromatic [4–6]. Although either HML or HMR can be used to repair a DSB at MAT, there is a strong mating type-dependent preference for the choice of the two donors. In MATa cells, HML is preferentially chosen for repair, about 85–90% of the time, whereas MATa cells strongly prefer HMR, about 95% [3,7–9]. Donor preference is not altered if the mating-type genes encoded in the Y region are changed, e.g. if HMR carries Ya instead of Ya or if HML is replaced with HMR [7,8].

Donor preference in MATa depends on an approximately 275-bp Recombination Enhancer (RE), located 17 kb to the right of HML [10,11]. One important aspect of donor preference is that MATa cells activate a large (~40 kb) region near the left end of chromosome III, so that a donor within this region is strongly preferred over HMR [8]. RE is responsible for this activation along the entire left arm of chromosome III [11,12]. Donor preference does not change if the cis-acting silencer sequences around HML or HMR are removed [13]. In addition, RE is not limited to the special features of MAT switching. If a leu2 allele is inserted in place of HML, its success in recombining with a different leu2 allele, either near MAT or even on another chromosome, is 20–30 times higher in MATa than in MATa and is RE-dependent [8,12]. RE is “portable”; that is, it will work in other chromosome contexts. When HML, HMR and MATa are all inserted on chromosome V, HML usage increases significantly when RE is inserted nearby [12]. In addition, in MATa cells where RE promotes HML, the usage of HMR can be markedly increased by placing a second RE near HMR [11,12].

In MATa cells, RE is inactivated by binding of the Matα2-Mcm1 repressor complex, which leads to formation of highly organized nucleosomes covering the RE region but not extending into adjacent gene regions [8,10,14]. In MATa cells, RE exhibits several nuclease hypersensitive sites when Mcm1...
Mating-type gene switching occurs by a DSB–initiated gene conversion event using one of two donors, HML or HMR. MATα cells preferentially recombine with HML whereas MATα cells choose HMR. Donor preference is governed by the Recombination Enhancer (RE), located about 17 kb from HML. RE is repressed in MATα cells, whereas in MATα RE binds several copies of the Fkh1 protein. We replaced RE with four LexA operators and showed that the expression of LexA-Fkh1 fusion protein enhances HML usage. Donor preference depends on the phosphothreonine-binding FHA domain of Fkh1. LexA-FHAαN1 physically associates with chromatin in the region surrounding the DSB at MAT. We propose that RE regulates donor preference by the binding of FHAαN1 domains to phosphorylated sites around the DSB at MAT, thus bringing HML much closer than HMR. FHAαN1 action partially depends on casin kinase II but not on the DNA damage checkpoint kinases Mec1 and Tel1. We also find that, when RE binds to the MAT region, phosphorylation of histone H2A (γ-H2AX) by Mec1/Tel1 not only surrounds the DSB but also spreads around RE. This is the first demonstration that γ-H2AX can spread to contiguous, but undamaged, chromatin.

Author Summary

Mating-type gene switching occurs by a DSB–initiated gene conversion event using one of two donors, HML or HMR. MATα cells preferentially recombine with HML whereas MATα cells choose HMR. Donor preference is governed by the Recombination Enhancer (RE), located about 17 kb from HML. RE is repressed in MATα cells, whereas in MATα RE binds several copies of the Fkh1 protein. We replaced RE with four LexA operators and showed that the expression of LexA-Fkh1 fusion protein enhances HML usage. Donor preference depends on the phosphothreonine-binding FHA domain of Fkh1. LexA-FHAαN1 physically associates with chromatin in the region surrounding the DSB at MAT. We propose that RE regulates donor preference by the binding of FHAαN1 domains to phosphorylated sites around the DSB at MAT, thus bringing HML much closer than HMR. FHAαN1 action partially depends on casin kinase II but not on the DNA damage checkpoint kinases Mec1 and Tel1. We also find that, when RE binds to the MAT region, phosphorylation of histone H2A (γ-H2AX) by Mec1/Tel1 not only surrounds the DSB but also spreads around RE. This is the first demonstration that γ-H2AX can spread to contiguous, but undamaged, chromatin.

Results

Measuring Donor Preference by Southern Blot or a PCR-Based Assay

All strains in this study are derived from XW652 [11], which carries HMLα, MATα and HMRα-B on chromosome III (Figure 1A). HMRα-B contains a single base pair change that creates a BamHI site [9]. After galactose-induced expression of HO, MATα can be repaired to MATα or MATα-B, using HMLα or HMRα-B, respectively. Following HO induction for 60 min, HO expression was repressed by the addition of 2% dextrose and the ratio of switching to MATα or MATα-B was checked after 24 h. Donor preference could be measured either by Southern blot [9] or by a PCR-based assay in which the combination of MATα or MATα-B PCR products is digested with BamHI (Figure 1B). PCR-based assay showed 85% usage of HMLα for XW632 but ≤10% for RE-deleted XW676 (Figure 1C).

A LexA System to Study the Regulation of Donor Preference

Fkh1 is involved in the regulation of donor preference through direct interaction with RE [15,16]. To further explore the role of Fkh1, we constructed a strain ECY406 by replacing RE with four LexA operators (Figure 2A). In an otherwise wild type background, HML usage in EY406 was less than 5% as expected for a deletion of RE (Figure 2B). We then constructed a plasmid pEC16 that constitutively expresses a LexA-Fkh1 fusion protein from an ADH1 promoter of pAT4 [21]. The LexA-Fkh1 sequences from pEC16 were stably integrated at the arg5,6 locus of EY406 to generate a new strain EY457 (Figure 2A). Expression of LexA-Fkh1 in EY457 was able to up-regulate donor preference to around 32% presumably by binding to four LexA operators replacing RE (Figure 2B), whereas the use of HML was less than 5% when LexA alone was expressed (data not shown). This result demonstrates that regulation of donor preference by Fkh1 does not require the binding of Mcm1 or Swi4/Swi6 to their specific sites in the normal RE sequences. We noted further that the Fkh1 moiety in the LexA-Fkh1 fusion remained functional even with normal RE, as it could complement a fkh1Δ mutant in YJL017 by up-regulating donor preference to 68% (Figure 2C).

The Phosphothreonine-Binding FHA Domain of Fkh1 Is Responsible for Donor Preference

Fkh1 contains two conserved domains: a forkhead-associated (FHA) and a forkhead DNA binding domain (Figure 3A) [22,23]. To understand roles of different domains of Fkh1 in the regulation of donor preference, we prepared three plasmid constructs by fusing LexA of pAT4 with different regions of Fkh1: pJL4 for LexA-FHA (aa 1–230 of Fkh1), pJL5 for LexA-interdomain (aa 163–302), and pJL6 for LexA-forkhead (aa 231–484) (Figure 3A). The LexA fused sequences from these plasmids were integrated at arg5,6 locus of EY406 to generate strains YJL019, YJL020, and YJL021, respectively (Figure 3A). These three strains and EY457 (Figure 2A) all have a wild-type Fkh1, which is not functional in donor preference because Fkh1 cannot bind to RE:LexAααN1. Southern blots revealed that only YJL019 could re-establish donor preference to 90%, whereas YJL020 and YJL021 failed to increase HML usage (Figure 3B). This result suggests that the FHA domain may play a critical role in the regulation of donor preference.

We noted that donor preference regulated by LexA-FHAαN1 (90% donor preference for YJL019; Figure 3B) was much higher than that by LexA-Fkh1 (32% donor preference for EY457; Figure 2B). We suggest two possible explanations for this
difference. First, two DNA binding domains (LexA and the forkhead DNA binding domain) are present in LexA-Fkh1, whereas only one (LexA) is present in LexA-FHAFkh1. Therefore, the LexA-Fkh1 fusion protein likely binds multiple sites in yeast genome, which could mean that less fusion protein is available for regulating donor preference. In contrast, because there is only one DNA binding domain for LexA-FHAFkh1, all fusion protein will be available for donor preference regulation. A second possible reason is that the FHAFkh1 domain is more exposed in LexA-FHAFkh1 than in LexA-Fkh1 when both fusion proteins bind to four LexA operators replacing RE. The presence of a forkhead domain in LexA-Fkh1 could interfere with regulation of the FHAFkh1 domain in donor preference, whereas this kind of interference is not present in LexA-FHAFkh1.

The FHA (forkhead-associated) domain is a small protein module that can preferentially bind to phosphothreonine residues on proteins [22,24,25]. FHA domains have been found in a wide range of proteins, such as kinases, phosphatases and transcription factors [23,26]. To confirm that the FHA_{Fkh1} domain was responsible for increasing HML usage, LexA-FHA-R80A from pJL8 was integrated into the arg5,6 locus of ECY406 to generate a strain YJL094 (Figure 3A). Preferential usage of HML was completely abolished using LexA-FHA-R80A (Figure 3C), which carried a non-functional FHA domain [22,23]. Thus, the phosphothreonine-binding motif of the FHA domain plays a critical role in the regulation of donor preference.

The FHA Domain of Fkh1 Physically Interacts with the MAT Region after DSB Induction

We employed Chromatin Immunoprecipitation (ChIP) to ask if LexA-FHA_{Fkh1} could associate with the region around MAT before or after induction of a DSB. Using an anti-LexA antibody, we showed that LexA-fused FHA_{Fkh1} physically interacted with the MAT region after DSB induction in a strain lacking HML and

Figure 1. Measure Donor Preference via a PCR-Based Assay. (A) Mating-type switch at the MAT locus. When RE is active in MATα cells, donor preference (HML usage) is 85–90%. In contrast, HML usage reduces to only 10–15% when RE is deleted. Donor preference is calculated using a formula \((MAT_a/(MAT_a+MAT_A-B))\). (B) A PCR-based strategy for measuring donor preference. Diagrams are shown for MATα and MATα-B. After galactose induction, DSBs at MAT can be repaired using either donor of HMLα and HMRα-B. A primer pair (Yalpha105F/MATdist-4R) can only amplify \(MATa\) or \(MATa-B\), but not \(HMLa\), \(HMRa-B\), \(MATa\) due to specificities of these two primers. (C) Measure donor preference via a PCR-based assay. Both \(MATa\) and \(MATa-B\) are PCR-amplified using the primer pair (Yalpha105F/MATdist-4R). The purified PCR products are digested with BamHI and checked on ethidium bromide stained agarose gel. RE is present in XW652, but deleted in XW676.

doi:10.1371/journal.pgen.1002630.g001
Figure 2. A LexA System to Study Donor Preference. (A) Illustration of strain genotypes for ECY406, ECY457, ECY399 and YJL017. (B) LexA-Fkh1 regulates donor preference by binding to REΔ::LexABD4. ECY457 was constructed by integrating LexA-Fkh1 (from pEC16) to arg5,6 of ECY406. Donor preference was measured by Southern blot using a Yα specific probe in panels B and C. XW652 serves as a wild-type control. (C) LexA-Fkh1 complements a fkh1Δ mutant (ECY399) to regulate donor preference presumably by binding to RE. The arg5,6::LexA-Fkh1 was crossed into ECY399 to generate a strain YJL017.

doi:10.1371/journal.pgen.1002630.g002
HMR (Figure 4A), so that DSBs could not be repaired by homologous recombination. We observed a >10-fold increase in ChIP signals within about 5 kb on either side of the HO cleavage site at the \( \text{MAT} \), whereas no significant signal could be detected using primer pairs that amplify regions further away from the HO site (Figure 4B). Therefore, the LexA-FHA\(_{\text{Fkh1}}\) fusion protein physically interacted with the DSB-cut \( \text{MAT} \) through a repair-independent mechanism, which suggests that LexA-FHA\(_{\text{Fkh1}}\) or

![Diagram](image)

**Figure 3. The FHA Domain of Fkh1 Is Responsible for Donor Preference Regulation.** (A) The strain construction strategy for YJL019, YJL020, YJL021 and YJL094. Fkh1 has two conserved domains: FHA and a forkhead DNA binding domain. We prepared three plasmid constructs by fusing LexA of pAT4 with different regions of Fkh1: pJL4 for LexA-FHA (aa 1–230 of Fkh1), pJL5 for LexA-interdomain (aa 163–302), and pJL6 for LexA-forkhead (aa 231–484), respectively. LexA-fused sequences from these plasmids were integrated to the \( \text{arg5,6} \) locus of ECY406 (Figure 2A) to generate yeast strains YJL019, YJL020 and YJL021, respectively. For YJL094, LexA-fused sequences (LexA-FHA-R80A) from pJL8 were integrated. (B) FHA domain of Fkh1 is responsible for the regulation of donor preference. Donor preference was measured by Southern blot in panels B and C. (C) The phosphothreonine binding motif of FHA domain plays a critical role in the regulation of donor preference. XW652 and ECY406 serve as positive and negative controls, respectively.

doi:10.1371/journal.pgen.1002630.g003
Figure 4. The FHA Domain of Fkh1 Physically Interacts with the MAT after DSB Induction. (A) Chromosome III and relevant strain genotypes for YJL110. This donorless strain is same as YJL019 (Figure 3A) except that HML and HMR are deleted. Both YJL019 and YJL110 have a wild-type Fkh1, which is not functional in donor preference because Fkh1 cannot bind to REΔ::LexABD4. (B) LexA-FHA fusion protein physically interacts with the MAT after DSB induction. YJL110 was grown in YP-galactose and subjected to ChIP using anti-LexA antibody. The primer pair L16.5 is 16.5-kb proximal (left) from the HO site of MATa, whereas R10 is located 10-kb distally (right). All other primer pairs are named and color-coded accordingly. The approximate position of each primer pair is shown in the above diagram. Immunoprecipitation (IP) signals were quantified via real-time PCR, and IP signal at each locus was normalized to that of a control locus CEN8. Y axis represents IP signal as fold increase relative to the IP signal at same locus before HO induction (time zero). Error bars are calculated from three repeated experiments. (C) The phosphothreonine binding motif of FHA domain

PLoS Genetics | www.plosgenetics.org 6 April 2012 | Volume 8 | Issue 4 | e1002630
is responsible for its physical interaction with the MAT region. Primer pairs in this panel are named similarly as in panel B, and the position of each primer pair is indicated in the above diagram. YJL094 used in the ChiP assay showed no physical interaction between LexA-FHA-R80A and the MAT region after DSB induction (Figure 4C). However, the LexA-FHA-R80A fusion protein still strongly associated with RE in the absence of the LexA domain (Figure 4C). These data strongly support the idea that the FHA domain of Fkh1 regulates donor preference by physically interacting with the MAT region during mating-type switch, and these interactions fully depend on the phosphothreonine binding motif of the FHA domain.

**RE Accelerates the Rate of DSB-Induced Ectopic Recombination for Non-MAT Sequences**

Because the FHA domain regulates donor preference via a repair-independent but break-dependent mechanism, it suggests that FHA domain or RE can be used to facilitate recombination between any two homologous sequences in yeast genome. Previously we showed that RE stimulates the lus2 heteroallelic spontaneous recombination when one of the alleles was situated in place of HML in JKM139 [11]. In that case, the nature and position of the initiating DNA lesions were unknown. Here we integrated a lus2::H0k construct at cam1 locus on chromosome V, so HO-induced DSBs can recombine with a LEU2 locus placed near RE on chromosome III (Figure 5) [30]. In one assay, LEU2 on chromosome III could be used as a donor to repair HO-induced DSBs on chromosome V in competition with a lus2-K donor inserted at ara3, which is 85 kb from the lus2::H0k (Figure 5A). The lus2-K allele was created by ablating the rpm1 site in LEU2 [31]. As shown in Figure 5A, the proportion of repair events using the interchromosomal donor was more than 50% when RE was present but fell to less than 10% when RE was deleted. In a second assay, the LEU2 on chromosome III was the only possible donor for DSB repair. This construct allowed us to ask whether RE stimulated recombination by facilitating the earliest step, the search for homology by Rad51 recombinase bound to the resected end of the DSB. We measured the time at which Rad51 became associated with the donor (i.e. when strand invasion had occurred) by a ChiP assay analogous to that used to assay strand invasion kinetics during MAT switching [32,33]. As seen in Figure 5B, the kinetics of Rad51 association with the LEU2 donor was significantly faster when RE was present. The presence of RE also increased the proportion of cells that completed repair was 72% compared to 37% when RE was deleted. The percentage of completed repair was determined by comparing survival on galactose plates with that on dextrose plates where HO was not induced.

**γ-H2AX Formation at RE in MATa Cells Provides Additional Evidence of Direct RE-to-MAT Contact**

γ-H2AX rapidly forms around sites of a DSB, dependent on either Mec1 or Tel1 checkpoint protein kinase [27,28]. If RE bound to regions around the DSB, would γ-H2AX also form around RE region? To address this question, we used ChiP with anti-γ-H2AX antibody to examine the phosphorylation of histones around RE following initiation of a DSB. γ-H2AX formed over a large region around MAT following the induction of a DSB within 15–60 min (Figure 6A). Surprisingly, γ-H2AX also appeared around RE at 1 hr after HO induction in MATa cells. As predicted, there was no similar modification around RE in MATa cells, where RE is repressed (Figure 6B). Moreover, the kinetics of γ-H2AX modification around RE was slower than around MAT, consistent with the idea that RE, first had to be recruited to the DSB before this modification could take place (Figure 6A, 6C). Finally, using both mec1Δ sml1Δ and tel1Δ derivatives of JKM139, we showed that either checkpoint kinase was capable of carrying out γ-H2AX modification around RE (Figure 6D). These data provide additional supporting evidence of a direct RE-to-MAT contact after DSB induction and support the model that the binding of RE to MAT is the basis of bringing HML into close proximity. In addition, these data show for the first time that a region not suffering a DSB can be modified by both checkpoint kinases if this region is brought close to the DSB site.

**Are Histones the Target of FHA Domain in Donor Preference Regulation?**

Our data strongly argue that the FHA domain of Fkh1, clustered at normal RE or FHA::LexA domain, interacts with phosphorylated residues in the region surrounding the DSB. The most obvious candidates are histones that are phosphorylated after DSB induction, including H4-S1 [29] and histone H2A-S129 (γ-H2AX). The possibility that H4-S1 could be involved was made more attractive by our finding that this modification is confined to the first 10 kb around a DSB, much more restricted than γ-H2AX (Figure 4D). We constructed a strain YJL102, carrying the h4-S1A in HIFF2 and deleted for HIFF1; however this alteration had no effect on donor preference (Figure 7A). In addition, phosphorylation of H2A-S122, H2A-T126 and H2A-S129 have been implicated after MMS-induced DNA damage [34]. To test these H2A modifications, we constructed a strain YJL121 by deleting endogenous HTA1-HTB1 and HTA2-HTB2 and complementing by a plasmid carrying hta1-S122A-T126A-S129A-HTB1, but donor preference was not affected (Figure 7A).

We have directly tested whether post-translational modifications of the N-terminal tail of histones H3 or H4 are implicated in donor preference. In addition to H4-S1, several other sites have been reported to be phosphorylated during the cell cycle, such as H3-T3, H3-S10 and H3-S28 [35–37], which might also be targets for modification after a chromosome break. In particular, we constructed a strain YJK340, in which H1F1-HT1 was deleted with MAT. Then, the remaining copy of H3 gene was modified to carry a deletion of the first 32 amino acids or HHF2 was modified...
Figure 5. The Presence of RE Promotes DSB–Mediated Interchromosomal Gene Conversion and Accelerates Rad51 Synapse Formation. (A) The presence of RE promotes the usage of its adjacent inter-chromosomal donor for DSB repair. An HO cut site was previously introduced to the Kpnl site of the LEU2 to generate leu2::HOcs [54]. YCSL001 (as depicted) contains the leu2::HOcs at the can1 locus on chromosome V.
HO-induced DSBs can be repaired via gene conversion using one of two donors: LEU2, inserted ~12 kb proximal to RE on chromosome III, and LEU2-K, lacking a KpnI site, integrated as part of a Yip5 plasmid at the ura3-52 locus. Cells were plated on YP-galactose to induce DSBs. The repaired region of each survivor was amplified by PCR, followed by KpnI digestion to determine which donor was used for repair. The bar represents the percentage of repair events using either donor. Light blue bars show the use of leu2-K while dark blue bars indicate the use of the ectopic LEU2 on chromosome III. YCSL003 is same as YCSL001, except that RE is deleted. For YCSL001 (RE'), error bars are calculated from three experiments; for YCSL003 (REA), values are the same for two experiments (20 colonies per experiment). (B) The presence of RE accelerates Rad51 synapse formation. In YSJ119 (as depicted), the LEU2 on chromosome III is the only homologous donor to repair the DSB on chromosome V. YCSL014 is same as YSJ119, except that RE is deleted. Both YSJ119 (RE') and YCSL014 (RE') were grown in galactose and subjected to ChiP with anti-Rad51 antibody. IP signal was amplified using a primer pair (YCL094p1-Leu2-91082), indicated by a red solid line, which is located at the left boundary of LEU2 on chromosome III. IP signal was normalized to that of a control locus CEN8. Y axis represents IP signal as fold increase relative to the IP signal at the same locus before HO induction (time zero). Error bars indicate the range of two experiments.

doi:10.1371/journal.pgen.1002630.g005

Discussion

We have shown that the phosphothreonine binding motif of the FHA domain of Fkh1 plays a critical role in the regulation of donor preference (Figure 3). A strong physical association between the FHA domain and HO cleavage domain at the RE region and MAT is readily seen, but only after a DSB is induced. This interaction is independent of the presence of an adjacent homologous HML donor (Figure 4). Conversely, the region surrounding RE can be phosphorylated by Mec1 and Tel1 kinases only after DSB induction in MAT but not in MAT strains (Figure 6), again suggesting that these regions can come into physical contact when there is a DSB at MAT and RE is active.

RE's activity does not depend on any of the special features of MAT switching such as HML or HMR silencing [13] or HO cleavage [11,15]. Consequently RE is able to improve the use of an ectopic donor in repairing a DSB on a different chromosome. Normally, a DSB will be preferentially repaired by a donor on the same chromosome in competition with an ectopic donor, but if the ectopic donor is located near RE, more than half of gene conversions use the interchromosomal donor (Figure 5A). Although our data and those from others show that HML is not constitutively much closer to MAT than HMR is (i.e. in the absence of HO cleavage) [41–43], the data we present here suggest that such a reorganization will occur after a DSB is created.

Taken together, our data suggest a simple model for RE action (Figure 7C). After the induction of a DSB, casein kinase II and possibly other kinases modify some proteins bound near the DSB. These modifications, most likely phosphothreonines, are clustered near the DSB and can be bound by FHA domains tethered at RE. This binding effectively tethers HML within about 20 kb of the DSB whereas HMR remains 100 kb away. Thermodynamic considerations argue that this close proximity is sufficient to explain why HML should be used 90% of the time for DSB repair in MAT cells [13]. This model also explains how RE can act over a long region of the left arm of chromosome III [8], although with diminishing effect [12], by this tethering mechanism.

The model we propose argues that RE should be portable and able to stimulate the use of any homologous donor in a DSB repair mechanism. Our previous work has shown that RE is portable, as it is able to activate HML use when both are inserted on chromosome V [12]. Moreover, if a copy of RE is inserted near HMR in a MAT strain that also has RE near HML, then HMR usage is increased to about 50% (E.C., S.-Y. Tay and J.E.H., unpublished). The ectopic recombination experiment presented here shows that RE can act efficiently on non-MAT sequences for DSB repair (Figure 5A).

We note that we have previously shown that RE could stimulate spontaneous recombination between leu2 heteroalleles when one of them was located close to the RE [11,12]. The results we report here suggest that a large proportion of spontaneous recombination events may be triggered by DSBs or that the same phosphorylated
protein attracting the attention of RE during DSB repair also accumulates at the lesions that stimulate spontaneous recombination.

At present, we have not yet identified the phosphothreonine target for the FHA domain of Fkh1. We have ruled out a number of candidates, including γ-H2AX, N-terminal tails of histones H3 and H4, as well as Mre11 and Sae2, two proteins involved in DSB end-binding and initiating 5' to 3' resection (C.-S. L., J.E.H., unpublished observations). Studies using peptide libraries and immunoprecipitation of the FHA_Fkh1 domain after DSB induction are underway.

Aparicio group has recently made the intriguing finding that Fkh1 and Fkh2 proteins play a key role in the activation and clustering of early origins of replication in budding yeast [44].

Figure 6. γ-H2AX Formation around MAT Spreads to the RE Region in MATα Cells. (A) γ-H2AX formed around the MAT locus after DSB induction by HO. JKM139 (MATα) lacking HML and HMR was grown in galactose and subjected to ChIP analysis with anti-γ-H2AX antibody. DNA was extracted from immune-precipitates with protein G-agarose, and IP signals around the MAT locus were quantified via real-time PCR using five primer pairs (−10 kb, 10 kb, 20 kb, 30 kb and 40 kb from the HO cut site). IP signal at each locus was normalized to that of a control locus CEN8. Y axis represents IP signal as fold increase relative to the IP signal at the same locus before HO induction (time zero). Each data point is the average of two separate experiments, with error bars representing the range of IP values. (B) γ-H2AX appeared around the RE region in MATα, but not in MATα cells. JKM139 (MATα) and JKM179 (MATα) cultured in galactose for an hour were subjected to ChIP with anti-γ-H2AX antibody as described in panel A. To test γ-H2AX PCR signals around the RE, primers pairs at various distances from RE were used. Error bars represent the range of IP values from two independent experiments. (C) Kinetics of γ-H2AX formation around RE in JKM139 (MATα). All experimental procedures are same as described in Panel A for primer pairs amplifying regions around RE. (D) The level of γ-H2AX signals around RE at 1 hr after HO induction was compared among the wild type (JKM139), tel1Δ, mec1Δsml1Δ or mec1Δsml1Δtel1Δ strains. doi:10.1371/journal.pgen.1002630.g006
Figure 7. Roles of Histones and Kinases in Donor Preference and a Model for FHA-Directed Regulation. (A) The effect of H4 or H2A phosphorylation sites on donor preference. HML usage was not altered in strains only containing mutated h4-S1A or hta1-S122A-T126A-S129A. Donor preference was measured using a PCR-based assay (Figure 1B). (B) The effect of Mec1/Tel1 or casein kinase II on donor preference. In a triple mutant strain (YJL054), donor preference is not different from wild-type control (XW652). The cka1::KAN, cka2::NAT, pRS315-cka2-8 (ts) are crossed into YJL019 (Figure 3A) to generate the YJL119 strain. Both strains are first cultured at 25°C for overnight and then transferred to 37°C for 3 hour incubation. Galactose induction is performed for 1 hour and stopped by the addition of 2% dextrose. (C) A model for FHA-directed regulation of donor preference. After the generation of a DSB at MATα, we propose that a physical interaction between the FHA domain of Fkh1 and phosphothreonines of histones or bound proteins around the MAT will bring HML to the vicinity of the DSB, therefore allowing HML to serve as the favored template for DSB repair. The tethering of HML approximately 20 kb from the MAT can account for an almost 10-fold preference of HML usage over HMR.

doi:10.1371/journal.pgen.1002630.g007
regulation involves a cis-acting association of these two forkhead proteins with proteins at origins. It will be interesting to ask if the FHA domain of Fkh1 plays an important role in this regulation.

Another important finding emerging from our work is that two DNA damage checkpoint kinases, Mec1/ATR and Tel1/ATM, can act to phosphorylate distant DNA sequences when they are tethered in the vicinity of the DSB. As shown in Figure 6, the γH2AX modification spreads around the RE region, but with significantly delayed kinetics compared with the modification around MAT, consistent with the idea that RE has to first recognize and bind to phosphorylated residues in the vicinity of the DSB at MAT. How these checkpoint kinases act on their target sequences is not yet firmly established. Mammalian ATM has been shown to be activated by intercellular autophosphorylation and dimer exchange, which would suggest that activated ATM would initially form a “cloud” of activated kinases around the DSB, when these two regions are brought together by Rad51-

**Materials and Methods**

**Yeast Strains**

All strains except when noted were derived from strain XW652 (ho ade3::GAL::HO HML \( a^+ \) RE MATa HMRx-B ura3-52 lys5 leu2-3,112 tp1::hisG) carrying a galactose-inducible HO endonuclease integrated at the \( ADE3 \) locus [11]. Strains are pre-cultured in YP-based medium until cell density reaches about \( 5 \times 10^6 \) per ml. Galactose induction is performed for 1 hour and stopped by the addition of 2% dextrose.

Construction of ECY406 (Figure 2A): Four LexA operators are amplified from pSH18-34 [32] using primers BglIIIlexAU (5′-cgaa cga gat cta tac ata tcc ata acct-a) and BglIIIlexAL (5′-gct gca gat ctc taa tcc cat ccc tga-3′). Then PCR products were digested with BglII and subcloned into the BamHI site of pKS88 to generate pEC15. The Splh-digested pEC15 (marked with “LEU2”) was transformed into XW676 (ho ade3::GAL::HO HML \( a^+ \) RE UA3 MATa HMRx-B ade1 leu2 tp1::hisG) to replace RE \( a^+ \) with four LexA operators to generate a strain ECY405. Then, RE \( a^+ \)-LexA-4 LEU2 from ECY405 was replaced with RE \( a^+ \)-LexA-4-LEUV to generate a strain ECY406 (Figure 2A) via transformation using PCR fragments amplified from pJH1894 with primers leu2KanU (5′-ggag aac tcg ttc gac cac caa att cag gag tcg-3′) and att att ggc tta tta aac att acg tga aag ttg ctc ggc-3′) and leu2KanL.
Plasmid Constructs
To study if Fkh1 can regulate donor preference in our LexA system, we construct a LexA-Fkh1 fusion plasmid (pEC16) carrying the coding sequence of Fkh1. Fkh1 coding sequence is PCR amplified from XW652 genomic DNA using primers XmalFkh1U (5'-ctg cgc cgg gat agg cgc ttt tgt cta tgt tta cgg ggg gat ccg cta gac gg aat gat ctt tat atc g-3') and PstIFkh1-906L (5'-cga cga cct gca gta ggc ggt cca gct gtt gta atc g-3'). The amplified PCR product is digested with XmaI and PstI and then subcloned into a pre-digested pAT4 [21] to generate the plasmid pEC16.

We also address different roles of Fkh1 domains in the regulation of donor preference, three regions of Fkh1 are subcloned into pAT4 (Figure 3A). The FHA domain of Fkh1 is amplified via PCR using donor preference, three regions of Fkh1 are subcloned into pAT4 into a pre-digested pAT4 [21] to generate three fusion plasmids pJL4, pJL5 and pJL6, respectively.

Site-Directed Mutagenesis of pJL4
Quickchange Multi Site-Directed Mutagenesis Kit (Catalog # 200315, Stratagene, La Jolla, CA) was used to mutate the FHA domain of pJL4. Two primers Fkh1-Arg80 (5'-tga ggt gta acc att ggt ggc aac gac gac age ttg aac-3') and pAT4-940R (5'-ctt tgc cag ccc ggg gat ccg ttt tgt cta tgt ttc cag tag) were used to synthesize mutant strand from pJL4. Fkh1-Arg80 shares two-base mismatches with Fkh1 and pAT4-940R perfectly matches pJL4. The mutated plasmid pJL8 (pLexA-FHA-R80A) was confirmed by direct sequencing.

References
1. Haber JE (1998) Mating-type gene switching in Saccharomyces cerevisiae. Annu Rev Genet 32: 361–399.
2. Haber JE (2012) Mating-type genes and MAT switching in Saccharomyces cerevisiae. Yeast Book Genetics Society of America, In press.
3. Klar AJ, Hicks JB, Strathern JN (1982) Directionality of yeast mating-type interconversion. Cell 29: 531–561.
4. Luo S, Eime J (1994) Silencers and domains of generalized repression. Science 264: 1768–1771.
5. Ravindra A, Weiss K, Simpson RT (1999) High-resolution structural analysis of chromatin at specific loci. Saccharomyces cerevisiae silent mating-type locus HMRa. Mol Cell Biol 19: 5944–5950.
6. Weiss K, Simpson RT (1998) High-resolution structural analysis of chromatin at specific loci: Saccharomyces cerevisiae silent mating-type locus HMLa18p. Mol Cell Biol 18: 5392–5403.
7. Weiler KS, Brousch JR (1992) Donor locus selection during Saccharomyces cerevisiae mating-type interconversion responses to distant regulatory signals. Genetics 132: 929–942.
8. Wu X, Haber JE (1995) MATa donor preference in yeast: mating-type switching activation of a large chromosomal region for recombination. Genes Dev 9: 1922–1932.
9. Wu X, Moore JK, Haber JE (1996) Mechanism of MAT alpha donor preference during mating-type switching of Saccharomyces cerevisiae. Mol Cell Biol 16: 637–648.
10. Wu C, Weiss K, Yang C, Harris MA, Ty B, et al. (1996) Mcm1 regulates donor preference controlled by the recombination enhancer in Saccharomyces mating-type switching. Genes Dev 12: 1726–1737.
11. Wu X, Haber JE (1996) A 700 bp cis-acting region controls mating-type dependent recombination along the entire left arm of yeast chromosome III. Cell 87: 277–285.
12. Coie E, Richard GF, Haber JE (2006) Saccharomyces cerevisiae donor preference during mating-type switching is dependent on chromosome architecture and organization. Genetics 173: 1197–1206.
13. Coie E, Martin J, Ryu T, Tay SY, Kendov J, et al. (2011) Dynamics of homology searching during gene conversion in Saccharomyces cerevisiae revealed by donor competition. Genetics 189: 1225–1233.
14. Weiss K, Simpson RT (1997) Cell-type-specific chromatin organization of the region that governs directionality of yeast mating type switching. Embryol 16: 4352–4360.
15. Sun K, Coie E, Zhou Z, Durrens P, Haber JE (2002) Saccharomyces forkhead protein Fkh1 regulates donor preference during mating-type switching through the recombination enhancer. Genes Dev 16: 2053–2066.

Plasmid Constructs
To study if Fkh1 can regulate donor preference in our LexA system, we construct a LexA-Fkh1 fusion plasmid (pEC16) carrying the coding sequence of Fkh1. Fkh1 coding sequence is PCR amplified from XW652 genomic DNA using primers XmalFkh1U (5'-ctg cgc cgg gat agg cgc ttt tgt cta tgt tta cgg ggg gat ccg cta gac gg aat gat ctt tat atc g-3') and PstIFkh1-906L (5'-cga cga cct gca gta ggc ggt cca gct gtt gta atc g-3'). The amplified PCR product is digested with XmaI and PstI and then subcloned into a pre-digested pAT4 [21] to generate the plasmid pEC16.

We also address different roles of Fkh1 domains in the regulation of donor preference, three regions of Fkh1 are subcloned into pAT4 (Figure 3A). The FHA domain of Fkh1 is amplified via PCR using donor preference, three regions of Fkh1 are subcloned into pAT4 into a pre-digested pAT4 [21] to generate three fusion plasmids pJL4, pJL5 and pJL6, respectively.

Site-Directed Mutagenesis of pJL4
Quickchange Multi Site-Directed Mutagenesis Kit (Catalog # 200315, Stratagene, La Jolla, CA) was used to mutate the FHA domain of pJL4. Two primers Fkh1-Arg80 (5'-tga ggt gta acc att ggt ggc aac gac gac age ttg aac-3') and pAT4-940R (5'-ctt tgc cag ccc ggg gat ccg ttt tgt cta tgt ttc cag tag) were used to synthesize mutant strand from pJL4. Fkh1-Arg80 shares two-base mismatches with Fkh1 and pAT4-940R perfectly matches pJL4. The mutated plasmid pJL8 (pLexA-FHA-R80A) was confirmed by direct sequencing.

Chromatin Immunoprecipitation (ChIP)
Procedures for ChIP analysis were described previously [15]. Rabbit anti-LexA polyclonal antibody (Catalog no. 39184) used in ChIP assay is purchased from “Active Motif” company (Carlsbad, CA). LexA ChIP signals are quantified with real-time PCR using a Chromo 4 machine from MJ Research. The linearity of PCR signals is monitored with r-square value of a calibration curve, which is prepared using a series of dilutions of the 0 hr input sample. IP signal is determined by comparing to the calibration curve, and then normalized to the IP signal of a control locus CEN8. PCR primer sequences around the MATT, RE and the ectopic "ten":HO sequences are available on request.

Acknowledgments
We are grateful for comments and suggestions from the Haber lab and Xiaohua Wu. We are grateful to Akira Shiohara for the gift of Rad51 antibody.

Author Contributions
Conceived and designed the experiments: JL EC KL C-SL J-AK QW JEH. Performed the experiments: JL EC KL C-SL J-AK QW. Analyzed the data: JL EC KL C-SL J-AK JEH. Contributed reagents/materials/analysis tools: JL EC KL C-SL J-AK QW JEH. Wrote the paper: JL JEH.
31. Lichten M, Borts RH, Haber JE (1987) Meiotic gene conversion and crossing over between dispersed homologous sequences occurs frequently in Saccharomyces cerevisiae. Genetics 115: 233–246.
32. Sugawara N, Wang X, Haber JE (2003) In vivo roles of Rad52, Rad54, and Rad55 proteins in Rad51-mediated recombination. Mol Cell 12: 209–219.
33. Wolfner B, van Komen S, Sung P, Peterson CL (2003) Recruitment of the recombinational repair machinery to a DNA double-strand break in yeast. Mol Cell 12: 221–232.
34. Moore JD, Yazgan O, Ataian Y, Krebs J (2007) Diverse roles for histone H2A modifications in DNA damage response pathways in yeast. Genetics 176: 15–25.
35. Hendzel MJ, Wri Y, Mancini MA, Van Hooser A, Ranalli T, et al. (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106: 348–360.
36. Goto H, Tomono Y, Ajiro K, Kosako H, Fujita M, et al. (1999) Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. J Biol Chem 274: 25543–25549.
37. Dai J, Higgins JM (2005) Haspin: a mitotic histone kinase required for metaphase chromosome alignment. Cell cycle 4: 665–668.
38. Kayne PS, Kim UJ, Han M, Mullen JR, Yoshizaki F, et al. (1998) Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell 95: 27–39.
39. Toh GW, Sugawara N, Dong J, Toth R, Lee SE, et al. (2010) Mec1/Tel1-dependent phosphorylation of Sbe1 stimulates Rad1-Rad10-dependent cleavage of non-homologous DNA tails. DNA repair 9: 718–728.
40. Hanna DE, Rethinaswamy A, Glover CV (1995) Casein kinase II is required for cell cycle progression during G1 and G2/M in Saccharomyces cerevisiae. J Biol Chem 270: 25865–25871.
41. Bressan DA, Vasquez J, Haber JE (2004) Mating type-dependent constraints on the mobility of the left arm of yeast chromosome III. J Cell Biol 164: 361–371.
42. Dekker J, Rippe K, Dekker M, Kleckner N (2002) Capturing chromosome conformation. Science 295: 1306–1311.
43. Miele A, Bystricky K, Dekker J (2009) Yeast silent mating type loci form heterochromatic clusters through silencer protein-dependent long-range interactions. PLoS Genet 5: e1000478. doi:10.1371/journal.pgen.1000478.
44. Knott SK, Peace JM, Ostrow AY, Gan Y, Rex AL, et al. (2012) Forkhead transcription factors establish origin timing and long-range clustering in S. cerevisiae. Cell 148: 99–111.
45. Bakkenist C, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 421: 499–506.
46. Bonilla CY, Melo JA, Toczyski DP (2008) Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage. Mol Cell 30: 267–276.
47. Lee JH, Paull TT (2003) ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science 308: 551–554.
48. Nakada D, Matsumoto K, Sugimoto K (2003) ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism. Genes Dev 17: 1957–1962.
49. Cimprich KA, Cortez D (2008) ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol 9: 616–627.
50. Zou L, Elledge SJ (2003) Sensing DNA damage through ATRIP recognition of RPA-RadDNA complexes. Science 300: 1542–1548.
51. Dekker J (2008) Mapping in vivo chromatin interactions in yeast suggests an extended chromatin fiber with regional variation in compaction. J Biol Chem 283: 14523–14540.
52. Estojak J, Brent R, Goleminis EA (1995) Correlation of two-hybrid affinity data with in vitro measurements. Mol Cell Biol 15: 5820–5829.
53. Lee SE, Moore JK, Holmes A, Umezu K, Kolodner RD, et al. (1998) Saccharomyces Ku70, Mre11/Rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage. Cell 94: 399–409.
54. Paques F, Leung WY, Haber JE (1998) Expansions and contractions in a tandem repeat induced by double-strand break repair. Mol Cell Biol 18: 2045–2054.