Rad5-dependent DNA Repair Functions of the *Saccharomyces cerevisiae* FANCM Protein Homolog Mph1

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**Background:** Yeast proteins homologous to human Fanconi proteins exist, but their cross-link repair functions are undefined.

**Results:** Mutants are cross-link-sensitive, and Mph1 overexpression protects yeast cells.

**Conclusion:** The yeast pathway is epistatic with *rad5* and *rad51*, and the Mph1 helicase stabilizes ICL-stalled replication forks in a Rad5-dependent manner.

**Significance:** Rad5 directs the yeast Fanconi-like interstrand cross-link repair pathway.

Interstrand cross-links (ICLs) covalently link complementary DNA strands, block DNA replication, and transcription and must be removed to allow cell survival. Several pathways, including the Fanconi anemia (FA) pathway, can faithfully repair ICLs and maintain genomic integrity; however, the precise mechanisms of most ICL repair processes remain enigmatic. In this study we genetically characterized a conserved yeast ICL repair pathway composed of the yeast homologs (Mph1, Chl1, Mhf1, Mhf2) of four FA proteins (FANCM, FANCJ, MHF1, MHF2). This pathway is epistatic with Rad5-mediated DNA damage bypass and distinct from the ICL repair pathways mediated by Rad18 and Pso2. In addition, consistent with the FANCM role in stabilizing ICL-stalled replication forks, we present evidence that Mph1 prevents ICL-stalled replication forks from collapsing into double-strand breaks. This unique repair function of Mph1 is specific for ICL damage and does not extend to other types of damage. These studies reveal the functional conservation of the FA pathway and validate the yeast model for future studies to further elucidate the mechanism of the FA pathway.

DNA damaging agents such as nitrogen mustard (NM), formaldehyde (FMA), and cisplatin generate many DNA lesions including the interstrand cross-link (ICL). ICLs covalently link complementary DNA strands and prevent their separation during replication and transcription. Accordingly, ICLs are considered the most toxic of all DNA lesions. It is estimated that one unrepaired ICL is sufficient to kill a yeast or bacterial cell (4) and ~40 unrepaired ICLs can kill a mammalian cell (5).

As a result of this high cytotoxicity, cross-linking drugs are commonly used as anticancer agents (6). Each drug is bifunctional, meaning a portion of monoadducts undergo a second reaction to form ICLs, intrastrand cross-links, or DNA-protein cross-links. Although these agents form many monoadducts in addition to the secondary outcomes, the ICL seems to be the critical cytotoxic lesion (7).

Cells have the capacity to repair ICLs through highly complex DNA repair mechanisms. In *Escherichia coli*, incisions on either side of the ICL unhook a short oligo that remains attached to the intact strand; the resulting gap is filled in by recombination or translesion synthesis, and the unhooked oligo is removed by nucleotide excision repair (8).

Eukaryotic mechanisms are more complex, involve multiple repair pathways, and can occur in multiple phases of the cell cycle. Several recent reviews artfully address this complexity (9–19), which we briefly summarize here. In budding yeast, three epistasis groups (PSO2, RAD52, and RAD18) have been suggested to function in yeast ICL repair (20), but each pathway is not fully defined. Pso2 is an endonuclease that likely cleaves ICL repair intermediates (21, 22). Homologous recombination (HR) repair proteins, including Rad52 and Rad51, fill in gaps post-incision and/or repair DSBs that arise during ICL repair. Proteins in the Rad18-mediated post-replication repair (PRR) pathway (Rad5 and Rev3) comprise two damage tolerance pathways that help fill in the gaps produced after the incision and unhooking of ICLs.

Highlighting the complexity of ICL repair in higher eukaryotes, defects in most known DNA repair pathways (including nucleotide excision repair, base excision repair, PRR, and anesulfonate; OE, overexpression; CTD, C-terminal domain of Mph1; GCR, gross chromosomal rearrangements; PCNA, proliferating cell nuclear antigen.)
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HR) result in ICL sensitivity. The Fanconi anemia (FA) DNA repair pathway (named for FA patients, which have mutations in 1 of 15 FA or FA-like proteins) is a major regulator of human ICL repair (9, 23). FA mutations confer developmental defects, cancer predisposition, and marked sensitivity to ICL-forming agents. In the FA repair pathway, FANCM and FAAP24 recognize blocked forks and recruit the FA core complex (FANC A-C, E-G, L, FAAP100), which ubiquitylates FANCD2 and FANCI. These events likely promote HR repair and other poorly understood downstream repair events mediated by BRC2/FANCD1, FANCN, and FANCJ.

In contrast to a G1-specific ICL repair pathway that involves nucleotide excision repair and translesion synthesis (24), eukaryotic ICL repair pathways in S-phase require complex mechanisms to resolve blocked replication forks (15). As repair proceeds from unhooking one side of the cross-link to gap-filling and to adduct removal, the blocked replication fork remains a fragile structure with exposed single-strand DNA (ssDNA) (Fig. 1A). The ssDNA at stalled forks is vulnerable to nucleases and endogenous agents that could nick the DNA and break the fork. Early fork breakage could inappropriately engage HR, which will fail in the presence of the cross-linked substrate. This situation would be uniquely problematic for unhooked ICLs as other types of damage would not irreparably block recombination pathways. To face the unique challenges of ICL repair at stalled replication forks, mechanisms have likely evolved to move the replication machinery away from the stalled fork and to protect the ssDNA at the stalled fork. DNA helicases are good candidates for this function. Such helicases would reverse the fork and create a “chicken foot” structure, thereby moving replication proteins and protecting exposed ssDNA during repair (25). Interestingly, the DNA helicase FANCM is recruited to ICL-stalled replication forks where it may induce fork regression (26).

FANCM is one of the few FA proteins with clear, evolutionarily conserved orthologs in lower organisms (Mph1, Saccharomyces cerevisiae; Fm11, Schizosaccharomyces pombe; Hef, Archaeabacteria) (27, 28). Each of these orthologs shares a conserved helicase domain, which is important for its role in DNA repair. The FANCM interacting proteins MHF1 and MHF2, which stabilize FANCM at ICL damage sites (29, 30), are also conserved in S. cerevisiae and S. pombe (29). Additionally, the S. cerevisiae protein, Chl1, shares sequence homology with FANCJ (31, 32).

Damage sensitivity assays have clearly illustrated that the yeast proteins Mph1 (33, 34), Mhf1 and Mhf2 (29, 30), and Chl1 (35) all play a role in DNA damage tolerance and genomic integrity. Despite their apparent DNA repair roles and the sequence similarities shared with the Fanconi proteins, an evolutionarily conserved yeast ICL repair pathway consisting of these proteins has not been characterized. In the studies presented here we genetically characterized a conserved repair pathway consisting of Mph1, Mhf1, Mhf2, and Chl1. This pathway is epistatic with the recombination-mediated bypass pathway regulated by Rad5 and distinct from the Rad18- and Pso2-mediated pathways. Furthermore, consistent with the FANCM role in stabilizing ICL-stalled replication forks, we present evidence for an ICL-specific role of Mph1 where Mph1 protects ICL-stalled forks and prevents their collapse into ICL-induced DSBs (Fig. 1B).

EXPERIMENTAL PROCEDURES

Please see supplemental Methods for detailed methods and information on yeast strains, plasmids, media and buffer recipes, protein alignments, antibodies, and statistical analyses.

Genetic Assays—For damage sensitivity assays, mid-log phase cells were acutely treated for 2 h in 1 ml of water or drug. Treated cells were diluted in water and plated on extract/peptone/dextrose for spot tests, cells were serially diluted 1:5. Gross chromosomal rearrangements (GCR) mutation rates (36) and intrachromosomal recombination (IR) rates (37) and drug-induced GCR and IR frequencies were calculated as previously described (38).

Pulse Field Gel Electrophoresis—Mid-log phase yeast cultures were treated for 2 h, and pellets were frozen at −80 °C until plugs were made. A 1% pulse field-certified agarose gel ran on the CHEF-DR® III system with a 90-s initial/final switch time, 24-h run time, 6 volts/cm, and 120° included angle. Gels were imaged and analyzed with the Kodak 1D image analysis software.

FACS Analysis—The W303 RAD5 + MATa strain was grown at 23 °C, and mid-log cells were synchronized in G1 with a 3 μg/ml α factor and released into yeast extract/peptone/dextrose (YPD) or YPD containing 200 μg/ml hydroxyurea or 100 μM NM (39). Western blot analysis of TCA extracts was previously described (40).

Fork Regression Assay—Mph1 and/or Rad5 was incubated with radiolabeled DNA substrate (26) in 10 μl of buffer for 5 min at 37 °C. The reaction was terminated with 0.5% SDS and 0.5 mg/ml proteinase K. The reactions were resolved in an 8% native polyacrylamide gel. Gels were dried onto Whatman DE81 paper and analyzed in a Personal Molecular Imager FX Phosphor Imager (Bio-Rad).

RESULTS

The Yeast FANC Homologs Mph1, Chl1, Mhf1, and Mhf2 Play a Role in ICL Repair—The yeast proteins Mph1 and Chl1 share sequence homology with the human proteins FANCM and FANCJ (Fig. 2A). Based on this homology, we hypothesized that Mph1 and Chl1 would play functionally conserved roles in yeast ICL (interstrand cross-link) repair. To test this hypothesis, we examined the sensitivity of null mutants to the ICL agents2 NM (nitrogen mustard, mechlorethamine), FMA (formaldehyde), and cisplatin. For space considerations, the results of NM sensitivity assays are shown, but similar results were obtained with the other ICL agents. Although MPH1 was previously identified in a homozygous diploid screen for cisplatin sensitivity (41), the haploid null mhf1 in the S288c and BY4741 backgrounds were largely not sensitive to each ICL agent (Fig. 2B, supplemental S1, A and B). Similarly, chl1 was not reproducibly sensitive to ICL agents (Fig. 2B, supplemental S1B).

4 The definitions of the genetic terms used are epistasis, a genetic interaction that suggests that two proteins work in the same pathway due to a common, non-additive phenotype; synergistic, a complex genetic interaction where the combined phenotype is more than additive, suggesting that the pathways can compensate for one another; additive, a genetic interaction where the expressed phenotype reflects the sum of the two independent phenotypes.
Many DNA helicases function redundantly in DNA repair. In particular, the DNA helicase Srs2 shows a synergistic interaction with Mph1 when double mutants are challenged with the DNA alkylation agent methyl methanesulfonate (MMS) (42–46). We hypothesized that redundancy with Srs2 might mask the ICL repair functions of Mph1 and Chl1. To address this, we measured the NM sensitivity of the double null mutants srs2, mph1, and srs2 chl1. Consistent with our hypothesis, srs2 was further sensitized by both mph1 and chl1 (Fig. 2B), and their sensitivity was dependent on their helicase domains (Fig. 2C). If Mph1 and Chl1 function in a common pathway for ICL repair, an epistatic relationship should be apparent. In support of epistasis, mph1 chl1 double mutants had NM sensitivity (Fig. 2D), an elevated CAN1 forward mutation rate (Table 1), and an ele-
vated NM-induced mutation frequency (Table 1) that was indistinguishable from either single mutant. Furthermore, the epistatic spontaneous CAN1 forward mutation rates (Table 1) suggest that Mph1 is upstream of Chl1 as would be expected based on the human pathway (47).

Physical interactions between FANCM, MHF1, and MHF2 are conserved with the yeast homologs (supplemental Fig. S2). Also, mhf1 and mhf2 are epistatic with mph1 for MMS sensitivity (29). Based on this work we postulated that Mhf1 and Mhf2 would also play a conserved role in ICL repair. Similar to Mph1, the single mutants were not appreciably sensitive to NM, but srs2 was sensitized by both mhf1 and mhf2 (Fig. 2D). Epistatic analysis with mph1 revealed no further increase in sensitivity to NM (Fig. 2D) or the CAN1 spontaneous mutation rate (Table 1). These results suggest that Mhf1, Mhf2, and Mph1 work in a functionally conserved pathway for ICL repair.

To determine if these genetic interactions extend to other non-ICL forms of DNA damage, we examined the sensitivity of

Table 1

| Genotype       | Mutation rate $\times 10^{-7}$ (STDEV) | Mutation frequency $\times 10^{-5}$ (STDEV) |
|----------------|----------------------------------------|--------------------------------------------|
|                | Spontaneous                            | Uninduced                                 | NM-induced                               |
| WT             | 2.8 (0.73)                              | 5.9 (2.8)                                 | 84 (20)                                  |
| mph1           | 23 (5.7)$^a$                           | 25 (12)$^a$                               | 120 (33)$^a$                            |
| chl1           | 6.1 (2.4)$^a$                           | 6.2 (1.3)                                 | 130 (24)$^a$                            |
| mph1 chl1      | 25 (2.2)$^{a,b}$                        | 19 (1.1)$^{a,b}$                          | 150 (21)$^{a,b}$                         |
| mhf1           | 4.2 (0.91)                              | 5.9 (2.3)                                 | 83 (15)                                  |
| mph1 mhf1      | 23 (5.8)$^{a,b}$                        | ND                                        | ND                                       |
| mhf2           | 3.7 (0.69)                              | 4.7 (2.4)                                 | 51 (7.3)$^a$                            |
| mph1 mhf2      | 25 (4.6)$^{a,b}$                        | ND                                        | ND                                       |

$^a$ Statistically different from WT.

$^b$ Not statistically different from mph1.
each strain combination to the alkylating agent MMS. Loss of the yeast FA homologs also sensitized *srs2* to MMS (supplementary Fig. S1). These similar sensitivity phenotypes suggest that these genetic interactions are not ICL-specific; rather, they apply to a general DNA damage response.

**Mph1 and Chl1 Belong to a Recombination-based Pathway of ICL Repair**—There are three reported epistasis groups for ICL repair in yeast: *RAD18, PSO2*, and *RAD51/RAD52* (20). To determine if Mph1 and Chl1 belong to any of the established pathways, we performed epistasis analysis with *rad18*, *pso2*, and *rad51*. *Srs2* is a member of the *RAD18* pathway (48–50). We already observed a synergistic relationship between *srs2* and both *mph1* and *chl1* (Fig. 2B); thus, we predicted similar results with *rad18*. As predicted, *rad18* was sensitized by both *mph1* and *chl1* (Fig. 3A). Similarly, the *pol30-K164R* PCNA mutant (which is defective in the *Rad18*-mediated pathway) was sensitized by *mph1* (supplemental Fig. S3A). *Pso2* was also sensitized by *mph1* and *chl1* (Fig. 3B). The sensitivity of the triple mutant *pso2 chl1 mph1* was not significantly different from the *pso2* double mutants (supplemental Fig. S3B), further supporting an epistatic relationship between Mph1 and Chl1. In contrast to *pso2* and *rad18*, *rad51* was not sensitized by either *mph1* or *chl1*, suggesting that Mph1 and Chl1 function in a Rad51-dependent pathway (Fig. 3C). To further confirm this, the *rad51* experiments were repeated in an *srs2* background where an *mph1* phenotype is apparent. *srs2 mph1 rad51* displayed a similar NM sensitivity as *srs2 rad51* (Fig. 3D). Collectively, these results suggest that Mph1 and Chl1 belong to a recombination-based ICL repair pathway. Interestingly, the high NM and MMS sensitivity of *srs2 mph1* is rescued by the loss of *rad51* (Fig. 3D and supplemental S3C). This result suggests that toxic recombination intermediates accumulate in the absence of Srs2 and Mph1. The implications of this finding are further addressed in the discussion.

*Rad5* is an E3 ubiquitin ligase that has traditionally been associated with the error-free, sister chromatid exchange branch of
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the RAD18 epistasis group (as defined for MMS-induced DNA damage tolerance) (50, 51). Previous reports have suggested that Mph1 may be epistatic with this Rad5-mediated pathway of recombination (34). To determine if Mph1 is involved in this recombination branch for ICL repair, we examined the genetic interactions between RAD5 and RAD18, PSO2, and MPH1. In Figs. 2B and 3, A–D, we demonstrate that mph1 synergistically sensitizes srs2, rad18, and ps02 but not rad51. If Mph1 works with Rad5 for ICL repair, then we would predict similar phenotypes for rad5 and mph1. Consistent with this, deletion of RAD5 sensitized rad18 and ps02 to NM (Fig. 3, A and B). Similarly, rad5 srs2 was slightly more sensitive than srs2 (Fig. 3E). This mild sensitization was confirmed quantitatively (supplemental Fig. S3D) is also apparent after MMS treatment (supplemental Fig. S3E) and does not occur in rad18 srs2 (supplemental Fig. S3F). The high sensitivities of rad5 and rad18 were rescued by a defect in srs2 (Fig. 3E, supplemental S3F). This is consistent with previous genetic results showing that the MMS sensitivity of rad5 and rad18 can be rescued by the loss of Srs2 by allowing damaged substrates to be funneled into a functional recombination pathway rather than the defunct PRR pathway (48, 52). 

rad5 was not further sensitized by mph1 either alone or in combination with srs2 (compare rad5 versus rad5 mph1 and rad5 versus rad5 mph1 srs2) (Fig. 3E). Interestingly, the synthetic rescue of rad5 by srs2 was dependent on mph1 for both MMS and NM (Fig. 3E, supplemental S3E). As described above, the rescue of rad5 sensitivity in srs2 rad5 occurs due to a relief in the Srs2-dependent block of a recombination pathway that can bypass the damage. This result further supports that Mph1 functions in a recombination-dependent pathway. Surprisingly, this result also suggests that at least some recombination roles of Mph1 are independent of Rad5. Additional studies are required to determine if this role reflects proposed Mph1 functions in D-loop dissolution and/or noncrossover formation (46). Despite that the recombination role of Mph1 apparent in srs2 rad5 can function in the absence of Rad5, the common genetic phenotypes when mph1 and rad5 are combined with rad18 and ps02 suggest that Mph1 and Rad5 can also work in a common pathway for ICL repair. Furthermore, the high sensitivity of rad5 compared with mph1 suggests that Rad5 directs several ICL repair pathways, one of which is mediated through Mph1.

Interestingly, a synergistic sensitization was observed in rad5 rad51 double mutants (Fig. 3C). This synergy highlights the redundant, overlapping functions of Rad51- and Rad5-mediated ICL repair pathways and leaves the possibility that some Rad5 ICL repair functions require Rad51 (or vice versa) as has been previously proposed for other types of damage (53). If the rad5 sensitivity reflects a simple overlap between the Rad18 and Rad51 pathways, then the sensitivity of rad18 rad51 rad5 should be equal to that of rad18 rad51. For NM sensitivity, it is apparent that Rad5, Rad18, and Rad51 have at least some non-overlapping functions because the triple mutant was more sensitive than any double mutant combination (supplemental Fig. S3G).

rad5-GAA contains mutations in the ATPase domain of Rad5 (54) and rad5-C932A,H934A contains mutations in the RING domain of Rad5 (54, 55). Consistent with previous data for other types of DNA damage (55–57), we observed an intermediate NM sensitivity of these strains compared with rad5 deletion strains (Fig. 3F) and both rad5 alleles sensitized rad51 (Fig. 3F). This suggests that for ICL repair, both Rad5 domains function in pathways that complement Rad51-mediated HR pathways.

The Shu complex is linked to some functions of Rad5 (58); however, for ICL repair, Shu complex mutants were not sensitive to NM and did not sensitize srs2 (supplemental Fig. S3H). The Smc5/6 complex is epistatic with Rad5 for GCR formation (59) and is required to resolve some Mph1-dependent intermediates (60, 61). The smc6–9 mutation sensitized rad18 and was epistatic with rad5, suggesting that the Smc5/6 complex functions in this Rad5-mediated branch of ICL repair (supplemental Fig. S3I).

Mph1 Fork-reversal Activity Is Not Stimulated by Rad5—Our genetic evidence suggests that Rad5-mediated ICL repair involves Mph1. We considered that Rad5 might physically recruit Mph1 to sites of ICL-stalled replication; however, co-immunoprecipitation experiments did not reveal a physical interaction between the proteins. In addition, a genome-wide yeast two-hybrid screen for Rad5 interacting proteins did not identify Mph1.5

We hypothesize that Mph1 helicase stabilizes the intermediates of the ICL repair pathway by catalyzing fork reversal (Fig. 1B). In support of this, purified Mph1 catalyzed migration of replication fork-like structures in vitro (Ref. 62; Fig. 4, B and C). Rad5 can also catalyze fork regression (Fig. 4, B and C, and Ref. 63). To determine if fork reversal is stimulated by the presence of both Mph1 and Rad5, both proteins were combined in the replication fork reversal assay. The combined treatment appeared to reflect an additive rather than synergistic, effect on product formation under conditions of both limiting (Fig. 4B) and excess (Fig. 4C) substrate, suggesting that Rad5 does not stimulate the replication fork reversal activity of Mph1 or vice versa. Similar results were obtained when purified Mhf1 and Mhf2 were included in the reaction (data not shown), ruling out the possibility that these proteins are required to mediate synergy between Mph1 and Rad5.

Mph1 Expression Protects Cells from ICL Damage in a Rad5-dependent Manner—Based on the helicase-dependent fork reversal functions of Mph1 and FANCM (26) and the helicase requirement of the role in Mph1 in ICL repair, we predicted that Mph1 stabilizes a DNA structure that facilitates repair. If this is the case, overexpression of Mph1 may increase the formation of these structures and further protect cells from ICL damage. To address this possibility, we overexpressed Mph1 and measured survival in the presence of cross-linking drugs. Mph1 overexpression (Mph1 OE) clearly improved the survival of cells exposed to FMA (Fig. 5A). This phenotype is in marked contrast to the enhanced sensitivity observed after MMS treatment of Mph1 OE cells (Ref. 42 and Fig. 5B). Interestingly, Mph1 OE did not protect cells from NM exposure (supplemental Fig. S4A). In addition to ICL formation, NM (like MMS) forms many alkylation monoadducts on DNA. Although FMA-

5 K. Myung, unpublished data.
specific protection cannot be ruled out, the intermediate difference between vector and Mph1 OE for NM (2-fold) exposure compared with MMS (8-fold) and FMA (80-fold) likely highlights the mixed adduct formation after NM exposure (supplemental Fig. S4A).

Mph1 contains a helicase domain and a short C-terminal domain (CTD) that is essential for replication protein A interactions (Fig. 2A) (42). To determine which domain is important for the improved survival observed with Mph1 OE, we examined the survival when alleles defective in either domain were overexpressed. Improved survival was not apparent when either the helicase dead (E210Q OE) or the CTD deletion (CTD OE) alleles were overexpressed (Fig. 5A). Improved survival was also dependent on Rad5 and PCNA ubiquitylation (Fig. 5C).

Notably, Mph1 OE actually sensitizes both pol30-K164R and rad5 backgrounds to FMA (Fig. 5C). The implication of this observation is addressed under “Discussion.”

The PCNA residue Lys-164 can be mono-ubiquitylated (Rad18-dependent), poly-ubiquitylated (Rad5-, Ubc13/Mms2-dependent), or sumoylated (Siz1-dependent). To ensure that improved survival is mediated by PCNA ubiquitylation and not sumoylation, we examined survival in siz1 and ubc13 backgrounds (Fig. 5C). The implication of this observation is addressed under “Discussion.”

Our group previously reported that Mph1 OE promotes GCRs through the CTD (42). GCR frequencies were measured to determine if Mph1-mediated GCRs promote FMA survival. FMA-induced GCR frequencies in mph1 were indistinguishable from WT (supplemental Table S1, p = 0.2). Furthermore, although Rad5 is required for the ICL resistance observed with Mph1 OE (Fig. 5C), Mph1-induced GCR frequencies remained high in rad5 (supplemental Table S1). This result suggests that Mph1-mediated GCRs and ICL protection are independent phenotypes. Thus, GCR formation is not a major contributor to the ICL protection observed with Mph1 OE.

**Mph1 Expression Protects DNA from ICL-induced Double-strand Breaks**—We found that G1-synchronized cells allowed to replicate in the presence of NM activated checkpoints (measured as Rad53 activation) and exhibited a prolonged arrest in G2, likely due to the presence of unrepaired DNA lesions (Fig. 6A). Furthermore, NM (but not hydroxyurea) treatment leads to accumulation of γ-H2A, a marker for DNA lesions and DSBs (Fig. 6A). These data suggest that NM treatment creates problems in S phase.

Rad5 bypass repair is initiated by replication problems (64, 65). Based on Mph1 genetic interaction with Rad5 for ICL damage (Fig. 5B). To further support this notion we examined ICL- and MMS-induced mutation frequencies. Although loss of mph1 and chl1 did not increase MMS-induced mutation frequencies, ICL-induced mutations were significantly elevated (Fig. 5D). Thus the Mph1 pathway uniquely prevents ICL-induced mutations. Furthermore, an epistatic relationship between Mph1 and Chl1 is supported as the double mutant ICL-induced mutation frequency was not significantly different from single mutant frequencies (Fig. 5D).
repair, we hypothesized that Mph1 also functions in a pathway initiated by ICL-stalled DNA replication. We examined whether Mph1 OE affects the response of S-phase cells to NM treatment by following the replication kinetics of WT cells with Mph1 OE. Compared with controls, Mph1 OE reproducibly slowed S-phase progression without affecting the Rad53 activation and H2A accumulation induced by NM treatment (Fig. 6A).

ICL-induced DSBs can be visualized by pulse field gel electrophoresis (66). To further test whether Mph1 OE may protect against deleterious lesions, we treated asynchronous, log-phase cells for 2 h with NM, released them into fresh media, and examined the ICL repair intermediates by pulse field gel electrophoresis. Although the overall kinetics of ICL repair seemed similar between Mph1 OE and vector controls (compare the reappearance of intact chromosomes at 6 and 24 h post NM, Fig. 6B), the “time 0” smear was consistently more elongated in Mph1 OE cells (Fig. 6B). We verified and quantified this phenomenon over multiple experiments by dividing image intensity of the “protected” smear by the image intensity of the “total” smear (Fig. 6C). Importantly, the protection phenotype was not due to cell cycle differences because the FACS profiles of the asynchronous vector and Mph1 OE cells were similar at time 0 (supplemental Fig. S5A). These results suggest that Mph1 may protect a portion of the intermediates from collapsing into fully broken chromosomes. This protection was dependent on the Mph1 helicase domain and Rad5 but not the CTD of Mph1 (Fig. 6C). Interestingly, this protection seems to be unique for ICL-induced DSBs as no protection was observed after MMS treatment (although there is not conclusive evidence that MMS causes DSBs (67)) (supplemental Fig. S5B).

Our results suggest that Mph1 may protect ICL repair intermediates from aberrantly engaging HR proteins before ICL unhooking. In support of this model, Mph1 OE reduced the formation of NM-induced Rad52 foci, and mph1 mutants have enhanced NM-induced Rad52 foci (supplemental Fig. S6).

**DISCUSSION**

The ICL damage sensitivity assays presented here (Fig. 2) support an epistatic ICL repair role for the yeast Fanconi pathway homologs Mph1, Chl1, Mhf1, and Mhf2. To our knowledge this is the first evidence that the Fanconi pathway is (at least partially) functionally conserved in budding yeast. These results are consistent with the current knowledge of the human
system where the FA pathway has general functions in response to replication stressors (68), FANCM functions upstream of FANCI (47), and MHF1 and MHF2 have milder phenotypes than FANCM (29). Importantly, these studies provide further mechanistic insight into the Fanconi pathway by demonstrating that the homologous yeast repair pathway functions through a novel Ubc13-independent mechanism (mediated by Rad5 and ubiquitylated-PCNA) and is resolved through a recombination-based mechanism. These studies also provide the first in vivo evidence that helicase-mediated fork reversal can protect ICL repair intermediates from inappropriate repair and/or processing. Finally, these results validate the yeast model for future studies of the enigmatic FA repair pathway and suggest that other functional FA homologs likely exist.

Over the years studies in human cells have reported conflicting results regarding the connection between the HR, PRR, and FA pathways. For example, HR repair capacities of human FA cells are reportedly decreased (69–71), increased (72, 73), and unchanged (74). Disparate findings also mar mutagenesis results looking at the connection between FA and translesion synthesis PRR pathways where hprt mutagenesis is reduced in FA lymphoblasts (75), spontaneous mutation frequencies at two autosomal loci are increased in FA patients (76), and FANCC promotes translesion synthesis in chicken DT40 cells (77). Our results help clarify this controversy by clearly demonstrating that Mph1 (and, by extension, the yeast FA pathway) functions in a Rad5-dependent recombination pathway that is at least partially distinct from Rad18-directed PRR (Figs. 3, 5, and 6). Furthermore, the synergistic sensitivity in mph1 srs2 (Fig. 2A) reveals a functional redundancy that clearly supports the conclusions of this study, whereas Srs2 actively blocks Rad51 filament formation (78), and Mph1 protects ICL-repair intermediates from being inappropriately engaged by HR proteins (supplemental Fig. S6). This conclusion is further supported by the rescue of the high NM sensitivity of mph1 srs2 by the loss of rad51 (Fig. 3D).

Although the PRR pathway is functionally conserved in human cells, it is still poorly defined by comparison to the yeast system. Currently two human Rad5 orthologs have been identified, SHPRH and HLTF (79–82). Although these two proteins contribute to PCNA poly-ubiquitylation in vitro and in vivo, they are not essential for in vivo PCNA polyubiquitylation. Thus, the existence of another unidentified Rad5 ortholog has been proposed (83). Accordingly, unlike yeast rad5 cells, SHPRH<sup>−/−</sup> and HLTF<sup>−/−</sup> are specifically sensitive to MMS but

FIGURE 6. Mph1 OE protects ICL-stalled replication forks. A, FACS profiles and checkpoint responses (Rad53 phosphorylation and γH2AX formation) of untreated, hydroxyurea (HU)-treated, or NM-treated Mph1 OE cells and vector controls are shown. B, shown is a representative pulse field gel with untreated (U) cells and cells treated 2 h with 100 µM NM and allowed to recover in fresh media for 0, 1, 6, or 24 h. C, The percent protected (as indicated in B) was calculated for each experiment. Error bars reflect the S.D.
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**TABLE 2**

ICL-specific phenotypes of Mph1
A summary of the Mph1-dependent phenotypes for MMS-induced alkylation damage and ICL-induced damage reveals the unique ICL-specific roles of the Mph1 pathway. NA, not applicable.

| Type of damage | mphp1Δ-induced mutagenesis | Cell survival with Mph1 OE | DSB protection with Mph1 OE |
|----------------|---------------------------|----------------------------|----------------------------|
|                |                           | Damage sensitivity | Domain requirements | Domain requirements |
| MMS            | No change                 | Sensitive            | CTD                  | NO                  |
| ICL            | Increased                 | Resistant            | Helicase, CTD        | YES                 |

not ICL-forming drugs (79). This suggests that the unidentified Rad5 ortholog must be important for ICL repair in human cells. It follows that this ortholog may direct the Fanconi pathway as well.

Despite our clear genetic evidence of a Rad5-dependent Mph1 pathway, the mechanism of how Rad5 directs Mph1 remains an open question. We were unable to detect a physical interaction between the proteins, and our biochemical fork reversal assay did not reveal stimulated activity when both proteins were combined. There are two non-mutually exclusive explanations for these results; 1) the mechanism through which Rad5 directs Mph1 is upstream of Mph1 helicase function (i.e. indirect recruitment), or 2) additional in vivo factors are required for Rad5 to mediate Mph1 helicase function or recruitment. Future studies are required to address these issues.

This study defines a novel yeast repair pathway composed of Rad5, PCNA, Rad51, and Mph1. Importantly, this pathway is independent of both Rad18 and Ubc13 (Fig. 3A and supplemental Fig. S4). This finding is surprising considering that Rad5-mediated polyubiquitylation of PCNA is thought to require both Rad18 and Ubc13. Our genetic evidence clearly demonstrates an increased sensitivity in rad18 rad5 mutants compared with either single mutant (Fig. 3A and supplemental S3G), indicating that these two proteins have some independent functions. Furthermore, the mph1 ICL phenotype is epistatic with rad5, not rad18 (Fig. 3). Recently, others have proposed that PCNA polyubiquitylation acts before PCNA monoubiquitylation in the DNA damage response (65). Further studies will be needed to determine if PCNA monoubiquitylation is accomplished through the action of Rad5 or other proteins implicated in PCNA modification. Importantly, a recent study showed that DNA ligase defects induce PCNA ubiquitylation at lysine 107 through a mechanism involving Rad5, Mms2, and Ubc4 but not Rad18 or Ubc13 (84). In addition, PCNA monoubiquitylation at lysine 164 can occur in yeast in an Asf1-dependent mechanism (85) and in unperturbed mammalian cells in a CRL4Cdt2-dependent mechanism (86).

Mph1 OE uniquely protects cells from FMA damage (Fig. 5) while also affecting S-phase progression (Fig. 6A). Considering that the helicase activity of Mph1 is required for this protection, it is possible that Mph1 may drive the formation of alternative structures at the stalled replication fork and protect the replication fork from collapsing before repair. Our results suggest a model where Mph1 is recruited by Rad5-mediated PCNA ubiquitylation (Fig. 1B). Interestingly, improved survival upon Mph1 overexpression is dependent on both the helicase and CTD of Mph1, whereas protection against NM-induced DSBs is only dependent on the helicase domain. The CTD of Mph1 is important for replication protein A interactions and GCR formation (42). Although it is possible that the CTD of Mph1 may improve survival by promoting GCR formation, this seems unlikely as Mph1 OE-mediated protection is completely lost in rad5 (Fig. 5C), but Mph1-OE-mediated GCRs remain high in this background (supplemental Table S1). We feel that the different domain requirements reflect the different end point of each experiment. In the pulse field gel electrophoresis analysis we see a CTD-independent/helicase-dependent protection of repair substrates. This reflects an early step in the repair process, where Mph1 prevents substrates from collapsing into DSBs. In contrast, the FMA survival experiments (Fig. 5) reflect successful completion of repair.

Completion of repair requires both the helicase (e.g. fork reversal by Mph1) and the CTD of Mph1. The CTD may be required to recruit the downstream components of ICL repair through protein-protein interactions.

In contrast to the resistance observed in WT cells, Mph1 OE actually sensitizes rad5 and pol30-K164R cells to ICL damage (Fig. 5C). One possible explanation is that Mph1 OE compromises the remaining ICL repair pathways in the absence of regulation by Rad5. In addition, Rad5 may be required to reset the replication fork after any ICL repair pathway. Without Rad5, replication may not be completed after repair, and cell death may be triggered.

These studies highlight a unique role for Mph1 in ICL repair that is distinct from its repair roles in other forms of DNA damage. Mph1 OE protects cells from ICL-forming agents through its helicase domain and CTD (Fig. 5A). In contrast, we previously reported that the CTD of Mph1 sensitizes cells to MMS under OE conditions (42). The disparities (summarized in Table 2) between the MMS and ICL responses highlight the unique challenges that the ICL imposes on DNA repair systems and the unique functions of Mph1 at ICLs. Clearly the covalent link between two complementary strands is a significant challenge for cells that requires a unique approach. For MMS damage, repair likely happens behind the fork (53, 65, 87), and translesion bypass and recombinational repair are viable options. Accordingly, MMS transiently stalls replication forks and thus does not impede replication as severely as ICL damage (which needs to be repaired through various time-consuming steps). Thus, MMS damage-stalled forks may not require stabilization by Mph1 fork reversal activity (61). The increased sensitivity of Mph1 OE cells to MMS suggests that Mph1 interaction with replication protein A (or other repair factors) actually hampers repair processes at an MMS-stalled fork (42). In marked contrast, MPH1 OE improves survival after ICL damage (Fig. 5B), and therefore, we speculate that Mph1-mediated fork reversal is a beneficial step for ICL repair.
The improved viability imparted by Mph1 OE may have far-reaching implications for chemotherapy treatments. ICL agents are commonly used chemotherapeutics, and often chemo-resistant tumor cells arise after these treatments. Our results suggest that the overexpression of FANCM may give rise to chemo-resistant cells and suggest that combinatorial therapies utilizing ICL-inducing agents in conjunction with FANCM inhibitors would improve the efficacy of tumor treatment. Our results specifically address the consequences of Mph1 OE, but the up-regulation of other Fanconi genes is also associated with melanoma (88) and cellular resistance to DNA damaging agents (89, 90). Thus, these therapeutic implications may also extend to other Fanconi proteins and their inhibitors.

Overall, these results contribute to a clearer understanding of a complex DNA repair pathway. Using this model, we now have a clearer appreciation of Mph1 unique function in ICL repair (and by extension FANCM function) and the importance of Rad5 as a mediator of the yeast Fanconi-like pathway.

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