Essential Domains of Schizosaccharomyces pombe Rad8 Required for DNA Damage Response

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ABSTRACT Schizosaccharomyces pombe Rad8 is a conserved protein homologous to S. cerevisiae Rad5 and human HLTs that is required for error-free postreplication repair by contributing to polyubiquitilation of PCNA. It has three conserved domains: an E3 ubiquitin ligase motif, a SNF2-family helicase domain, and a family-specific HIRAN domain. Data from humans and budding yeast suggest that helicase activity contributes to replication fork regression and template switching for fork restart. We constructed specific mutations in the three conserved domains and found that both the E3 ligase and HIRAN domains are required for proper response to DNA damage caused by a variety of agents. In contrast, mutations in the helicase domain show no phenotypes in a wild-type background. To determine whether Rad8 functionally overlaps with other helicases, we compared the phenotypes of single and double mutants with a panel of 23 nonessential helicase mutants, which we categorized into five phenotypic groups. Synthetic phenotypes with rad8Δ were observed for mutants affecting recombination, and a rad8Δ helicase mutation affected the HU response of a subset of recombination mutants. Our data suggest that the S. pombe Rad8 ubiquitin ligase activity is important for response to a variety of damaging agents, while the helicase domain plays only a minor role in modulating recombination-based fork restart during specific forms of replication stress.

Proper response to DNA damage during S phase requires that stalled replication forks are protected and efficiently restarted (Lambert and Carr 2013; Mirkin and Mirkin 2007). Evidence suggests that arrest and restart depend on management of single-stranded DNA and recombination. For example, on release from hydroxyurea (HU), which starves for nucleotides, S. pombe cells show a burst of foci of the single stranded DNA binding protein RPA, followed by foci of homologous recombination (HR) protein Rad52 (Carr and Lambert 2013; Meister et al. 2005; Sabatinos et al. 2012). This is consistent with HR involvement in fork restart (Bailis et al. 2008; Lambert et al. 2010; Meister et al. 2005). HR intermediates are processed and managed by specific helicases (e.g., Rqh1; Fml1) and nucleases (e.g., Mus81) to prevent inappropriate rearrangements (Dehe et al. 2013; Doe et al. 2002; Nandi and Whitby 2012; Sun et al. 2008; Willis and Rhind 2009).

There is good evidence from several systems that one form of fork restart works through a template switching pathway, with recovery through a Holliday junction-like structure (Atkinson and McGlynn 2009; Lambert and Carr 2013). Accumulation of RPA on ssDNA regulates this reaction (Betous et al. 2013; Sirbu et al. 2013). Several lines of evidence link the S. cerevisiae Rad5 to this activity. ScRad5 functions in the error-free branch of postreplication repair (PRR) by promoting the polyubiquitilation of PCNA (Branzei et al. 2004). It also has helicase-dependent fork reversal and restart activity, suggesting that it functions in fork regression downstream of PCNA ubiquitination (Blastyak et al. 2007; Minca and Kowalski 2010). Cells with mutations that disrupt the ATP binding site in ScRad5 are sensitive to DNA damage and disrupt the ligation of broken ends in an MRN-dependent pathway (Chen et al. 2005; Minca and Kowalski 2010). ScRad5 mutants are also HU-sensitive, consistent with a role in fork recovery (Kapitzky et al. 2010; Kats et al. 2009). Rad5 has two human homologs, SHPRH and HLT (Figure 1B) (Unk et al. 2010). SHPRH promotes PCNA polyubiquitilation (Motegi et al. 2006b). HLT

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facilitates template switching via its double-stranded DNA translocase activity (Blasztaky et al. 2010). It also displaces RPA and PCNA from a modeled replication fork in vitro and contributes to Rad51-independent D-loop formation (Achar et al. 2011; Burkovics et al. 2014). SHPRH and HLTF respond to different forms of damage (Lin et al. 2011). Together, these data suggest that the Rad8/HLTF helicase domain contributes to replication fork stability and restart, possibly in an alternative pathway to Rad51.

In S. pombe, the orthologous protein Rad8 (Figure 1B) has been shown to extend the ubiquitin chain on mono-ubiquitinated PCNA and contributes to replication fork stability and restart, possibly in an alternative pathway to Rad51.

**MATERIALS AND METHODS**

**Strain construction**

All S. pombe strains were constructed and maintained in yeast extract minimal media (EMM) with appropriate supplements using standard techniques (Sabatinos and Forsburg 2010).

S. pombe strains (listed in Supporting Information, Table S1) were from our collection or purchased from the Bioneer Corporation and the Korea Research Institute of Biotechnology and Bioscience. The \( \Delta rad8::kanMX-Bioneer \) (FY5132) deletion was isolated from the Bioneer S. pombe Deletion Mutant Library (V2-11-F11). It was backcrossed twice with laboratory wild-type strains. Both strains were retained (FY5216: \( h^+ \) \( \Delta rad8::loxP-ura4^+::loxM3 ura4-D18 leu1-32 ade6-M210 can1-1 \)) or backcrossed twice to laboratory wild-type strains. Both strains were retained (FY5217: \( h^+ \) \( \Delta rad8::loxP-ura4^+::loxM3 ura4-D18 leu1-32 ade6-M210 can1-1 \)).

**Serial dilution assays**

Cells were grown to mid-log phase in YES. Five-fold serial dilutions were prepared in YES and spotted on drug containing rich medium. The plates were incubated 5 d at 25\(^\circ\)C or 3 d at 32\(^\circ\)C. Experiments were repeated two to five times.

**Protein extracts and immunoblotting**

Whole cell extracts (WCE) were prepared using trichloroacetic acid (TCA) extraction as described (Catlett and Forsburg 2003). Eighty micrograms of WCE were separated by SDS-PAGE in a 6% acrylamide gel. FLAG tag was detected with mouse anti-FLAG M2 (1:1000; Sigma) and anti-mouse-IgG-HRP (1:2000; Sigma). Mcm7 was detected with antibody purified from rabbit serum 6184 (1:1000) (Liang and Forsburg 2001) and anti-rabbit-HRP (1:2000; BD Biosciences).

**GFP fusions**

Two types of GFP fusion were made. For Western blot, the C-terminus of \( rad8^+ \) was tagged with a GFP fragment at its native locus as described (Bahler et al. 1998). For microscopy (overexpression), linearized plasmids that carry \( rad8^+\)-GFP (under the nmt1 promoter), either wild-type or mutant derivatives, were integrated at \( leu1-32 \) locus in \( rad8^+ \) background. The nmt1 promoter was repressed in the presence of 15 \( \mu \)M thiamine (Maundrell 1993).

**Cloning of rad8^+ gene and plasmid construction**

The \( rad8^+ \) gene was cloned in two steps. First, two independent fragments, one from ATG to a naturally occurring SpeI site within the ORF (\( rad8A \)) and the other from the SpeI site to the TAA codon (\( rad8B \)), were amplified by PCR and cloned into a pBluescript vector. The SpeI site in the \( rad8B \) was silenced by site-directed mutagenesis. The complete \( rad8^+ \) ORF was generated by ligating the \( rad8A \) fragment into \( rad8B-noXhoI \) containing plasmid at XhoI and SpeI. Mutations were introduced separately in \( rad8A \) or \( rad8B-noXhoI \) containing plasmids prior to generation of the full gene. Phusion Site-Directed Mutagenesis Kit (Finzymes) was used for site-directed mutagenesis. Plasmids are listed in Table S2.

**Constructing rad8 mutants at its endogenous locus**

The \( rad8^+ \) mutants were created as described (Watson et al. 2008). The base strain (FY5622: \( h^+ \) \( \Delta rad8::loxP-ura4^+::loxM3 ura4-D18 leu1-32 ade6-M210 can1-1 \)) was constructed by replacing the entire \( rad8^+ \) ORF (from start to stop codon) with the \( loxP-ura4^+::loxM3 \) cassette that was amplified from the pAW1 plasmid (EUROSCARF-P30537) using primers TATACATGTTTTTATATTTTCACTAGTTTTTACGTTAAGTTTGGGATGAACAAACATTCAAAACGAGATCCCGGGGTATTTTAAATD2 and GTAAGATTGCGATTCAATGAATATATATATTAAAATTTTATATTTTATGAGTCTTTTAAAATTGTCGTTTGAACACCGTTGGAATTCGAACTGCTGTTTAAAC. Bold sequences indicate the sequences homologous to pAW1. The wild-type (pLD35) and mutated \( rad8^+ \) coding plasmids (pLD36, pLD37, pLD38, and pLD39) were digested with XhoI and SacI and cloned into the pAW8-XhoI plasmid (EUROSCARF-P30585). The resulting plasmids (pLD45: \( rad8^+ \); pLD46: \( rad8^+\)-
H\( \Delta \)IRIAN; pLD47: \( rad8^+\)-K355A, T536A (\( rad8^+\)-HD); pLD48: \( rad8^+\)-I879A (\( rad8^+\)-LD); and pLD49: \( rad8^+\)-K355AT536AI879A (\( rad8^+\)-HDLD)) were transformed into the base strain and selected on EMM+Ade+thiamine plates. Transformants were grown in nonselective thiamine-free medium at 32\(^\circ\)C for 1 d, plated onto 5′-FOA plates, and incubated at 32\(^\circ\)C for 4 d. The 5′-FOA-resistant and LEU− candidates were confirmed by colony PCR and verified by sequencing.
RESULTS

Rad8 is required for response to a subset of genome damaging agents

To assess the role of Rad8 in damage response, we examined rad8Δ sensitivity to different genotoxins (Figure 1A) and compared this to a commonly used rad8 truncation allele, rad8-190, which we determined encodes a protein product with a premature stop codon at amino acid 315. We compared these to a nonubiquitatable mutant pcn1-K164R, which has increased sensitivity compared with rad8Δ (Frampton et al. 2006). Surprisingly, and in contrast to S. cerevisiae ScRad5, rad8Δ mutants are not sensitive to HU. We found that rad8Δ is also not sensitive to CPT, a topoisoasemerase toxin that causes S phase-specific DNA breaks (Wan et al. 1999). However, pcn1-K164R is very CPT-sensitive, possibly due to a sumoylation-mediated pathway involving the same residue (Kai et al. 2007).

Interestingly, hΔΔrad8 has increased sensitivity compared with hΔΔrad8 when exposed to MMS (Figure 1A). Mutants lacking homologous recombination proteins often are sick in hΔΔ than in hΔΔ configuration, which is presumed to reflect the absence of a template for repair of the mating type imprint and break required for switching in the hΔΔ strain (Khasanov et al. 1999). This could suggest a role for Rad8 in aspects of HR repair. We compared hΔΔ strains throughout the remainder of this study to avoid differences attributable to mating type.

Domain structure of Rad8

S. pombe Rad8 was first identified as a member of the SNF2 helicase family based on sequence homology (Doe et al. 1993) and has distinct domains matching those in ScRad5 and HLTF (Minca and Kowalski 2010; Unk et al. 2010). There is a SNF2 helicase domain including an ATP binding site. Mutations that change the lysine and threonine to alanine (K538A, T539A) in the ATP binding site abolish the helicase activity in ScRad5 (Chen et al. 2005; Minca and Kowalski 2010). Embedded in the helicase domain, there is a RING-type zinc finger ubiquitin E3 ligase domain that polyubiquitinates Pcn1 (ScPol30, hPCNA) in concert with the Mms2/Ubc13 E2 heterodimer (Frampton et al. 2006). A point mutation from isoleucine to alanine (I916A) in this motif in ScRad5 abolished the E3 ubiquitin ligase activity by eliminating its interaction with Ubc13 (Ulrich 2007). Near the N-terminus is an uncharacterized HIRAN domain, a motif shared by all the members of this family (HIP116, Rad5p N-terminal domain). It has been suggested that this domain recognizes specific DNA damage or stalled replication forks (Iyer et al. 2006), but it has not been analyzed. The Rad8 HIRAN domain spans amino acid 206 to 319 and contains a potential NLS (nuclear localization signal) RKKSK between amino acids 245 and 251. The rad8-190 truncation allele expresses most of this domain (amino acid 1-314) while deleting the helicase and RING-finger E3 ligase domains. We constructed mutations (Figure 1B) in all three of these conserved domains to assess their function in S. pombe. A large-scale fission yeast phosphorylation analysis (Wilson-Grady et al. 2008) suggested that a serine residue (S18) of Rad8 is a potential phosphorylation site. However, we observed no phenotype of an S18A mutation in DNA damage response, so this was not investigated further (Figure S1).
The HIRAN domain contributes to nuclear localization

To assess protein location, we tagged endogenous Rad8 with GFP at its C-terminus. Under the endogenous promoter, there was not sufficient signal to visualize the protein (data not shown). Therefore, we engineered overproduction strains to increase the GFP signal. We integrated Rad8-GFP at the at leu1* locus, under the control of the nmt1* promoter. This promoter allows modest levels of expression in thiamine, and a dramatic overproduction in the absence of thiamine (Forsburg 1993). We observed a distinctive Rad8-GFP signal in the nucleus within 16 hr of removing thiamine (Figure 2A). Prolonged Rad8-GFP overproduction over several days is slightly toxic (Figure S2A).

We constructed a 3′ truncation allele rad8-H mutant that expresses only the N-terminus of Rad8 up to the end of the HIRAN domain, and a HIRAN deletion mutant (rad8-ΔHIRAN) that precisely deletes aa206-319. Rad8-H-GFP (integrated into the leu1* locus under the nmt1 promoter in the rad8Δ strain) localizes in the nucleus (Figure 2B), whereas Rad8-ΔHIRAN-GFP remains in the cytoplasm (Figure 2C). Next, we replaced the entire HIRAN domain with the SV40NLS (PKKKRKV) (Pasion and Forsburg 1999), and this restored the nuclear localization of Rad8 (Figure 2D). However, inserting the putative Rad8NLS (RKKSK) (Doe et al. 1993) in the same configuration only partially restored the nuclear localization (Figure 2E). A smaller deletion (amino acids 246 to 250) removed the RKKSK sequence (Rad8-ΔNLS). The majority of this protein localized properly, indicating this is not the primary NLS (Figure 2F).

To test the drug sensitivity of the HIRAN mutants, we integrated them without GFP under the endogenous rad8* promoter at the native locus using the Cre recombinase-mediated cassette exchange (RMCE) system (see Materials and Methods) (Watson et al. 2008). Both rad8-H and rad8-ΔHIRAN phenocopied rad8Δ in damage sensitivity (Figure 2G). Importantly, restoration of nuclear localization with SV40NLS did not rescue the drug sensitivity. The Rad8-ΔNLS strain was modestly sensitive to damage, consistent with the slight defect in localization (Figure 2G). Taken together, these results suggest that nuclear localization is necessary but not sufficient for Rad8 function, and that the HIRAN domain provides additional functions beyond nuclear localization.

Mutations in the E3 ligase and helicase domains have different phenotypes

Using the same strategies, we examined localization and drug sensitivity of Rad8 proteins containing point mutations in the putative helicase or ring finger domains. The point mutants correspond to the HD and LD mutants. The Rad8 ubiquitin ligase domain proved to be the major contributor to all damage responses (Figure 3E and F). The Rad8 ubiquitin ligase domain proved to be the major contributor to all damage responses (Figure 3E and F). The amount of Rad8 protein in these mutants is similar (Figure 3G).

Figure 2 Nuclear localization is necessary but not sufficient for Rad8 function. (A–F) Schematic representation and localization of Rad8-GFP proteins, overproduced from a single copy transgene integrated at the leu1-32 in Δrad8 background. Pictures were taken 16 hr after the removal of thiamine: (A) Rad8-GFP; (B) Rad8-HIRAN-GFP; (C) Rad8-ΔHIRAN; (D) Rad8-ΔHIRAN::SV40NLS; (E) Rad8-ΔHIRAN::rad8NLS; and (F) Rad8-ΔNLS (with schematic representations of Rad8 HIRAN-related constructs on the right; not drawn to scale). (G) Drug sensitivity of indicated mutants. These mutants were integrated without a GFP tag at the native locus under the endogenous promoter. Strains were grown overnight at 32°C, 1:5 serially diluted, and spotted to plain YES rich medium (Control) and YES with indicated drugs. (1) Wild-type. (2) loxp-rad8Δ-loxM3. (3) loxp-Δrad8-loxM3. (4) loxp-rad8-ΔHIRAN-loxM3. (5) loxp-rad8ΔHIRAN::SV40NLS-loxM3. (6) loxp-rad8ΔHIRAN::rad8NLS-loxM3. (7) loxp-rad8-ΔNLS-loxM3. (8) loxp-rad8-ΔHIRAN-loxM3. Plates were incubated at 32°C for 3 d.

Figure 3 Breakdown of the HIRAN domain

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Previous work with S. pombe showed that SpRad8 is required for PCNA polyubiquitylation (Frampton et al. 2006). We compared the phenotypes of rad8Δ and the rad8 point mutations in combination with different PRR mutants and examined their sensitivity to MMS, HU, and UV. As expected, rad8Δ is in a common epistasis group with rhp18Δ and mms2Δ (Figure S3A), whereas it has increased sensitivity to MMS when combined with mutations in the TLS polymerases in the error-prone arm of the pathway (Figure S3B).

As seen previously (Frampton et al. 2006), pen1-K164R (which cannot be ubiquitylated) is epistatic to rad8Δ. We observed a similar phenotype for rad8-LD, which lacks the ubiquitin ligase domain (Figure 4). Although we observe no obvious phenotype of the helicase mutant alone, the pen1-K164R rad8-HD double mutant has reduced viability in response to MMS compared with pen1-K164R rad8Δ (Figure 4). Moreover, pen1-K164R rad8-HD is slightly sensitive to HU. This suggests that the helicase domain may become important in response to replication stress only if PCNA cannot be modified at K164.

A damage fingerprint of helicase mutants

Given the observations with the budding yeast and human orthologs, we were surprised at the absence of any phenotypes associated with the rad8-HD mutant. We investigated whether Rad8 is redundant with other helicases in S. pombe, which has 23 annotated nonessential helicases. First, we isolated mutants available in the Bioneer deletion collection (Deshpande et al. 2009) and compared their responses to DNA-damaging drugs or treatment. Based on their patterns of sensitivity to different DNA damaging agents, we identified five distinct groups (summarized in Figure 5; see references in Table S3 and data in Figure 6, Figure 7, Figure S4, and Figure S5).

The first group is specifically sensitive to MMS, consisting only of rad8Δ itself and rhp26Δ, the ortholog of ScRad26 associated with transcription-coupled repair (Kanamitsu and Ikeda 2011). Group 2 mutants were only sensitive to CPT treatment, but not the other agents. This group included two chromatin remodelers and an uncharacterized RNA/DNA helicase. Group 3 contained smf22Δ, another member of the SFR2 family, srs2Δ, and an uncharacterized putative RNA helicase ASPC694.02. They were sensitive to HU and CPT, which are specific to stress during S phase. The group 4 strains, sensitive to all agents tested, have been linked to aspects of replication fork stabilization and recombination. The last group of mutants showed no sensitivity to any of the agents we tested.

We examined the sensitivity of these mutations in combination with rad8Δ (summarized in Figure 5; see references in Table S4 and data in Table S3).
Not surprisingly, the \( \Delta \text{rhp26} \) double mutant was profoundly sensitive to MMS. There were no significant synthetic phenotypes observed in combination with group 2 mutants or the group 3 mutant \( \Delta \text{SPAC694.02} \). Synthetic phenotypes were observed with most of the group 4 strains, generally showing increased sensitivity to all damaging agents (indicated in red). In a few cases, the double mutant improved growth on one agent (shown in blue): \( \Delta \text{srs2} \) and \( \Delta \text{snf22} \) improved growth on HU (Figure 6E and Figure S4), whereas \( \Delta \text{fml1} \) and \( \Delta \text{fml2} \) show improved growth on CPT. We also examined conditional alleles of several essential helicases, including \( \Delta \text{dna2} \) (discussed below) and \( \Delta \text{pfh1} \), which is required to replicate through particular structures in the genome (Pinter et al. 2008; Sabouri et al. 2012; Steinacher et al. 2012; Tanaka et al. 2002). \( \Delta \text{pfh1-R20} \) is a cold-sensitive allele (Boule and Zakian 2006). The \( \Delta \text{pfh1-R20} \) double mutant showed increased sensitivity to MMS and UV, no effect on CPT, and a partial rescue on HU (Table S4 and Figure S4).

Based on the results from this analysis, we took a candidate approach to investigate whether the \( \Delta \text{rad8} \) helicase domain is responsible for any of the synthetic phenotypes we identified.

### Identifying an effect of the Rad8-helicase mutant in recombination-deficient backgrounds

Homologous recombination is a central component of the response to replication stress (Bailis et al. 2008; Lambert et al. 2010; Meister et al. 2005). \( \Delta \text{Rad5} \) and human HLTF are implicated in formation of recombination structures between sister chromatids that can promote replication fork restart, independent of Rad51 (Burkovics et al. 2014; Hu et al. 2013; Minca and Kowalski 2010; Mott and Symington 2011). We examined mutations affecting \( \Delta \text{Rad51} \), which coats an invading ssDNA filament (Heyer et al. 2010). Filament formation is promoted by mediator complexes including Rad55/Rad57 and Sfr1/Swi5, and is opposed by the helicase Srs2 (Haruta et al. 2008; Heyer et al. 2010). Invasion and D loop formation are promoted by recruitment of the Rad54 helicase (Heyer et al. 2010).

Consistent with previous observations (Frampton et al. 2006), we find that \( \Delta \text{Rad51} \Delta \text{Rad8} \) is growth-impaired even without external genotoxins (Figure 6A). This growth defect was not as dramatic in the \( \Delta \text{Rad51} \Delta \text{Rad8-LD} \) double mutant lacking the ubiquitin ligase domain and not apparent at all in the \( \Delta \text{Rad51} \Delta \text{Rad8-HD} \) double mutant lacking the helicase motif, thus implicating the E3 ligase domain as an important contributor to genome stability in the absence of Rad51.

Both \( \Delta \text{Rad51} \Delta \text{Rad8} \) and in \( \Delta \text{Rad51} \Delta \text{Rad8-LD} \) show a dramatic increase in MMS sensitivity, which is consistent with loss of two pathways of repair (template switching and recombination). Increased HU...
were incubated at 32°C for 3 d. (D) and (E) Strains were grown overnight
/C176. [Image 58x311 to 285x735]

were incubated at 25°C for 5 d. (Figure S5). These showed no obvious growth defect on plate assays in
HU. However, phenotypes of these additional rad mutants were all
different on HU. First, rad54Δ is not quite as HU-sensitive as
rad51Δ, whereas rad55Δ and rad57Δ are not sensitive at all (Figure
S6). Second, there was a strikingly increased HU sensitivity of rad54Δ in
all the rad8 double mutants, suggesting that both ligase and helicase
activity are important in the absence of Rad54. In contrast, rad55Δ showed
increased HU sensitivity combined with rad8Δ or especially
rad8-LD, but no effect of rad8-HD, whereas rad57Δ showed only a
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mutants. These data suggest that Rad8 helicase activity may be
specifically important for the response in HU if Rad54 is missing, and
despite separable roles of the Rad55/57 mediator components.

Srs2 helicase is an anti-recombinase that opposes Rad51 filament
formation (Macris and Sung 2005). However, Srs2 also promotes fork
reversal in repetitive sequences (Kerrest et al. 2009) and contributes to
fork restart and template switching at stalled forks (Lambert et al.
2010). In MMS, Srs2 is implicated in restraint of the HR response
rather than in budding yeast, but not in fission yeast (Doe and
Whitby 2004; Kai et al. 2007). We observed that rad8Δ, srs2Δ rad8Δ,
and srs2Δ rad8-HD all showed similar sensitivity to MMS and UV,
whereas srs2Δ rad8-HD resembled the more modest phenotype of
srs2Δ (Figure 6E and Figure S4). Intriguingly, loss of rad8 partly
suppressed the HU sensitivity observed in srs2Δ, and this suppression
was strongest in the srs2Δ rad8-HD double mutant lacking the helicase.
This is opposite the phenotype observed for rad51Δ rad8, rad54Δ
rad8, and rad55Δ rad8 double mutants, and suggests that the Rad8
helicase may antagonize Srs2.

Based on this result, we examined two additional proteins
implicated in fork restart and HU response: the Mus81 resolvase and
and its paralogue, Fml2, which plays a minor role (Sun et al.
2005; Schurer et al. 2004; Sun et al. 2008; Zheng et al. 2011). Recent work suggests that budding
yeast Mph1/FancM operates downstream of ScRad5 in repair of inter-
strand crosslinks (Daee et al. 2012). S. pombe has two Fml proteins: Fml1 and
its paralogue, Fml2, which plays a minor role (Sun et al. 2008).

We examined the drug sensitivity of double and triple mutants
(Figure S5). These showed no obvious growth defect on plate assays in
HU, MMS, and UV (Figure 6F and Figure S4).

Fml1 operates in an independent pathway

Another interacting helicase revealed in our screen is Fml1 (ScMph1/
FANCm). This protein family is capable of fork reversal and promotes
recombination at stalled replication forks (Blackford et al. 2012; Gari et
al. 2008; Nandi and Whitby 2012; Prakash et al. 2005; Schurer et al. 2004;
Sun et al. 2008; Whitby 2004; Kai et al. 2007; Roseaulin et al. 2008). The double
mutants with rad8Δ showed increased defects in growth on HU, MMS, and UV (Figure 6F and Figure S4).

Genetic evidence that Rad8 contributes to resection

Homologous recombination initiates from DNA ends, and broken
ends are a target for resection by the MRN complex. There is evidence

sensitivity was also observed for both rad51Δ rad8Δ and rad51Δ rad8-HD
compared with rad51Δ (note the relatively low dose used, because
rad51Δ is extremely HU-sensitive). Although the effect was modest,
we observed a slight increase in MMS sensitivity in the rad51Δ rad8-HD double mutant. However, there was no synthetic phenotype on
HU of rad51Δ rad8-HD.

We examined other members of the HR pathway: rad54Δ, rad55Δ, and
rad57Δ. On MMS, we saw similar phenotypes to those for
rad51Δ: a significantly increased sensitivity in double mutants with
rad8Δ or rad8-LD lacking the ligase domain and a very slightly in-
creased sensitivity with the rad8-HD mutation in the helicase domain
(Figure 6, B–D). This suggests that they all act in a common pathway
with Rad51 for MMS response, and this implicates the helicase
domain for a minor contribution in the MMS response when the other
pathways are compromised.

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compared with rad51Δ (note the relatively low dose used, because
rad51Δ is extremely HU-sensitive). Although the effect was modest,
we observed a slight increase in MMS sensitivity in the rad51Δ rad8-HD double mutant. However, there was no synthetic phenotype on
HU of rad51Δ rad8-HD.

We examined other members of the HR pathway: rad54Δ, rad55Δ, and
rad57Δ. On MMS, we saw similar phenotypes to those for
rad51Δ: a significantly increased sensitivity in double mutants with
rad8Δ or rad8-LD lacking the ligase domain and a very slightly in-
creased sensitivity with the rad8-HD mutation in the helicase domain
(Figure 6, B–D). This suggests that they all act in a common pathway
with Rad51 for MMS response, and this implicates the helicase
domain for a minor contribution in the MMS response when the other
pathways are compromised.
that ScRad5 binds to ssDNA as part of an end-joining pathway that involves the MRN complex (Chen et al. 2005). In contrast to the phenotype of Rad51 pathway members, we found only slight increase in MMS or UV sensitivity in double mutants between rad8Δ and some MRN components (rad50Δ and nbs1Δ), suggesting a possible common function (Figure 7 and Figure S4).

MRN and the associated Ctp1 protein promote short-range resection, whereas long-range resection is promoted by Exo1 (Langerak et al. 2011). We observed that exo1Δ rad8Δ double mutants have a substantial increase in MMS sensitivity, as does the mrc1Δ exo1Δ double mutant (Figure 7B). Similar results have been reported for rad50Δ exo1Δ (Tomita et al. 2003). The exo1Δ rad8Δ-LD with a mutation in the E3 ligase domain was also more sensitive, whereas exo1Δ rad8Δ-HD resembles exo1Δ alone. Finally, the rad8Δ rad8Δ and exo1Δ rad8Δ-LD double mutants also showed increased sensitivity to hydroxyurea. These data suggest a role for Rad8 ubiquitin ligase in early stages of fork resection.

Long-range resection in budding yeast has also been linked to the ScSgs1 helicase and the DNA2 helicase/nuclease, in concert with RPA, and in opposition to fork regression (Gejka et al. 2010; H. Chen et al. 2013; Hu et al. 2012; Karanja et al. 2012; Niu et al. 2010). The corresponding pathway involved in fission yeast is reported to play only a minor role in resection (Langerak et al. 2011), but we examined interactions with mutations in dna2 (Figure 7, C and D and Figure S4) and rqh1 (ScSgs1) (Figure 6F and Figure S4). The dna2Δ-K961T mutant lacks helicase activity (Hu et al. 2012), and the single mutant is not sensitive to DNA-damaging drugs at the dosages used here. The dna2Δ-K961T rad8Δ double mutant is sensitive to HU and even more so to MMS (Figure 7C). dna2Δ has defective nuclease activity, and dna2Δ-K961T complements its growth (Hu et al. 2012). The dna2Δ rad8Δ double mutant has slightly increased sensitivity to MMS (Figure 7D). In S. pombe, rad11Δ is the essential gene that encodes the largest subunit of the trimeric RPA (Parker et al. 1997). Even at permissive temperatures, rad11Δ is sensitive to all genotoxins tested. rad8Δ increased rad11Δ MMS and UV sensitivity, but partly rescued its HU sensitivity (Figure 7E and Figure S4).

**Interactions with the fork protection complex**

Finally, we examined activities known to be involved with stabilization of the replication fork and resolution of stalled structures, which are required for MMS response (Figure 8 and Figure S4). The checkpoint kinase Cds1 physically interacts with Mus81 and regulates its function by phosphorylation-induced chromatin dissociation (Boddy et al. 2000; Kai et al. 2005). Both Mus81 and Cds1 contribute to slowing replication in the presence of MMS (Willis and Rhind 2009).

We observed that cds1Δ rad8Δ mutants have increased sensitivity to MMS (Figure 8A). The cds1Δ rad8Δ and cds1Δ rad8Δ-LD showed a similar decline in viability, indicating that this sensitivity depends on the E3 ligase. Consistent with our other data, cds1Δ rad8Δ-HD did not change the sensitivity of cds1Δ on MMS. Curiously, rad8Δ-LD, but not rad8Δ or rad8Δ-HD, modestly rescued both cds1Δ CPT and HU sensitivity (Figure 8A and Figure S4).

Mrc1, Swi1, and Swi3 form the fork protection complex (FPC), which stabilizes the replication fork in the presence of stress and is required for MMS response (Noguchi et al. 2004; Sommariva et al. 2005). In addition to the function in fork protection, Mrc1 is also a replication checkpoint adaptor protein that facilitates Cds1 activation mediated by Rad3-Rad26 (Tanaka and Russell 2001; Zhao and Russell 2004; Zhao et al. 2003). The replication checkpoint activity of Mrc1 is abolished in mrc1-AQ mutant (Xu et al. 2006). Deletion of rad8 increased the sensitivity of swi1Δ, swi3Δ, and mrc1Δ to MMS and UV (Figure 7, B and C and Figure S4). Loss of Rad8 ubiquitin ligase activity was the major contributor to these phenotypes. Consistent with this, there was a strong synthetic phenotype of pcn1-K164R swi1Δ, consistent with the primary effect being through the ubiquitin ligase-mediated modification of PCNA.

**Discussion**

Several lines of evidence implicate the HTLF/Rad5 family of proteins in helicase or translocase function. Human HTLF is capable of displacing RPA and PCNA from a modeled replication fork in vitro and promotes fork regression (Achar et al. 2011; Blastyak et al. 2010). Recent evidence suggests it can promote formation of a D-loop independent of Rad51 or Rad54 (Burkovics et al. 2014). Helicase activity associated with ScRad5 is thought to promote fork regression downstream of PCNA ubiquitylation in the error-free PRR pathway (Blastyak et al. 2007; Minca and Kowalski 2010). ScRad5 helicase is also required to restrain duplication-associated rearrangements (Putnam et al. 2010). Mutations that disrupt the ATP binding site in ScRad5 result in sensitivity to DNA damage (Chen et al. 2005; Minca and Kowalski 2010). However, in contrast to S. cerevisiae rad5 (Kapitzky et al. 2010; Kats et al. 2009), S. pombe rad8Δ mutants show no HU sensitivity (Figure 1A). This suggests that Rad8 is not required for response to, or recovery from, HU in otherwise wild-type cells; this may reflect the different requirements for HU response in these two fungi (Sabatinos et al. 2012).

We used a structure-function analysis to examine three conserved domains in S. pombe rad8Δ. Deletion of the HIRAN domain (found specifically in this family of proteins) disrupted nuclear localization.
and damage response in vivo. We restored localization by adding the nuclear localization sequence from SV40 large T antigen, but this did not restore the normal DNA damage response (Figure 2). The simplest conclusion is that nuclear localization is necessary but not sufficient for Rad8 function and the HIRAN domain makes a unique contribution to the damage response, although we cannot eliminate the possibility that our deletion has an indirect effect that disrupts protein structure.

We mutated the same residues as those shown in ScRad5 to disrupt the highly conserved ATP-binding site of the SNF2-related helicase domain and the E3 ubiquitin ligase domain. (Chen et al. 2005; Minca and Kowalski 2010; Ulrich 2003). The strongest effects observed were linked to the E3-ligase domain mutation (Figure 3). Although the null was worse in some conditions, the rad8-HDLD mutant generally resembled the rad8-LD mutant, suggesting that there may be a noncatalytic, structural role associated with the physical presence of the protein. We found that the mutation of the E3-ligase domain (rad8-LD) was responsible for all of the damage sensitivity observed in rad8Δ single mutants. We found no evidence for the helicase domain functioning in otherwise wild-type cells.

We used the rad8 point mutations as separation-of-function alleles to see which domains were required for the phenotypes. Epistasis with other components of the PR pathway gave results as expected for Rad8 playing an essential role in PCNA ubiquitylation. The phenotype of pcn1-K164R rad8Δ and pcn1-K164R rad8-LD on MMS were only slightly worse than the single mutant (Figure 4), suggesting that the predominant role of Rad8 ubiquitin ligase is via the Pcn1 K164 residue. Curiously, however, the pcn1-K164R rad8-HD double mutant was more sensitive to MMS than pcn1-K164R rad8Δ or pcn1-K164R alone, and showed a slight sensitivity to HU. Thus, there is a more complex genetic interaction between rad8 and pcn1 mutants than would be suggested by a simple linear epistasis model, but deciphering this exceeds the generally qualitative nature of serial dilution assays.

We screened candidate helicases to assess any evidence for functional overlap with Rad8. A panel of strains from the Bioneer collection or our collection, which disrupted nonessential helicases, was "fingerprinted" for damage sensitivity (Figure 6, Figure 7, Figure S4, and Figure S5). Some candidates are missing from the version of the Bioneer collection we used and are not represented here. A substantial number of the mutants had no sensitivity to the agents we tested (HU, MMS, CPT, and UV), either alone or in combination with rad8Δ, and were not investigated further. Of the four remaining phenotypic groups, the first group is defined by rad8Δ, and is sensitive primarily to MMS. It contains one other helicase mutant, rhp26Δ, required for transcription-coupled repair (Kanamitsu and Ikeda 2011). Not surprisingly, a double mutant rad8Δ rhp26Δ showed dramatically increased sensitivity. The second group was only sensitive to CPT, an agent that causes S-phase–specific damage due to covalent coupling of topoisomerase to the ends of DNA (Wan et al. 1999). These mutants, which include several chromatin remodeling proteins, showed no synthetic interaction with rad8Δ. Group 3 is represented by srs2Δ, snf22A, and a putative RNA helicase, and is only sensitive to CPT and HU, which cause defects specifically during S phase. We did not observe MMS sensitivity under these conditions with this allele of snf22A, although there are other alleles that are reportedly MMS-sensitive (Dolan et al. 2010).

We observed a mixed interaction between rad8Δ and srs2Δ or snf22Δ, in which MMS and CPT sensitivity were increased relative to the single mutants, but HU sensitivity was suppressed (Figure 6E and Figure S4), suggesting different roles for Rad8 in response to HU and MMS in these mutant backgrounds. The fourth group includes recombination-associated activities that are associated with replication fork restart. chl1Δ has genetic interactions with an alternative RFC complex and with the fork protection complex (Ansbach et al. 2008); the double mutant increases MMS sensitivity but decreases CPT sensitivity (Figure S4). The other mutants define genes linked to HR and replication fork stability (Figure 6, B and F, Figure S4, and Figure S5): Fmh1 promotes recombination at stalled forks while preventing crossovers (Nandi and Whitby 2012; Sun et al. 2008); Fhit1 directly antagonizes Rad51 (Lorenz et al. 2009) and Rqh1 limits Rad51-mediated fork restart (Lambert et al. 2010). In contrast to these activities limiting recombination, Rad54 promotes Rad51-mediated strand invasion (Muris et al. 1997; Heyer et al. 2010) and stimulates fork regression (Bugreev et al. 2011).

There is a striking growth defect in rad51Δ rad8Δ strains that is not observed in double mutants with rad54Δ, rad55Δ, or rad57Δ (Figure 6, A–D). The sensitivity in rad51Δ can be attributed to the ubiquitin ligase domain, because rad51Δ rad8-LD, but not rad51Δ rad8-HD, showed increased sensitivity on HU. Because rad51Δ mutants show evidence of genome instability and DNA damage in the absence of damaging agents (Nakamura et al. 2008; Sabatinos et al. 2012), these results suggest that Rad8 ubiquitin ligase activity is important to respond to the intrinsic stress in rad51Δ mutants.

Double mutants between rad8Δ and rad54Δ, rad55Δ, or rad57Δ all had severe synthetic drug sensitivity to MMS treatment, and the same was observed for rad8-LD (Figure 6, A–D), which again was consistent with the central role of PCNA ubiquitylation in response to alkylation damage. However, the rad8-HD double mutants show modestly increased sensitivity relative to the HR single mutants, showing that there is a subtle contribution from the helicase domain when HR is affected.

Curiously, the results in HU were different for each of the separation of function alleles, suggesting that there are distinct differences between them. rad54Δ rad8-LD and rad54Δ rad8-HD had similar levels of sensitivity, which was almost as pronounced as rad54Δ rad8Δ (Figure 6B), suggesting there may be a structural role or function for the HIRAN domain even in the absence of a catalytic activity. We conclude that both catalytic domains of Rad8 are important in the absence of Rad54 in HU, which could implicate Rad8 helicase as an alternative mechanism to Rad54 activity in HU. The rad57Δ mutant was similar to rad54Δ, although the degree of synthetic sensitivity was not as dramatic (Figure 6D). Surprisingly, because Rad55 and Rad57 work together (Liu et al. 2011; Sung 1997), rad55Δ rad8-LD and rad55Δ rad8Δ were both HU-sensitive but rad55Δ rad8-HD was not (Figure 6C). Together, these data suggest a role for the Rad8 helicase domain in the absence of the typical Rad51 recombination pathway, which may be related to the capacity of the human HLTF protein to produce D-loops in a Rad51-independent and Rad54-independent pathway (Burkovics et al. 2014). They also suggest that the Rad55 and Rad57 proteins may not contribute equally to replication fork restart in HU.

Given these results in HU, we examined other mutations that disrupt replication fork restart. Srs2 contributes to fork reversal in repetitive sequences (Kerrest et al. 2009), and to fork restart and template switching at stalled forks (Lambert et al. 2010; Lorenz et al. 2009). On MMS, there was no synthetic interaction in srs2Δ rad8Δ double mutants (Figure 6E). However, rad8-HD and rad8Δ strongly rescued srs2Δ when treated with HU. Because Srs2 antagonizes recombination, the opposite effects on HU sensitivity in the absence of rad8 are consistent with Rad8 promoting HR, and opposing Srs2. This would be consistent with the modest increase in sensitivity in the rad54Δ rad8Δ mutants. We propose that the helicase domain of Rad8 plays a minor role in Rad51-independent replication fork restart, particularly in HU.
The FANCM homolog Fml1 is already characterized as a translo-
case that regulates recombination at stalled forks (Nandi and Whitby 2012; Sun et al. 2008), and ICL repair by FANCM/Scmophil depends on ScRad5 ubiquitin ligase activity (Daee et al. 2012). We observed synthetic phenotypes between rad8Δ and fml1Δ and showed these were linked to the E3-ligase domain (Figure S5). Similarly, double mutants between fork protection complex proteins and rad8Δ showed strikingly increased MMS sensitivity that depended on the E3 ligase domain and the PCNA K164 residue that is ubiquitylated. Again, phenotypes on HU suggest that in this condition, both Rad8 domains make a modest contribution to survival.

The requirement for the ubiquitin ligase activity of Rad8 is consistent with evidence that PCNA is required at multiple points of repair (Frampton et al. 2006). The modification of PCNA in the PRR pathway in budding yeast is linked to completion of replication (Branzei et al. 2004). The PRR pathway is also required to suppress gross chro-
mosome rearrangements and repeat associated expansions in budding yeast (Daee et al. 2007; Motegi et al. 2006a; Putnam et al. 2010). PCNA also limits D-loop extension during recombination (Sebesta et al. 2013). During resection, PCNA promotes processivity of Exo1 (X. Chen et al. 2013) and promotes end-joining (Chen et al. 2005).

The initial stages of resection work through an MRN-Ctp1 complex (Limbo et al. 2007). In turn, this initial event promotes Exo1-mediated bulk resection (Mimitou and Symington 2008). We see no obvious phenotype in mre11Δ rad8Δ (Figure 7A and Figure S4), and little additional phenotype of a double mutant combining rad8Δ with the other components of the MRN complex suggest that they function in a common epistasis group. However, a strong MMS-sensitive phenotype was observed when rad8Δ or rad8-LD was combined with exo1Δ (Figure 7B). This implicates PCNA ubiquitylation in resection pathways independent of Exo1, perhaps indicating a contribution to early stages of resection. Recent work shows that the single-
stranded DNA binding protein RPA also plays a significant role in promoting resection. We observe a pronounced sensitivity to MMS in a rad11Δ rad8Δ double mutant (Figure 7E).

This study suggests that while the general pathways and enzymes for genome stability are similar across eukaryotes, the activities of specific DNA regulators have diverged within those modules. Among the SNF2-specific helicases, for example, metazoans have significantly expanded the family (Hauk and Bowman 2011). Our data suggest that the helicase function of S. pombe Rad8 is potentially required under specialized circumstances, as a minor redundant pathway with the HR proteins most notably in the restart of replication forks in HU, or in the absence of ubiquitin-PCNA. The primary function of this enzyme in fission yeast in the maintenance of genome stability appears to be due to its E3 ubiquitin ligase activity and the HIRAN domain, indicating a fundamental role for PCNA modification in preserving genomic integrity.

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