Fission Yeast Cut8 Is Required for the Repair of DNA Double-Strand Breaks, Ribosomal DNA Maintenance, and Cell Survival in the Absence of Rqh1 Helicase

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Schizosaccharomyces pombe Rqh1 is a member of the RecQ DNA helicase family. Members of this protein family are mutated in cancer predisposition diseases, causing Bloom’s, Werner, and Rothmund-Thomson syndromes. Rqh1 forms a complex with topoisomerase III and is proposed to process or disrupt aberrant recombination structures that arise during S phase to allow proper chromosome segregation during mitosis. Intriguingly, in the absence of Rqh1, processing of these structures appears to be dependent on Rad3 (human ATR) in a manner that is distinct from its role in checkpoint control. Here, we show that rad3 rqh1 mutants are normally committed to a lethal pathway of DNA repair requiring homologous recombination, but blocking this pathway by Rhp51 inactivation restores viability. Remarkably, viability is also restored by overexpression of Cut8, a nuclear envelope protein involved in tethering and proper function of the proteasome. In keeping with a recently described function of the proteasome in the repair of DNA double-strand breaks, we found that Cut8 is also required for DNA double-strand break repair and is essential for proper chromosome segregation in the absence of Rqh1, suggesting that these proteins might function in a common pathway in homologous recombination repair to ensure accurate nuclear division in S. pombe.

Eukaryotic cells engage a variety of protective responses following DNA damage. These include enzymes which repair specific DNA lesions after DNA damage as well as regulators that coordinate DNA repair and cell cycle progression. Mutations abolishing many of these processes result in genome instability and cause cancer-prone syndromes in humans. One important class of these syndromes is caused by defects in RecQ helicases (1). These enzymes unwind DNA in a 3’ to 5’ direction and are proposed to function in DNA recombination repair. Another example of this is the phosphatidylinositol 3-kinase-like protein kinases ATM (mutated in ataxia telangiectasia) (12) and ATR (ATM and Rad3 related). These proteins are associated with DNA damage surveillance and control of cell cycle checkpoints. Three checkpoint protein complexes have been suggested to play roles in sensing DNA damage. The components of these complexes in Schizosaccharomyces pombe are the checkpoint Rad proteins, including the Rad3-Rad26 complex (human ATR and ATRIP, respectively), the PCNA-like Rad9-Rad1-Hus1 (9-1-1) complex, and the Rad17-replication factor C clamp loader complex (4). They are involved in the activation of two downstream effector kinases, Chk1 and Cds1 (19, 29). The Cds1-dependent S-phase checkpoint is required for arresting the cell cycle, stabilizing replication forks, and preventing late origin firing. The Chk1-dependent DNA damage checkpoint prevents entry into mitosis until the completion of DNA repair.

DNA double-strand breaks (DSBs) are the most severe damage to DNA caused by environmental factors and occur spontaneously during normal cellular metabolism. In eukaryotes, DSBs are repaired by homologous recombination (HR) or nonhomologous end-joining (NHEJ) mechanisms (22). In both Schizosaccharomyces pombe and Saccharomyces cerevisiae, HR is the preferred pathway for DSB repair and is under the control of the RAD52 epistasis group of genes, including rhp51+ (RAD51 homolog) (25). Proteins belonging to the RecQ helicase family, such as S. pombe Rqh1, S. cerevisiae Sgs1, and human BLM (mutated in Bloom’s syndrome), appear to play a role in DNA recombination repair at a later step. Interestingly, Sgs1, Rqh1, and BLM form functional enzyme complexes with DNA topoisomerase III (3, 14, 33). In vitro studies have also shown that RecQ-like helicase and TopIII can act together to resolve double Holliday junction structures, which probably constitute a late recombination intermediate (34). RecQ helicases may also function to disrupt potential substrates for HR by preventing replication fork collapse, thus preventing high levels of recombination and ensuring subsequent fidelity of chromosome segregation (32). Additional factors required for DSB repair include S. cerevisiae Sem1, a 19S proteasome lid subunit and an ortholog of human DSS1, which binds the breast cancer susceptibility protein BRAC2 (9). Chromatin immunoprecipitation showed that Sem1 is recruited along with the 19S and 20S proteasome subcomplexes to a DSB in vivo, and this recruitment is dependent on components of both the HR and NHEJ repair pathways, suggesting a direct role of the proteasome in DSB repair (13).

Previously, we and another group identified a specific interaction between Rqh1-Top3 and Rad3 (20, 31). In this study, we further extend these findings and show that rad3 rqh1 mutants are normally committed to a lethal pathway of DNA repair
requiring HR, but blocking this pathway by 
Rhp51 inactivation restores viability. We describe a function of Cut8 in DSB repair that is required for the survival of 
rhp1 mutants when Rad3 is also inactivated. Cut8 is a nuclear envelope protein involved in proteasome tethering and its proper function (26, 27). These results are consistent with a direct role of the proteasome in DSB repair (13), and the proteasome might function with other proteins in a common pathway in HR repair to ensure accurate nuclear division in S. pombe.

MATERIALS AND METHODS

Fission yeast strains and methods. We constructed all strains according to standard procedures (18). The original cut8 (27), gar2-GFP (23), rad22-CFP (6), 

Figure 1. Rad3 is required for the survival of rhp1Δ cells. (A) Five tetrads derived from diploid h” h” rhp1Δ::ura4+ rad3Δ::ura4+ rad3Δ::ura4+ (left) and rhp1Δ::ura4+ cds1::ura4+ cds1::ura4+ chk1::ura4+ chk1::ura4+ (right) strains were microdissected onto yeast extract (YE) agar, and the resulting colonies were photographed after 5 days of growth at 30°C. The genotypes of the segregants were determined by replica plating and are indicated schematically (W, rhp1Δ::ura4+; R, rad3Δ::ura4+; Q, rhp1Δ::ura4+; S, cds1::ura4+; K, chk1::ura4+). Boxes indicate the positions of rhp1 rad3 double (left) and rhp1Δ::ura4+ cds1::ura4+ chk1::ura4+ (right) mutants. (B) The strains, as indicated, were streaked in parallel onto YE agar plates and photographed after 3 days of incubation at 26°C or 36°C, as indicated. (C) The strains, as indicated, were grown in liquid culture to mid-logarithmic phase at 26°C and shifted to 36°C, the restrictive temperature. Samples of 500 cells taken at the indicated times after the shift to 36°C were plated in duplicate onto YE agar and incubated at 26°C. After 5 days of growth, viability was scored as a percentage of the number of colonies formed by the sample taken at time zero. Samples taken at the same time points were fixed, DAPI stained, and examined by fluorescence microscopy. The percentage of each sample exhibiting aberrant mitosis was scored as a total of at least 200 cells for each time point. (D) Fluorescence micrographs represent representative fields of DAPI-stained cells of the indicated strains grown at 26°C (right panels) or 9 h after the shift to 36°C (left panels). Cells exhibiting aberrant mitosis are indicated (arrowheads). Bar, 10 μm.
**RESULTS**

Synthetic lethality in **rqh1Δ rad3Δ** double mutants. It was previously found that the **rqh1Δ** mutant caused synthetic lethality in **rad3Δ** and **rad26Δ** mutants but not in other check-
point mutants, affecting the checkpoint sliding clamp (rad9, rad1, and hus1) or clamp loader rad17 (20). This indicates that rad3 together with rad26 might have a function in addition to their role in checkpoint control that is required for the survival of rqh1Δ mutants. This interpretation is further substantiated by the fact that simultaneous deletion of the two downstream effector kinases, Chk1 and Cds1, has no detrimental effect on the growth of rqh1Δ cells (Fig. 1A). Similar results have been found for top3 mutants. Consistent with the genetic interaction between top3 and rqh1, the top3 mutant is synthetically lethal in combination with rad3Δ or rad26Δ mutants but not with other checkpoint mutants (31).

To examine the basis of the growth defect in the rqh1 rad3 double mutant, we created a conditional mutant combining a rad3 temperature-sensitive (ts) mutation (16) with the rqh1 deletion. The rqh1Δ rad3Δ double mutant grew at 25°C but was unable to grow at 36°C, whereas single parental mutants remained fully viable at both temperatures (Fig. 1B). Given that deletion of rqh1 suppresses lethality in top3 mutants, we tested an rqh1Δ top3Δ rad3Δ triple mutant for viability and found that it is not viable at the restrictive temperature. These results suggest that the synthetic lethality between rqh1Δ and rad3Δ mutants is not due to inappropriate regulation of topoisomerase III activity. Examination of nuclear morphology revealed that the loss of viability in rqh1Δ rad3Δ cells correlates with defective mitoses, in which septation occurs without proper chromosome segregation (Fig. 1C and D). Given that simultaneous deletion of the two downstream effector kinases, Chk1 and Cds1, has no detrimental effect on the growth of rqh1Δ cells, these results suggest that the growth defect in the rqh1 rad3 double mutant arises from the failure to process DNA damage, which is manifested upon entry into mitosis as aberrant chromosome segregation. The fact that the viability drops steadily over 12 h, as seen in top3 mutants, suggests that the lethality is due to spontaneous damage caused by the loss of Rqh1 or Rad3 functions.

**Inactivation of HR suppresses rad3 rqh1 synthetic lethality.** One possible explanation for the lethality of the rqh1 rad3 double mutant is that DNA damage lesions are processed via a pathway dependent on HR, but intermediates are not properly resolved, leading to chromosome segregation defects and lethality. To examine this possibility, we analyzed the formation of Rad22 (ScRad52) foci, which allows the identification of subnuclear sites where recombination proteins are loaded onto DNA (6). Following the inactivation of Rad3, the number of rqh1Δ cells with Rad22 foci increases (Fig. 2A and B), consistent with either an increased rate of HR or a block in the recombination pathway. To examine how the inactivation of HR affected the rqh1 rad3 mutant, we introduced an rhp51 deletion (ScRAD51) into this mutant background (Fig. 2C and D). We performed the tetrad analysis using mat1ΔA17::LEU2 and smn7 mutations that abolish the formation of a DSB at mat1 and suppress the undesired growth defects in rad3Δ rhp51Δ mutants (10). Analysis of spore colonies indicated that while no...
rad3Δ rqh1Δ progeny was recovered, as expected from previous results, several rad3Δ rqh1Δ rhp51Δ triple mutants grew into small- or intermediate-sized colonies that could be propagated. Similar results were observed with the other RAD52 epistasis group mutants, although the rhp54 and rhp55 triple mutants grew more slowly than the rhp51 mutant (Fig. 2D). Taken together, these results suggest that rad3 Δ rqh1 Δ mutants are normally committed to a lethal pathway of DNA repair requiring HR, but blocking this pathway restores viability.

**Cut8 is required for the survival of rqh1Δ cells.** To learn more about the defects in rqh1 Δ rad3 Δ double mutants, we screened for genes capable of suppressing rqh1Δ rad3Δ lethality when overexpressed (Fig. 3A). In this way, cut8Δ, which encodes a nuclear envelope protein involved in proteasome tethering (26, 27), was identified. The specificity of this interaction is further substantiated by synthetic lethality of a strain carrying both cut8Δ and rqh1Δ deletions (Fig. 3B). After prolonged incubation, cut8Δ rqh1Δ spores germinated (Fig. 3C), but these cells were very slowly growing and generally highly elongated with extensive nuclear DNA fragmentation (Fig. 3D). As with the genetic interaction between rad3 Δ and rqh1 Δ, cut8 Δ and rqh1 Δ synthetic lethality was suppressed by deletion of rhp51 Δ (Fig. 3C).

**Cut8 is required for DNA repair.** Further experiments were performed to determine the role of Cut8 in Rqh1 deficiency. Consistent with the results described above, we found that cut8Δ mutants are hypersensitive to the ribonucleotide reductase inhibitor hydroxyurea (HU) in addition to DNA-damaging agents (26) (Fig. 4A). This sensitivity is not due to checkpoint defects, as cut8Δ mutants, like wild-type cells and unlike rad3Δ mutants, could arrest cell cycle progression and became highly elongated in response to HU and bleomycin, a radiomimetic drug that causes DSBs (Fig. 4B and data not shown). We thus investigated whether cut8Δ cells were able to repair DSBs efficiently by using pulsed-field gel electrophoresis to follow repair after exposing cells to bleomycin (Fig. 4C). After bleomycin treatment, chromosomes were damaged as shown by the low-molecular-weight smear of DNA. Fragmented chromosomes were mostly rejoined in wild-type cells by 3 h, but in contrast, chromosomes in the cut8Δ mutant remained fragmented even after 6 h. Thus, cut8Δ cells are defective in DSB repair.

In cycling S. pombe cells, HR is the dominant pathway for DSB repair whereas NHEJ is dispensable for cell survival (7). To explore the role of Cut8 in DNA repair, we examined the formation of Rad22 (ScRad52) foci in cut8Δ mutants. As shown in Fig. 4D, the rapid formation of Rad22 foci in response to bleomycin treatment occurred in wild-type cells as well as cut8Δ mutants, indicating that the initial recruitment of Rad22 is independent of Cut8. Taken together, these results suggest that Cut8 is required for the completion but not the initiation of HR repair. Like the rhp51Δ mutant, the cut8Δ mutant is viable in combination with rad3Δ mutation (Fig. 4E and F).

**Cut1 and Cut2 in Rqh1-associated defects.** The results presented above suggest a function of cut8Δ in DNA damage but do not clarify the molecular basis of this defect. The degradation of two proteins, namely, the mitotic cyclin Cdc13 and the securin Cut2, is dramatically delayed in a cut8Δ mutant, and this is apparently due to the mislocalization of the proteasome (26). It is not obvious why a delay in Cdc13 degradation would lead to a defect in DNA repair, but misregulation of cohesin cleavage, via Cut2, could be relevant since cohesin has been implicated in DSB repair in a number of studies (11). In particular, Nagao et al. (21) showed that cleavage of cohesin/ Rad21 by separase/Cut1 is needed for efficient DNA repair, and this could be related to the enhanced loading of cohesin that is seen at DSBs (24, 28). Since Cut1 is regulated by the securin Cut2, it is possible that the proper function of cohesin in DNA damage repair is aberrant if Cut2 degradation is defective. To test this, we overexpressed cut2Δ and also cut1Δ in wild-type cells and examined their ability to form colonies on plates containing HU. As shown in Fig. 4G, cells overexpressing cut2Δ were unable to grow at the concentration of HU that allowed colony formation in the control strain or cells overexpressing cut1Δ. We also tested the effects of Cut1 overexpression, since this might have similar effects to the promotion of Cut2 degradation by overexpression of Cut8. Indeed, as shown in Fig. 4H, overexpression of cut1Δ suppressed the temperature sensitivity of rqh1Δ rad3Δ cells. Taken together, these results suggest that Cut8 functions in a common pathway with Cut2 and Cut1 in the cellular response to HU inhibition of DNA replication as well as DNA damage that is important for cell survival in the absence of Rqh1.

![Figure 4](https://example.com/fig4.png)
Accumulation of Cut8 in response to DNA damage. Next, we examined how Cut8 responds to DNA damage and found that the accumulation of Cut8 is dependent on Rad3. This is not due to an indirect effect of cell cycle arrest, as rad3 mutants, unlike wild-type cells, proceeded with cell cycle progression regardless of DSBs generated by bleomycin (Fig. 5B), and in any case, Cut8 levels are constant during the cell cycle (data not shown). We also examined the cellular localization of GFP-tagged Cut8. Cut8 is enriched in the nucleus, particularly at the nuclear periphery after the addition of bleomycin (Fig. 5B), in keeping with its function in promoting the nuclear enrichment of the proteasome (26, 27).

Cut8 is important for rDNA maintenance. During the course of analysis of the chromosome structure in cut8Δ mutants, we consistently observed anomalous migration of chromosome III from cut8Δ cells, which had a significantly faster mobility than the wild-type chromosome (Fig. 6A, lanes 1, 2, and 4). Similar results were recently described for rap1 and top3 mutants, in which the reduction of chromosome III length is caused by the loss of ribosomal DNA (rDNA) repeats (5, 31). Consistent with the genetic interaction between cut8 and rap1, these results suggest that Cut8 might have a role in the maintenance of the rDNA repeats located at the ends of chromosome III. This was further supported by the genetic interaction between cut8Δ and slx1Δ, which encodes a structure-specific endonuclease that maintains rDNA copy number (5).

As shown in Fig. 6B, similar to that in rap1Δ mutants, the deletion of cut8 resulted in synthetic lethality in slx1Δ mutants, probably due to the failure to repair stalled replication forks in rDNA loci. Furthermore, cut8Δ slx1Δ synthetic lethality was suppressed in the presence of minicircles, confirming the importance of Cut8 in maintaining rDNA integrity.
pressed by the deletion of rhp51, in keeping with the genetic interaction between cut8 and rqh1 (Fig. 6C).

**Rad3 is required for proper segregation of rDNA.** Having established that Cut8 has a role in rDNA maintenance, we were interested to see whether Rad3 plays a role in the maintenance of rDNA structure. As shown in Fig. 7A, we consistently observed anomalous migration of chromosome III from rad3Δ cells, which also showed a greater reduction in the intensity of signal than chromosome I or II (40% reduction compared with that of wild-type cells), perhaps indicating the accumulation of abnormal structures that cannot enter the gel. This was further explored by examining the segregation of rDNA in rad3Δ cells by using a fusion protein between GFP and the nucleolar protein Gar2, which localizes to the rDNA where transcription occurs (23). Although not extremely frequent, abnormal anaphase progression, monitored in living cells by Gar2-GFP fluorescence, was observed in 10% of binucleated cells (n = 200) (Fig. 7B). In some rad3Δ cells, this took the form of two unequal masses of Gar2-GFP (Fig. 7C, middle panels). In other cells, an extended bridge of Gar2-GFP often persisted for some time between the nascent daughter nuclei (Fig. 7C, right panels), as previously described for rqh1 and top3 (31, 32). This was not observed in wild-type cells or checkpoint mutants, such as the rad9Δ mutant. Taken together, these results suggest that Rad3 is required for the proper segregation of rDNA.

**DISCUSSION**

Two main points emerge from our analysis of rad3 and rqh1. First, consistent with the independent assembly of different checkpoint protein complexes in response to DNA damage, our results suggest that Rad3 regulates more proteins than the checkpoint effectors and might directly participate in repair activity. Secondly, we describe a function of Cut8 in DSB repair that is required for cell survival in the absence of Rqh1. We also present evidence that these proteins function in a common pathway in HR repair that is essential for ensuring fidelity of chromosome segregation.

The results presented here, together with our previous data on the interaction between rqh1 and top3, suggest that Rqh1-Top3 has a function that is essential for accurate chromosome segregation (31, 32). Rqh1, with Top3, is presumably required to process or disrupt aberrant recombination structures that arise during S phase to allow proper chromosome segregation during mitosis. Intriguingly, in the absence of Rqh1, the processing of these structures appears to be dependent on Rad3 in a manner that is distinct from its role in checkpoint control. In *S. cerevisiae*, the ATR homolog Mec1, together with Rad53 (Cds1 homolog), has been implicated in facilitating DNA replication by stabilizing replication forks (15). However, the requirement of Rad3 function in the absence of Rqh1 is independent of Cds1, suggesting that it acts through a different pathway. Like mammalian ATM/
ATR, Rad3 might have an additional function in the regulation of DNA damage repair (8), and consistent with this, we identified cut8, which is required for DSB repair, as a multicopy suppressor of rad3Δ rhp1Δ mutants could arise from the failure to process certain DNA lesions, such as an unresolved recombination intermediate from a collapsed replication fork (34). Entering mitosis with sister chromatids entangled by unresolved recombination intermediates would impair subsequent chromosome segregation. This hypothesis is further supported by the fact that the inactivation of HR by the deletion of rhp51Δ is able to suppress the lethality of rad3Δ rhp1Δ mutants, suggesting a function of Rad3 in repair dependent on HR. Consistent with this interpretation, Mochida and Yanagida (17) recently showed that Rad3 was needed for the repair of DSBs after UV irradiation in cells arrested at G0 as well as G2 phase. Also, Nagao et al. (21) showed that modification of the cohesin subunit Rad21 by Rad3 might be involved in the recognition of damaged lesions that must be repaired. In line with our analysis of rad3Δ and rhp1Δ, phosphorylation of Rad21 after UV irradiation was dependent on Rad3 but not on Chk1 or Cds1. Taken together, these results suggest a function of Rad3 in the regulation of DNA damage repair that is distinct from those of other checkpoint proteins.

An alternative explanation of the ability of multicopy Cut8 (and separase/Cut1) to rescue rad3Δ rhp1Δ synthetic lethality is that precocious cleavage of cohesin reduces spontaneous recombination events between sister chromatids, thereby suppressing the hyper-recombination effect of the rhp1Δ mutants. However, the cut8Δ defect in DSB repair is unlikely to be an indirect negative effect on HR, as sister chromatids remain closely associated during interphase and this persists even after cells enter mitosis in the absence of Cut8 (27). A more direct role is suggested by the DNA repair function of separin-separase acting through the cleavage of cohesin (21), which could be needed following the enrichment of cohesin at DSB sites (24, 28). Cut8 might function with the separin-separase complex to aid DNA repair by removing local cohesin in interphase, thereby suppressing the synthetic lethality between rad3Δ and rhp1Δ. Alternatively, proteasome function mediated by Cut8 could be required for efficient DSB repair. It will therefore be of considerable interest to determine whether particular repair proteins are subjected to degradation by the proteasome. Further experiments are required to explore these possibilities and determine the requirement of Rad3 in these activities.

In summary, our data are consistent with a function of Rad3 and Cut8 in the modification of chromatin structure that is required for efficient DNA repair. Together with Rqh1-Top3, these functions are presumably required to process or disrupt aberrant recombination structures that arise during S phase to allow proper chromosome segregation during mitosis. Homologues of Cut8 have been identified in Metazoa (26); thus, it is likely that inhibition of Cut8 activity could generally be detrimental to genome stability.

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REFERENCES


