RESEARCH COMMUNICATION

Selective modulation of the functions of a conserved DNA motor by a histone fold complex

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Budding yeast Mph1 helicase and its orthologs drive multiple DNA transactions. Elucidating the mechanisms that regulate these motor proteins is central to understanding genome maintenance processes. Here, we show that the conserved histone fold MHF complex promotes Mph1-mediated repair of damaged replication forks but does not influence the outcome of DNA double-strand break repair. Mechanistically, scMHF relieves the inhibition imposed by the structural maintenance of chromosome protein Smc5 on Mph1 activities relevant to replication-associated repair through binding to Mph1 but not DNA. Thus, scMHF is a function-specific enhancer of Mph1 that enables flexible response to different genome repair situations.

Supplemental material is available for this article.

Received January 23, 2015; revised version accepted April 14, 2015.

DNA motor proteins fulfill critical roles in genome maintenance by processing different DNA structures or nucleoprotein complexes in an ATP-dependent manner. The budding yeast motor protein Mph1 and its orthologs in other eukaryotes possess several activities pertinent to genome replication and repair (Kee and D'Andrea 2010;

Whitby 2010). When replication is blocked by template lesions, their replication fork regression activity provides an important tool for remodeling the stalled fork to allow lesion bypass (Gari et al. 2008a,b; Sun et al. 2008; Luke-Glaser et al. 2010; Kang et al. 2011; Zheng et al. 2011; Blackford et al. 2012). During DNA double-strand break (DSB) repair, they can dissociate D loops made by the homologous recombination protein machinery, leading to a noncrossover (NCO), as opposed to crossover (CO), outcome (Sun et al. 2008; Prakash et al. 2009; Kang et al. 2011; Crismani et al. 2012; Lorenz et al. 2012; Mazón and Symington 2013). Additional functions have also been demonstrated for some family members. For example, the human FANCM protein, which is linked to the cancer-prone disease Fanconi anemia (FA), facilitates the repair of interstrand cross-linked DNA in conjunction with other FA proteins (e.g., Xue et al. 2008; Wang et al. 2013). Conceptually, differential regulation of the activities of Mph1 family members would be necessary for achieving a flexible response to unique situations occurring during DNA replication versus DNA repair.

The histone fold proteins Mhf1 and Mhf2 form the MHF complex that structurally resembles the histone H3 and H4 heterotetramer and interacts with Mph1 and its orthologs (Singh et al. 2010; Yan et al. 2010; Daee et al. 2012; Tao et al. 2012; Yang et al. 2012; Bhattacharjee et al. 2013; Fox et al. 2014; Zhao et al. 2014). Recent work in fission yeast and human cells suggests that MHFs positively influence Mph1 family proteins. Fission yeast MHF enhances NCO generation and replication fork processing, while human MHF (hMHF) promotes FANCM chromosomal association and DNA motor activities (Singh et al. 2010; Yan et al. 2010; Lorenz et al. 2012; Bhattacharjee et al. 2013; Fox et al. 2014). The mechanisms underlying MHF functions are not completely understood, but the distinct effects of MHF in different organisms suggest that it may be tailored to the specific needs in an organism. Here, we examine how the budding yeast MHF (referred to as scMHF) influences Mph1 functions.

Like its orthologs, Mph1 promotes NCO formation during DSB repair via its D-loop-disruptive activity and supports recombinational repair during replication using its fork regression and branch migration activities (Schurer et al. 2004; Chen et al. 2009; Mankouri et al. 2009; Prakash et al. 2009; Choi et al. 2010; Ede et al. 2011; Kang et al. 2011; Zheng et al. 2011; Xue et al. 2014). The latter functions are selectively inhibited by the Smc5/6 complex, a structural maintenance of chromosomes (SMC) family member important for genome maintenance. Smc5 binds to the Mph1 regulatory domain and selectively inhibits its DNA fork regression and branch migration activities but not its D-loop-disruptive attribute (Chen et al. 2009; Xue et al. 2014). In addition, cellular defects associated with *smc5/6* mutants, including sensitivity to replication stress and accumulation of recombination intermediates, are alleviated by mutations or the removal of Mph1 (Chen et al. 2009; Choi et al. 2010; Chavez

[**Keywords:** homologous recombination; genome replication; replication fork repair; DNA motor proteins; histone fold proteins; structural maintenance of chromosome proteins]

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Article published online ahead of print. Article and publication date are online at <http://www.genesdev.org/cgi/doi/10.1101/gad.259143.115>.

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et al. 2011). These findings indicate that Smc5/6 negatively regulates select Mph1 functions. In order to understand the biological roles of scMHF, we examined how it is related to this negative regulator of Mph1 and whether it also selectively influences Mph1 functions.

We show that scMHF is epistatic with Mph1 in suppressing defects caused by Smc5/6 mutations. In contrast, scMHF, unlike Mph1, does not affect NCO and CO levels during DSB repair. Biochemical evidence reveals that scMHF specifically relieves the negative influence of Smc5 on Mph1-mediated DNA fork regression and branch migration reactions. Our findings establish scMHF as a function-specific regulator that assists Mph1 in DNA replication fork repair and highlight the functional versatility of histone fold protein complexes.

Results and Discussion

Association of scMHF and Smc5 with the Mph1 regulatory domain

We first examined whether the two Mph1 regulators scMHF and Smc5 interact with Mph1 simultaneously. In vivo tests were performed using epitope-tagged Mph1, Mhf1, and Smc5 expressed at endogenous levels from their chromosomal loci. Mhf1 coimmunoprecipitated with Mph1, consistent with their reported interaction (Fig. 1A, top; Daee et al. 2012). In addition, Mhf1 coimmunoprecipitated with Smc5, suggesting that scMHF also associates with Smc5 in vivo (Fig. 1A, bottom). Next, recombinant scMHF, Mph1, and Smc5 were purified from insect cell or *Escherichia coli* expression systems (Supplemental Fig. S1A,B; see the Materials and Methods) for in vitro pull-down assays. The results showed that Mph1 interacts with scMHF (Supplemental Fig. S1A, lane 3). In addition, Smc5 pulled down Mph1 in complex with scMHF (Fig. 1B, lane 3). As Smc5 directly interacts with Mph1 (Chen et al. 2009; Xue et al. 2014) but not with scMHF (Supplemental Fig. S1B, lane 3), we concluded that Mph1 plays a bridging role in the trimeric complex with Smc5 and scMHF.

Using yeast two-hybrid (Y2H) and biochemical analyses, we showed previously that Smc5 interacts with the Mph1 C-terminal regulatory domain (residues 754–993) (Fig. 1C; Xue et al. 2014). Here, we found that scMHF also interacts with this Mph1 domain in Y2H analysis (Fig. 1C). In vitro pull-down with purified proteins confirmed this finding, as this domain of Mph1, but not its helicase domain (residues 1–754), binds scMHF (Fig. 1D, lane 3; Supplemental Fig. S1C, lane 9). Taken together, these results show that scMHF and Smc5 can simultaneously interact with Mph1 through its regulatory region.

mhf suppresses smc6 mutant defects in epistasis with mph1

The *mph1Δ* mutation as well as the *mph1* helicase-dead allele suppress the sensitivity of *smc6* mutants to replication-blocking agents, such as methyl methanesulfonate (MMS) (Chen et al. 2009; Choi et al. 2010; Chavez et al. 2011). This suppression correlates with a reduction in DNA recombination intermediates that stem from processing of stalled or injured replication forks (Chen et al. 2009; Choi et al. 2010; Chavez et al. 2011). The correlation suggests that suppression is related to the loss of Mph1-

Figure 1. scMHF interacts with Mph1 and Smc5 in vivo and in vitro. (A) Coimmunoprecipitation of Mhf1 with Mph1 and Smc5 from cell extracts. TAP-tagged Mhf1 was immunoprecipitated, and the presence of Flag-tagged Mph1 and Myc-tagged Smc5 in the immunoprecipitates was revealed by Western blot analysis. Extracts containing untagged Mhf1 were included as controls. (B) Smc5 associates with the Mph1-scmMHF complex. Mph1-scmMHF was retained on amylose resin preloaded with MBP-Smc5 (lanes 1–3) but not MBP (lanes 4–6). Supernatant (S), wash (W), and eluate (E) fractions of the pull-down reactions were analyzed. (C) The Mph1 regulatory region interacts with Smc5 and scMHF. (Top) schematic of Mph1 helicase and regulatory domains. (Bottom) Mph1 (residues 1–993) and its regulatory region (residues 754–993) show two-hybrid interactions with Smc5, Mhf1, and Mhf2, as indicated by growth on the $-H+3AT$ medium. Empty indicates vector-alone situations. (D) The Mph1 regulatory domain binds scMHF. GST-tagged scMHF (lanes 1–3) or GST (lanes 4–6) was incubated with the Mph1 regulatory domain (residues 754–993). Various fractions from the pull-down reactions were examined as in B. The arrow denotes the Mph1 regulatory domain being pulled down by GST-scmMHF.

mediated replication fork processing. We thus examined *smc6* suppression to assess whether scMHF affects this Mph1-mediated function. Removal of either MHF subunit suppressed the MMS sensitivity of the *smc6-P4* mutant (Fig. 2A) and the *smc6-9* and *smc6-56* mutants (Supplemental Fig. S2A). The *mhf1Δ mhf2Δ* double mutant (*mhfΔ*) exhibited suppression similar to that of the single mutants (Fig. 2A; Supplemental Fig. S2A), suggesting that the Mhf proteins work together as a functional entity within this context.

The strength of suppression of *smc6* by *mhf1Δ* is less than that by *mph1Δ*, and the suppression conferred by the *mhf1Δ mph1Δ* double mutant is similar to that of *mph1Δ* (Fig. 2B). The same results were obtained for *mhf2Δ* (data not shown). Our interpretation is that scMHF facilitates Mph1-dependent replication-associated repair, but Mph1 retains partial function in its absence. Consistent with this premise, removal of either or both of the Mhf proteins engendered moderate MMS sensitivity but did not sensitize *mph1Δ* (Fig. 2C; Supplemental Fig. S2B). Collectively, the genetic analyses support the notion that Mhf1 and Mhf2 act together to promote replication-associated repair by Mph1.

Figure 2. scMHF acts in the same pathway with Mph1 to regulate recombination intermediates generated during replication. (A) *mhf1Δ*, *mhf2Δ*, and *mhf1Δ mhf2Δ* (*mhfΔ*) suppress the MMS sensitivity of *smc6-P4* (marked as *smc6*) cells. (B) *mhf1Δ* suppression of *smc6-P4* MMS sensitivity shows epistasis with *mph1Δ*. (C) *mhf1Δ* and *mhf2Δ* exhibit mild MMS sensitivity and are epistatic with each other and with *mph1Δ*. *mhf1Δ* and *mhf2Δ* sensitivity is seen at the higher MMS concentration, and their epistatic relationship with *mph1Δ* is clear at the lower MMS concentration. (D) Two-dimensional gel analyses show reduced X-shaped molecule (X-mol) levels when scMHF was removed in *smc6-P4* cells. Log-phase cells were arrested in G2 by nocodazole and released to medium containing 0.03% MMS. Cells were collected at the indicated times with MMS treatment. X-mols are indicated by white arrows. (E) *mhf1Δ* does not affect the MMS sensitivity of *rad51Δ* cells.

scMHF, like Mph1, influences replication-associated recombination structures

To further assess the role of scMHF in replication-associated repair, we examined whether its removal, like *mph1Δ*, would reduce the level of recombination intermediates in *smc6* mutants. As reported previously, *smc6-P4* mutant cells accumulated these intermediates, detected as X-shaped molecules (X-mols) in two-dimensional (2D) gel analysis, when cells replicated in the presence of MMS-induced damage (Fig. 2D, arrows). Consistent with its suppression of the MMS sensitivity of *smc6-P4* (Fig. 2A), *mhfΔ* reduced the level of X-mols in *smc6-P4* cells (Fig. 2D). These results provide evidence that the *smc6*-suppressive effect of *mph1* and *mhf* can be partly attributed to a reduction in toxic replication-associated recombination intermediates. Further substantiating this notion, *mhf1Δ* or *mhfΔ* did not enhance the MMS sensitivity of cells lacking the recombinase Rad51 (Fig. 2E; data not shown). We note that the suppression of the *smc6* mutant sensitivity to MMS through *mph1Δ* or *mhfΔ* is not complete, likely because Smc5/6 has additional functions in the replication stress response (Kegel and Sjogren 2010).

scMHF, unlike Mph1, does not affect CO control

Having established that scMHF positively influences Mph1 in replication-associated functions, we tested whether it also affects the ability of Mph1 to regulate the CO:NCO ratio during DNA break repair by homologous recombination (Prakash et al. 2009; Mazón and Symington 2013). We used an established ectopic recombination system to assess whether scMHF also affects the CO:NCO ratio (Prakash et al. 2009). Here, a DSB induced by the HO endonuclease in the *MATa* sequence on chromosome V is repaired by recombination using the *MATa-inc* donor sequence on chromosome III (Fig. 3A). The CO and NCO recombination products can be easily discerned by size upon restriction enzyme digestion and Southern blotting (Fig. 3A). Consistent with the published results (Prakash et al. 2009), *mph1Δ* showed a five-fold greater CO:NCO ratio than wild-type upon DSB induction (Fig. 3B,C). In contrast, *mhf1Δ*, *mhf2Δ*, and *mhfΔ* cells gave the wild-type ratio of CO and NCO recombinants, and introduction of *mhf1Δ* did not alter the CO:NCO ratio in *mph1Δ* cells (Fig. 3B,C). DSB repair also proceeds with wild-type efficiency in *mhf* single and double mutants (Fig. 3B). Thus, unlike Mph1, scMHF has no role in CO versus NCO control. Taking into con-

sideration results presented earlier, these findings reveal that scMHF selectively regulates specific functions of Mph1.

scMHF promotes specific Mph1 functions by relieving Smc5 inhibition

Given that Smc5 exhibits the same selectivity as scMHF in Mph1 regulation and that they both interact with Mph1, we tested the possibility that scMHF may counteract the inhibitory effect of Smc5. As reported previously (Xue et al. 2014), Smc5, when added in an amount stoichiometric to Mph1, reduced Mph1-mediated regression of the mobile replication fork (MRF) (Fig. 4A) and branch migration of the mobile Holliday junction (MHJ) (Fig. 4B). The addition of a similar amount of scMHF restored these activities to an intermediate level, indicative of an ability of scMHF to relieve the inhibitory effect of Smc5 on these Mph1 activities (Fig. 4A,B). Control experiments showed that, in the absence of Smc5, scMHF had no effect

Figure 3. scMHF does not affect CO levels or DSB repair. (A) Schematic of the ectopic recombination assay as described previously (Prakash et al. 2009). (B) Southern blot analysis of gene conversion with and without COs in the indicated strains. (C) Quantification of CO frequency in ectopic recombination. Plotted are the mean values $\pm$ SD from at least three independent experiments.

Figure 4. scMHF relieves Smc5 inhibition on Mph1. (A,B) Smc5 inhibits Mph1-mediated regression of a MRF (A) and branch migration of a MHJ (B). The addition of scMHF in the reaction relieves Smc5's inhibitory effect in both reactions. The incubation time was 4 min. The asterisks denote the 32 P label. Plotted are the mean values $\pm$ SD from three experiments. (C) scMHF competes with Smc5 for Mph1 binding. MBP-tagged mph1 (754–993) prebound to amylose beads was used to pull down Smc5 in the absence or presence of an increasing amount of scMHF. Pull-down reactions were analyzed as in Figure 1C. The relative ratios of Smc5 versus Mph1 in the eluates are indicated. Arrows denote Smc5 bands. (D) mhf-mut does not interact with Mph1 in vitro. Flag-tagged Mph1 was pulled down in combination with scMHF or mhf-mut. Arrows denote scMHF bands or the lack of them.

on these Mph1 activities (Supplemental Fig. S3A–D). Consistent with a lack of involvement in CO:NCO control, scMHF either alone or in combination with Smc5 did not influence D-loop dissociation by Mph1 (Supplemental Fig. S4A–C). We note that, similar to scMHF, Smc5/6 does not affect CO:NCO control (Lilienthal et al. 2013; S Gangloff, pers. comm.) or D-loop dissociation by Mph1 (Supplemental Fig. S4A–C; Xue et al. 2014). Taken together, the biochemical data indicate that scMHF selectively promotes Mph1 functions by relieving the inhibitory effects of Smc5 in replication fork processing.

scMHF attenuates Mph1–Smc5 interaction in vitro

Considering that both scMHF and Smc5 bind to the regulatory domain of Mph1, scMHF may relieve Smc5 inhibition of Mph1 through attenuating Smc5–Mph1 interaction. To test this idea, we examined whether scMHF could affect the interaction of Smc5 with the Mph1 regulatory domain with which both proteins associate. Importantly, in a concentration-dependent manner, the addition of scMHF led to a reduction of Smc5 brought down by the Mph1 regulatory domain (Fig. 4C). This finding provides a plausible mechanism for how scMHF may counteract the Smc5-mediated Mph1 inhibition.

Interaction with Mph1 is indispensable for scMHF function

The above results predicted that scMHF regulation of Mph1 requires its interaction with Mph1. However, as

hMHF binds DNA (Singh et al. 2010; Yan et al. 2010), we first clarified whether DNA binding is also involved in scMHF functions. By comparing scMHF and hMHF structures (Yan et al. 2010; Zhao et al. 2014), we found that the former lacks both the C-terminal arm region and positively charged surface residues on hMHF subunits involved in DNA binding (Supplemental Fig. S5A, circled regions). Indeed, DNA mobility shift assays showed that, unlike hMHF, scMHF does not bind DNA fork, HJ DNA, or dsDNA (Supplemental Fig. S5B–D). Moreover, the Mph1–scMHF complex has the same affinity as Mph1 for these DNA substrates (Supplemental Fig. S6A–C). Thus, scMHF does not bind DNA or affect the affinity of Mph1 for DNA.

Next, we tested whether scMHF's selective regulatory effect depends on its interaction with Mph1. Based on the structure of the hMHF–FANCM complex and the sequence alignment of MHF proteins (Singh et al. 2010; Yan et al. 2010; Tao et al. 2012), five residues in scMHF were predicted to be involved in Mph1 interaction (Supplemental Fig. S7A,B). These include three scMhf1 residues (R36, L44, and S54, located in helix 2) and two scMhf2 residues (E70 and L75, located in helix 3). These residues were mutated to create mhf1-R36A/L44A/S54A and mhf2-E70A/L75E mutants (designated as mhf-mut). The mutant proteins expressed well and formed a stable complex that could be readily purified, indicating that the mutations do not affect general protein folding or subunit interactions. In vitro pull-down tests showed that mhf-mut does not bind Mph1 (Fig. 4D, lane 6), suggesting that hMHF–FANCM and scMHF–Mph1 complexes share conserved protein interfaces. Importantly, mhf-mut, unlike the wild-type counterpart, failed to overcome the inhibitory effect of Smc5 on Mph1-mediated catalysis of fork regression and HJ branch migration (Fig. 5A,B), indicating scMHF's function as being reliant on its interaction with Mph1.

We then examined mhf-mut in vivo to understand the biological importance of the scMHF and Mph1 interaction. Consistent with the in vitro finding, mhf-mut expressed from the endogenous *MHF* loci failed to coimmunoprecipitate Mph1 (Fig. 5C). Importantly, *mhf-mut*, like *mhf* deletions, suppressed the MMS sensitivity of *smc6* mutants (Fig. 5D) and furthermore reduced the levels of X-mols in these mutants in the presence of MMS (Fig. 5E). Taken together, the in vivo and in vitro findings reveal that scMHF promotes specific Mph1 functions during replication-associated repair through its interaction with Mph1.

Mph1 and its orthologs are indispensable for the cellular response to replication stress and DNA break repair, and mutations in FANCM can lead to a predisposition to oncogenesis (Kee and D'Andrea 2010; Whitby 2010). Recent studies identified the histone fold complex MHF as a conserved regulator of these motor proteins (Singh et al. 2010; Yan et al. 2010; Lorenz et al. 2012; Tao et al. 2012; Yang et al. 2012; Bhattacharjee et al. 2013; Sugahara et al. 2013; Wang et al. 2013; Fox et al. 2014; Zhao et al. 2014). Here we provide genetic and biochemical evidence that the budding yeast MHF selectively regulates Mph1 functions to highlight the functional versatility of this family of histone fold complexes.

Several findings suggest that scMHF facilitates Mph1 functions pertaining to replication-associated repair. Like *mph1* mutations, *mhf* mutations suppress the MMS sensitivity of *smc6* cells (Fig. 2A,B; Supplemental Fig.

Figure 5. scMHF regulation of Mph1 requires its association with Mph1. (A,B) *mhfl-mut* does not relieve Smc5 inhibition on Mph1. The indicated proteins were incubated with the MRF (A) or MHJ (B) substrate for 4 min. (C) *mhfl-mut* fails to interact with Mph1 in cells. Strains contained Flag-tagged Mph1. (WT) Cells containing strep-tagged Mph1; (mut) cells containing strep-tagged *mhfl-mut*; (untag) cells containing untagged scMHF. Cell lysates (right) and immunoprecipitation fractions (left) were examined by Western blotting using anti-Flag (top) and anti-Strep (bottom) antibodies. (D) *mhfl-mut* suppresses the MMS sensitivity of *smc6-P4* cells. (E) *mhfl-mut* reduces the level of X-mols in *smc6-P4* cells, as revealed in 2D gel analysis. (F) Model depicting the role of scMHF in Mph1 regulation. See the text for details.

S2A); importantly, this correlates with decreased replication-associated recombination intermediates (Fig. 2D). The *mhfl* and *mhfl2* mutations are epistatic with each other and with *mph1Δ* (Fig. 2A–C; Supplemental Fig. S2), indicating that scMHF collaborates with Mph1 in replication-associated repair. Interestingly, scMHF does not affect the CO:NCO ratio (Fig. 3) even though Mph1 is an important regulator in this regard (Prakash et al. 2009; Mazón and Symington 2013). Thus, scMHF is a selective regulator of Mph1 functions during replication-associated repair but surprisingly has no role in DSB repair. Our study thus provides a new example that distinct functions of multitasking DNA motor proteins can be differentially regulated by histone fold proteins. Our data suggest that scMHF enables versatile and fine-tuned Mph1 responses to genome replication versus DSB repair situations.

Our work also provides mechanistic insights into the function of scMHF. Consistent with our genetic finding, scMHF does not affect D-loop dissociation by Mph1 (Supplemental Fig. S4) but relieves Smc5-mediated inhibition of Mph1 replication fork regression and DNA branch migration activities (Fig. 4A,B). As scMHF alone has no effect on these latter activities of Mph1 (Supplemental Fig. S3), our results indicate that scMHF serves as a specific regulator that relieves the inhibitory function of Smc5/6 on Mph1. Considering the finding that both Smc5 and scMHF interact with the Mph1 regulatory region (Fig. 1C,D; Xue et al. 2014), scMHF may render Mph1 more resistant to Smc5 inhibition through its association with Mph1. This premise is supported by both in vitro binding assays and mutagenesis studies (Figs. 4C,D, 5A–E). Our findings thus provide the first evidence that Mph1-mediated replication fork repair function is subject to regulation by both positive and negative regulators, underscoring the intricate control of this repair pathway (schematized in Fig. 5F). In cells, the role of Smc5 requires the integrity of the Smc5/6 complex. As *smc6-P4*, *smc6-9*, and *smc6-56* all affect the coiled-coil part of the protein, a region critical for intersubstrate interplay and thus complex function, they likely diminish the ability of Smc5 to bind and/or inhibit Mph1. Testing these ideas would entail biophysical characterization of the Smc5/6 complex and its mutant forms.

Mph1 and its regulators are important for preventing the genome alterations associated with cancer predisposi-

tion and other human pathologies. Our work provides mechanistic insights into how antagonistic regulators functionally intersect to affect the engagement of a conserved replication fork repair pathway. These and studies of MHF in other organisms suggest a model that this type of histone fold complex is a situation-dependent regulator of Mph1/FANCM family DNA motors tailored to the specific needs of a given eukaryotic organism. The intellectual and experimental frameworks that we and others have established should facilitate the continuing dissection of functions of these motor proteins in genome maintenance and cancer avoidance.

Materials and methods

Plasmids and yeast strains are listed in Supplemental Table. *smc6-P4* contains a K239R mutation (Chen et al. 2009), *smc6-9* contains Q903G and S908P mutations, and *smc6-56* contains A287V, H379R, and I421T mutations (Sollier et al. 2009). Strain construction and yeast culturing were performed using standard procedures. For damage sensitivity tests, 10-fold serial dilutions of log-phase cultures were spotted on plates with or without MMS. For Y2H assays, the bait plasmids containing GBD fusions (based on the pOBD vector) were cotransformed with the prey plasmids containing GAD fusions (based on the pOAD vector). Methods for Mph1, Smc5, and hMHF expression and purification have been described (Prakash et al. 2009; Zheng et al. 2011; Xue et al. 2014). Expression and purification of scMHF and mutants and assays for DNA mobility shift, coimmunoprecipitation, affinity pull-down, competition pull-down, 2D gel, CO assessment, and DSB repair followed published procedures (Chen et al. 2009; Xue et al. 2014) and/or are detailed in the Supplemental Material.

Acknowledgments

We thank Xiao Peng and Prabha Sarangi for their help in editing the manuscript, Xiaofeng Zheng for her help in protein purification, and Bingbing Wan for his help in preparing Supplemental Figure S7. This study was supported by U.S. National Institutes of Health (NIH) grants GM057814, ES007061, ES015632, and ES015252 to P.S.; NIH grant GM080670, American Cancer Society grant RSG-12-013-01-CCG, and a Leukemia and Lymphoma Society Scholar Award to X.Z.; NIH grant GM80600 to G.I.; the Italian Association for Cancer Research (AIRC IG 14171), Fondazione Telethon (GGP12160), and European Research Council (REPSUBREP 242928) grants to D.B.; NIH grant K99/R00 ES021441 to H.N.; and NIH grant T32 GM008539 to J.N.B. as a trainee.

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Genes Dev. 2015, **29**: originally published online May 8, 2015
Access the most recent version at doi:[10.1101/gad.259143.115](https://doi.org/10.1101/gad.259143.115)

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