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A Postsynaptic Role for Rhp55/57 That Is Responsible for Cell Death in Δ*rqh1* Mutants Following Replication Arrest in *Schizosaccharomyces pombe*

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ABSTRACT

Following replication arrest, multiple cellular responses are triggered to maintain genomic integrity. In fission yeast, the RecQ helicase, Rqh1, plays a critical role in this process. This is demonstrated in Δ*rqh1* cells that, following treatment with hydroxyurea (HU), undergo an aberrant mitosis leading to cell death. Previous data suggest that Rqh1 functions with homologous recombination (HR) in recovery from replication arrest. We have found that loss of the HR genes *rhp55*$^+$ or *rhp57*$^+$, but not *rhp51*$^+$ or *rhp54*$^+$, suppresses the HU sensitivity of Δ*rqh1* cells. Much of this suppression requires Rhp51 and Rhp54. In addition, this suppression is partially dependent on *swi5*$^+$. In budding yeast, overexpressing Rad51 (the Rhp51 homolog) minimized the need for Rad55/57 (Rhp55/57) in nucleoprotein filament formation. We overexpressed Rhp51 in *Schizosaccharomyces pombe* and found that it greatly reduced the requirement for Rhp55/57 in recovery from DNA damage. However, overexpressing Rhp51 did not change the Δ*rhp55* suppression of the HU sensitivity of Δ*rqh1*, supporting an Rhp55/57 function during HR independent of nucleoprotein filament formation. These results are consistent with Rqh1 playing a role late in HR following replication arrest and provide evidence for a postsynaptic function for Rhp55/57.

REPLICATION arrest is a common occurrence even in unperturbed cells. Studies in *Escherichia coli* have shown that spontaneous replication arrest occurs in 18% of cells and could be as high as 50% (Cox *et al.* 2000; Maisnier-Patin *et al.* 2001; McGlynn and Lloyd 2002). We can assume that this problem is even greater in eukaryotic cells where the genomes are generally much larger and multiple origins of replication are used. When the replication machinery encounters DNA damage, the S-phase checkpoint is induced, allowing time for the cell to repair or bypass the DNA damage prior to entry into mitosis (Diffley *et al.* 2000; Michel 2000; Carr 2002; Nyberg *et al.* 2002). What has become increasingly evident is the need for homologous recombination (HR) in the recovery and restart of replication following arrest (Michel *et al.* 2001; Saintigny *et al.* 2001; Lundin *et al.* 2002). It remains unclear how HR functions in replication restart but several models have been proposed (Cox *et al.* 2000; McGlynn and Lloyd 2002; Helleday 2003). One model favors branch migration of the stalled fork, leading to the formation of a pseudo-Holliday junction (HJ) known as a chicken foot structure (Cox *et al.* 2000; McGlynn and Lloyd 2002; Helleday 2003; Heyer *et al.* 2003). Alternatively HR can act in the process of template switching (Liberi *et al.* 2000). The structure recognized as a substrate for HR following replication arrest has not been definitively established although it has been shown that double-strand breaks (DSBs) form during replication arrest (Michel *et al.* 1997; Rogakou *et al.* 1999). However, in at least one study, replication restart by HR was shown to occur in the absence of detectable DSBs (Lundin *et al.* 2002).

In *Saccharomyces cerevisiae*, HR proteins were initially identified as conferring resistance to ionizing radiation (IR), although increasingly their main function appears to be in maintaining genomic integrity during replication (Michel 2000; Michel *et al.* 2001; Helleday 2003). Following the formation of a DSB, a complex of three proteins, Mre11p, Rad50p, and Xrs2p (MRX complex), is thought to be recruited to the site (Nelms *et al.* 1998). The MRX complex participates in the production of a 3′ single-stranded end particularly during meiosis (Bressan *et al.* 1999; Paques and Haber 1999; D'Amours and Jackson 2002; Symington 2002; Helleday 2003; Trujillo *et al.* 2003). The single-strand binding protein, RPA, rapidly coats this 3′ single strand. Rad52 aids in the loading of Rad51 onto the 3′ single-strand end. Rad51 binds DNA weakly so the obligate heterodimer, Rad55/Rad57, acts to stabilize its binding, leading to Rad51 polymerization along the 3′ tail, forming a nucleoprotein filament (Johnson and Symington 1995; Sung 1997; Paques and Haber 1999; Fortin and Symington 2002; Helleday 2003). Next, aided by Rad54,

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the Rad51 filament invades its homologous sequence either on its sister chromatid or, in diploid cells, on its homologous chromosome, forming a heteroduplex (Van Komen *et al.* 2000, 2002; Solinger *et al.* 2001). This creates a joint molecule that either can be resolved by HJ resolvase or is simply displaced by collapse of the D-loop, restoring the original duplex (Kuzminov 1993; Sharples *et al.* 1999; Haber and Heyer 2001).

Rad55 and Rad57 are referred to as Rad51 paralogs because of their close sequence homology to Rad51 (Symington 2002). *rad55* and *rad57* mutants are only mildly sensitive to IR at 30° but are as sensitive as *rad51* mutants at low temperatures (23°) (Lovett and Mortimer 1987; Johnson and Symington 1995). This, along with suppression of *rad55* and *rad57* by overexpression of Rad51, was the original basis for predicting their role as mediators (Hays *et al.* 1995; Johnson and Symington 1995). Cold-enhanced sensitivity is also seen in *Schizosaccharomyces pombe* Δ*rhp55* and Δ*rhp57* mutants (Tsutsui *et al.* 2000). A recent article showed that a *rad51* mutant with increased DNA binding could also suppress a *rad55* mutant (Fortin and Symington 2002). These results further support the role of rad55/57 as mediators of Rad51 function. Recent data have implicated Rad51 paralogs in post-strand invasion events. In two reports on human Rad51 paralogs, Rad51b protein was shown to preferentially bind HJ and Rad51c and Xrcc3 were shown to be necessary for HJ resolution (Yokoyama *et al.* 2003; Liu *et al.* 2004). This role for Rad51c has been shown only in cell extracts and was not demonstrated *in vivo*.

Homologs of all of the *S. cerevisiae* HR proteins have been identified in *S. pombe* (Muris *et al.* 1993, 1997; Khasanov *et al.* 1999; Wilson *et al.* 1999; Fukushima *et al.* 2000; Tsutsui *et al.* 2000; Ueno *et al.* 2003). While it is generally assumed that the *S. pombe* homologs will carry out functions similar to those of their *S. cerevisiae* counterparts, significant differences have been reported between HR in these two organisms. For example, while *rad52* mutants are the most sensitive of the HR mutants to DSBs in *S. cerevisiae*, the equivalent mutation in *S. pombe*, *rad22*, has only a slight sensitivity to IR (Muris *et al.* 1997; Suto *et al.* 1999; van den Bosch *et al.* 2001). This discrepancy may be due to the existence of a second Rad52 homolog in *S. pombe* known as Rti1/Rad22B (Suto *et al.* 1999; van den Bosch *et al.* 2001), the function of which becomes important in Δ*rad22* mutants. In *S. cerevisiae*, mutations in members of the *RAD52* epistasis group (*RAD51, RAD52, RAD54, RAD55*, and *RAD57*) confer only slight sensitivity to ultraviolet (UV) radiation. By contrast, mutants of the *S. pombe* homologs (Δ*rhp51*, Δ*rad22*, Δ*rhp54*, Δ*rhp55*, and Δ*rhp57*, respectively) are sensitive to UV radiation as well as to other DNA-damaging agents and hydroxyurea (HU). This suggests that in *S. pombe* various types of DNA damage may be converted into substrates recognized by HR proteins, such as nicks, gaps, or DSBs (Caspari *et al.* 2002; Laursen *et al.* 2003).

Rqh1, the *S. pombe* RecQ homolog, has been linked to homologous recombination in several studies. Evidence indicates that HR and Rqh1 respond to DSBs and replication arrest through a common process (Murray *et al.* 1997; Caspari *et al.* 2002). *rqh1*$^+$ mutants are sensitive to DNA damage and replication arrest (Murray *et al.* 1997; Stewart *et al.* 1997; Davey *et al.* 1998). While showing a normal or near normal checkpoint response during S-phase arrest, upon release Δ*rqh1* cells do not properly complete mitosis (Stewart *et al.* 1997; Davey *et al.* 1998; Marchetti *et al.* 2002). The mitotic defect is observed as an accumulation of cells with "cut" chromosomes or with an uneven distribution of nuclear material between daughter cells. Also, Δ*rqh1* cells show dramatically increased rates of HR following replication arrest or DNA damage (Stewart *et al.* 1997; Doe *et al.* 2000). When the *E. coli* Holliday junction resolvase, RusA, was expressed in Δ*rqh1* cells, their UV and HU sensitivities were partially suppressed, suggesting that in the absence of Rqh1, stalled replication forks accumulate unresolved Holliday junctions (Doe *et al.* 2000).

Mutants of the *S. cerevisiae* RecQ homolog, *SGS1*, show synthetic lethality with *mus81/mms4*, which forms a complex that cleaves a 3′ flap structure that mimics a stalled replication fork (Bastin-Shanower *et al.* 2003). Two studies reported that the synthetic lethality between Δ*mus81* and Δ*rqh1* is conserved in *S. pombe*, but two different interpretations of the data were offered for the activity of Mus81/Mms4 (Eme1): it acts in the resolution of regressed forks (HJ) or it acts on stalled replication forks (Boddy *et al.* 2001; Doe *et al.* 2002). It is conceivable that both interpretations are correct. A recent article reported that loss of HR suppressed the synthetic lethality between *mus81* and *sgs1* (Fabre *et al.* 2002), suggesting that the critical functions of these proteins are downstream of HR. Mutants defective for the yeast RecQ helicases also show synthetic interaction with Δ*srs2* (*srs2*$^+$ encodes another DNA helicase), which is also suppressed by loss of HR genes (Gangloff *et al.* 2000; Fabre *et al.* 2002; Maftahi *et al.* 2002; Doe and Whitby 2004). Together these findings have led to the speculation that yeast RecQ helicases act to prevent the deleterious effects of HR following replication arrest, either by suppressing the formation of DSB (or other structures that HR acts upon) or by participating in a process that leads to the resolution of recombination intermediates. Two recent articles have supported a role for RecQ helicases in restricting crossovers at DSBs during HR by acting on joint molecules, further supporting the role of this helicase family in recombination (Ira *et al.* 2003; Wu and Hickson 2003).

Here we report on studies that support a role for Rqh1 downstream of joint molecule formation during HR. We made a series of double mutants between Δ*rqh1* and deletions of HR genes. We found that loss of *rhp55*$^+$/*57*$^+$ dramatically suppressed the HU sensitivity of Δ*rqh1* mutants. This suppression was largely dependent on

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
sz472	*h*$^+$, *ade6-210, ura4-D18, leu1-32*	Laboratory stock
sz662	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rqh1::kanMX4*	Maftahi *et al.* (2002)
sz215	*h*$^+$, *ade6-704, ura4-D18, leu1-32, rhp51::ura4*	Jang *et al.* (1995)
sz231	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rhp54::ura4*	Muris *et al.* (1996)
sz844	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rhp55::ura4*	Khasanov *et al.* (1999)
sz664	*h*$^-$, *smt-0, ura4-D18, leu1-32, his3-D, arg3-D1, swi5::his3*	Hiroshi Iwasaki
sz384	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rqh1::kanMX4, rhp51::ura4*	This study
sz521	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rqh1::kanMX4, rhp54::ura4*	This study
sz843	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rqh1::kanMX4, rhp55::ura4*	This study
sz638	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rqh1::kanMX4 rhp55::ura4, rhp51::ura4*	This study
sz640	*h*$^+$, *ade6-210, ura4-D18, leu1-32, rqh1::kanMX4 rhp55::ura4, rhp54::ura4*	This study
sz694	*h*$^-$, *smt-0, ura4-D18, leu1-32, his3-D, arg3-D1, rqh1::kanMX4 swi5::His3*	This study
sz868	*h*$^-$, *smt-0, ura4-D18, leu1-32, his3-D, arg3-D1, rqh1::kanMX4, rhp55::ura4, swi5::His3*	This study

Rhp51 and Rhp54, suggesting that the deleterious function of Rhp55/57 was acting downstream of joint molecule formation. This was further supported by our results showing that complementing the defect of Δ*rhp55* in the Rhp51 nucleation step did not affect the suppression of the HU sensitivity in the Δ*rqh1* Δ*rhp55* double mutant. Loss of *rhp55*$^+$ decreased the number of aberrant chromosomes (showing torn nuclear material) seen in Δ*rqh1* cells following replication arrest, supporting the idea that these events are the result of unresolved recombination intermediates. These data imply that Rqh1 plays a late role in HR and that Rhp55/57 has a postsynaptic function.

MATERIALS AND METHODS

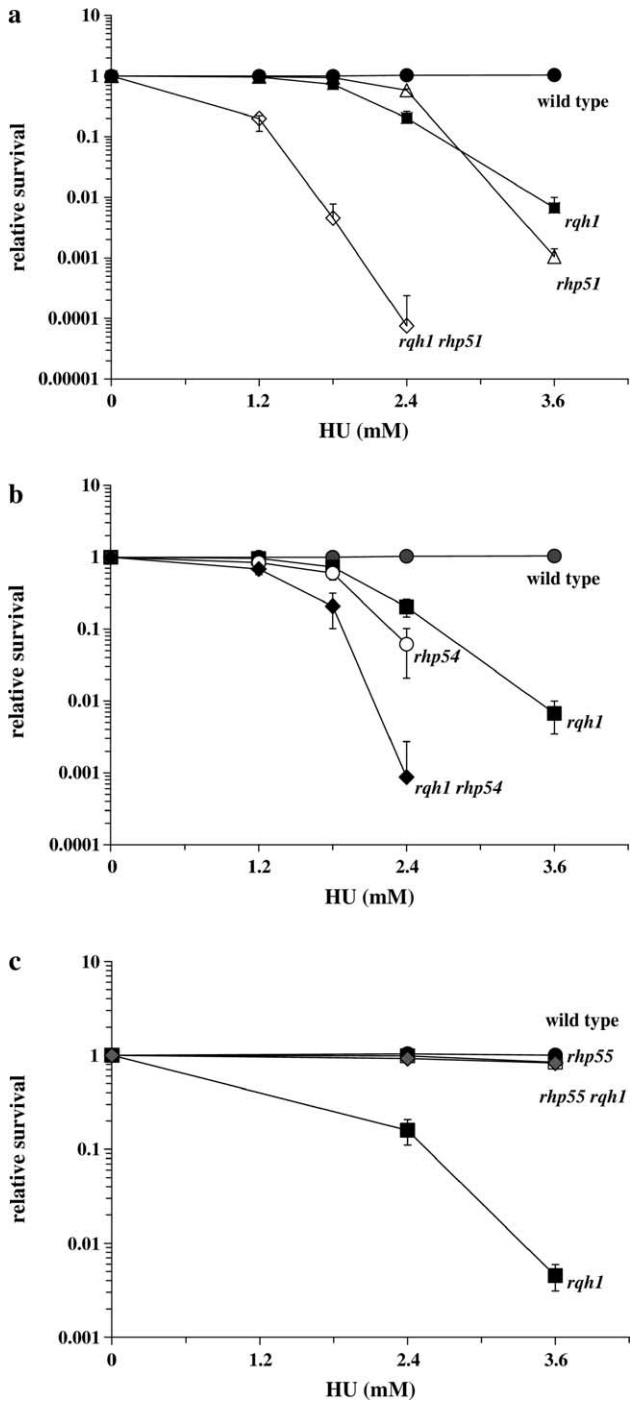
Media and construction of plasmids and mutant strains: Unless indicated, cells were grown in YEA media (0.5% yeast extract, 3% glucose, and 150 mg/liter adenine). Minimal medium was EMM (QBiogene) with the appropriate supplements. G418 selection was carried out with 150 mg/liter of Geneticin (GIBCO, Grand Island, NY) in YEA. Strains containing multiple mutations were generated from crosses. Double mutants were generally isolated from tetrads and occasionally from random spores. In either case, strains containing multiple mutations were tested individually by PCR analysis and, when necessary, sequenced. Table 1 lists the strains used in this study. The Rhp51 overexpression plasmid was constructed by PCR amplification of *rhp51*$^+$ from genomic DNA using primers rhp51 5′ *Sal*I AGATCGTCGACATGGCAGATA CAGAGGTGG and rhp51 3′ *Bam*HI AGATCGGATCCTTAGA CAGGTGCGATAATTTCC. The PCR product was gel purified and cloned into PCR2.1-TOPO using the TOPO TA cloning system (Invitrogen, Carlsbad, CA). The resulting plasmid pTOPO-Rhp51 was sequenced. The rhp51 fragment was then isolated from the pTOPO-Rhp51 by digestion with *Sal*I and *Bam*HI and ligated into *Sal*I and *Bam*HI or *Xho*I and *Bam*HI digested pREP-3x, pREP-41x, or pREP-81x (obtained from Susan Forsburg). The resulting plasmids were designated pREP-3x-Rhp51, pREP-41x-Rhp51, and pREP-81x-Rhp51, respectively.

Survival studies: Cultures were grown overnight to midlog (10^6–10^7 cells/ml). For UV survival, cells were plated onto YEA plates and irradiated with the indicated dose of UV light. Plates were incubated at 30° for 3–4 days and colonies were counted. For HU survival, cells were counted and plated onto YEA plates containing the appropriate concentration of HU. The plates were incubated at 30° except for the cold-enhancement studies where plates were initially incubated at 22°. After 4–6 days colonies were counted.

Studies using overexpressed Rhp51: pREP plasmids expressing Rhp51 were transformed into the various strains and selection was maintained on EMM plates with appropriate supplements including 8 μM thiamine. The presence of 8 μM thiamine suppressed the expression of Rhp51 from the *nmt* promoter. Previous studies of Rhp51 overexpression from the *nmt* promoter (Kim *et al.* 2001) had demonstrated that peak expression of Rhp51 occurred at 17–20 hr after the removal of thiamine. Strains containing pREP-81x-Rhp51 were grown for 20 hr in the presence or absence of 8 μM thiamine. HU (15 mM) was added to each culture and cells were collected at 3, 6, and 9 hr after addition. These cells were diluted and plated onto YEA plates and incubated at 30° for 4–6 days when colonies were counted.

Confirmation of Rhp51 overexpression: Overnight cultures of wild-type (sz472) and Δ*rqh1* Δ*rhp55* (sz843) cells containing pREP81x-Rhp51 were grown (20 hr) in the presence of thiamine. These cells were washed and then added to media with or without thiamine. Whole-cell extracts were prepared from cells following 20 hr of growth. Cell extracts (150 μg) were separated on a 12% PAGE-SDS gel and blotted onto ECL nitrocellulose paper (Amersham, Arlington Heights, IL). Rhp51 was detected using a rabbit anti-human rad51 antibody (Santa Cruz H-92), which was previously shown to cross-react with Rhp51 (Caspari *et al.* 2002). The presence of antibody was detected using ECL (Amersham).

Pulse field gel electrophoresis (PFGE): Cells were harvested at 9000 rpm in a microcentrifuge and washed in 1 ml of stop buffer (50 mM EDTA/1 mM NaN_3). Cells were counted using a hemacytometer and 4.0×10^7 were resuspended in 30 μl of stop buffer. Thirty-five microliters of warm (50°) 1.5% InCert agarose in stop buffer was added to the cell suspension and the entire volume was gently transferred into a plug mold. Plugs were allowed to solidify for 20–30 min at 4° followed by incubation in spheroplasting solution (1 ml 1 M sorbitol, 40 μl 0.5 M EDTA, 10 μl 1 M Tris pH 7.5, 1 μl β-mercaptoethanol, 2 mg/ml Zymolyase, 2 mg/ml Novazyme) for 2.5 hr at 37° with gentle shaking. Spheroplasting solution was removed and plugs were incubated with 2 ml ETS (0.25 M EDTA, 50 mM



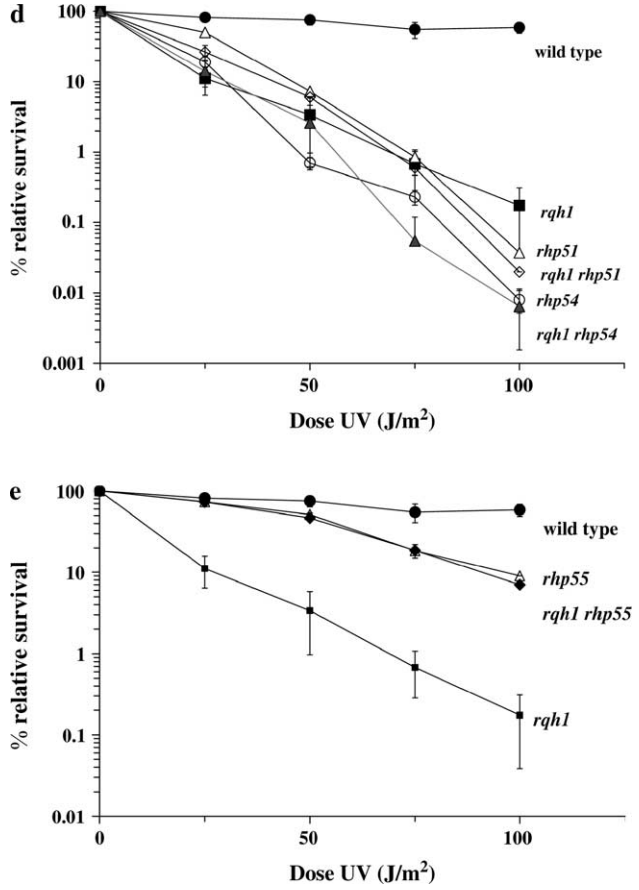


FIGURE 1.—*Continued.*

FIGURE 1.—HU and UV sensitivity of HR mutants alone and combined with Δ*rqh1*. Double mutants between Δ*rqh1* and mutants of the *RAD52* epistasis group were created. To measure HU sensitivity, cells were grown to midlog and then each single and double mutant was plated onto plates containing HU of varying concentrations and colonies were counted after 4–6 days of incubation at 30°. To measure UV sensitivity, midlog cultures were grown and cells were spread onto YEA plates at varying concentrations and irradiated with the indicated dose of 254 nm UV light. The results are shown. ●, wild type; ■, Δ*rqh1*. (a) △, Δ*rhp51*; ◇, Δ*rqh1* Δ*rhp51*. (b) ○, Δ*rhp54*; ◆, Δ*rqh1* Δ*rhp54*. (c) △, Δ*rhp55*; ◆, Δ*rqh1* Δ*rhp55*. (d) △, Δ*rhp51*; ◇, Δ*rqh1* Δ*rhp51*; ○, Δ*rhp54*; ▲, Δ*rqh1* Δ*rhp54*. (e) △, Δ*rhp55*; ◆, Δ*rqh1* Δ*rhp55*. (Note that some error bars are smaller than the symbols.)

Tris pH 7.5, 1% SDS) at 55° for 2 hr with one change of buffer. ETS solution was removed and plugs were incubated with 2 ml of 2 mg/ml proteinase K in SEP buffer (0.5 M EDTA, 1% lauryl sarcosine) for 1 hr at 55°. Fresh buffer was added and plugs were incubated overnight at 55°. Plugs were washed three times with 1× TE and loaded into the wells of a 0.6% agarose gel [Bio-Rad (Hercules, CA) PFGE grade] made with 1× TAE. Gels were run on a Bio-Rad CHEF-DR-II PFGE system for 72 hr at 15° at 2.0 V/cm, with switch times of 20 and 30 min. Gels were stained overnight in 1× TAE + SYBR green DNA stain (Molecular Probes, Eugene, OR) at the recommended concentration of 1:10,000 and visualized on a UV transilluminator.

RESULTS

Δ*rhp55* and Δ*rhp57* suppress HU and UV sensitivity of Δ*rqh1* cells: Previous studies have reported that Δ*rqh1* cells are sensitive to HU treatment and, although they arrest in S phase, they undergo an aberrant mitosis where the nuclear material has a "cut" appearance and is often unevenly distributed between daughter cells (STEWART *et al.* 1997; DAVEY *et al.* 1998; DOE *et al.* 2000). One explanation for the nuclear phenotype is that HU treatment induces HR intermediates to form between sister chromatids, which are not resolved in Δ*rqh1* cells. We reasoned that if this were the case, loss of HR should improve viability and suppress the formation of these aber-

rant nuclei. We first examined the HU sensitivity of the HR mutants corresponding to the *S. cerevisiae RAD52* epistasis group *RAD51, RAD54, RAD55*, and *RAD57*, which in *S. pombe* are *rhp51*$^+$, *rhp54*$^+$, *rhp55*$^+$, and *rhp57*$^+$, respectively. We did not pursue studies using the *RAD52* homolog, *rad22*$^+$, as we found Δ*rad22* to be synthetic lethal with Δ*rqh1* as was previously reported (Wilson *et al.* 1999). Cells were plated onto media containing various concentrations of HU and incubated for 4–6 days before colonies were counted to determine their sensitivities to replication arrest. We found that Δ*rhp55* and Δ*rhp57* mutants showed identical sensitivities to HU and DNA damage. This was expected as Rhp55 and Rhp57 act as an obligate heterodimer. Thus, for simplicity we primarily present the Δ*rhp55* data here. In Figure 1, a and b, Δ*rhp51* and Δ*rhp54* single mutants are shown to be sensitive to HU, particularly at higher doses. Δ*rhp55* cells showed essentially no sensitivity to HU exposure in the dose range examined (Figure 1c). These results show that Rhp51 and Rhp54 play a more central role in recovery from HU-induced replication arrest in *rqh1*$^+$ cells than does Rhp55.

Next we tested the HU sensitivity of double mutants made between the HR mutants and Δ*rqh1* (Figure 1, a–c). We found that Δ*rqh1* Δ*rhp51* and Δ*rqh1* Δ*rhp54* double mutants were actually more sensitive to HU than the single Δ*rqh1* mutant (Figure 1, a and b). However, loss of *rhp55*$^+$ significantly suppressed the HU sensitivity of Δ*rqh1* cells, to essentially the levels seen in the single Δ*rhp55* mutant (Figure 1c). These data suggest that the action of Rhp55/57 leads to the sensitivity in replication-arrested cells lacking Rqh1. To make certain that the losses of Rhp55 and Rhp57 were equivalent, we created a triple mutant, Δ*rqh1* Δ*rhp55* Δ*rhp57*, and tested its sensitivity to HU. As expected, the triple mutant showed levels of sensitivity identical to those seen in the Δ*rqh1* Δ*rhp55* and Δ*rqh1* Δ*rhp57* double mutants (data not shown).

Δ*rqh1* and HR mutants are sensitive to exposure to UV radiation (Muris *et al.* 1993, 1996; Ostermann *et al.* 1993; Murray *et al.* 1997; Davey *et al.* 1998; Gangloff *et al.* 2000; Fabre *et al.* 2002; Maftahi *et al.* 2002; Doe and Whitby 2004). When we tested the UV sensitivity of double mutants between Δ*rqh1* and genes of the HR pathway we found a pattern of suppression similar to that seen with HU treatment. Δ*rqh1* Δ*rhp51* and Δ*rqh1* Δ*rhp54* double mutants showed sensitivities to UV damage identical to those of the Δ*rhp51* and Δ*rhp54* single mutants, which are more sensitive than the Δ*rqh1* single mutant (Figure 1d). By contrast, deletion of *rhp55*$^+$ in a Δ*rqh1* background significantly suppressed the sensitivity of Δ*rqh1* mutants (Figure 1e). These data are consistent with our findings with HU treatment and suggest that Rqh1 has a role in recovery from DNA damage and replication arrest that acts downstream of Rhp55/57 function. A recent study by Doe and Whitby (2004) also showed that loss of *rhp55*$^+$ suppressed both the HU and the DNA damage sensitivity of Δ*rqh1* mutants.

Δ*rhp55* partially suppresses the presence of torn nuclear material and speeds the formation of intact chromosomes in HU-treated Δ*rqh1* cells: Since the loss of *rhp55*$^+$ improved the HU resistance of Δ*rqh1* cells, we speculated that its loss would also suppress the cut phenotype of Δ*rqh1* cells following replication arrest, supporting the hypothesis that these could represent unresolved recombination intermediates. To test this hypothesis, wild-type, Δ*rqh1*, Δ*rhp55*, and Δ*rqh1* Δ*rhp55* strains were incubated in HU for 5 hr, sufficient time to achieve 100% arrest of cells in S phase, based on PFGE results shown in Figure 2c and FACS analysis (not shown).

Cells were allowed to recover for various times from the HU block and then stained with 4′,6-diamidino-2-phenylindole (DAPI) to examine their nuclear material by fluorescence microscopy. Dividing cells were observed at times from 2 to 5 hr after HU release. The 3-hr time point had the greatest number of cells in the process of cell division, so we picked this time point for quantitative analysis. Photographs depicting representative examples of the four strains from the 3-hr time point are shown in Figure 2a. The presence of cells with cut nuclei and unevenly distributed chromosomal material is evident in dividing Δ*rqh1* cells.

For quantitative analysis, we counted only cells that had clearly undergone mitosis, where either a septum was present or daughter cells were still attached following cell division. Dividing cells were grouped into three categories: (1) cells undergoing normal cell division where nuclear material appeared normal and was equally distributed between daughter cells, (2) cells with torn nuclei and an unequal distribution of nuclear material, and (3) cells where torn chromosomes were not evident but where there was clearly an unequal distribution of nuclear material. These results, obtained from scoring >200 dividing cells from each strain, are summarized in Figure 2b. The data indicate that while 95% of dividing wild-type cells showed normal cell division, only 20% of Δ*rqh1* cells showed normal segregation of nuclear material. By comparison, 76% of Δ*rhp55* cells appeared normal, indicating that Δ*rhp55* cells are somewhat compromised in their ability to recover from HU. As predicted, loss of *rhp55*$^+$ significantly improved the ability of Δ*rqh1* cells to undergo normal mitosis; 52% of Δ*rqh1* Δ*rhp55* cells were found to divide normally. These results demonstrate that there is a correlation between the cut phenotype seen in HU-treated Δ*rqh1* cells and HR and, while not conclusive, are consistent with these nuclear aberrations representing HR intermediates. If these are recombination intermediates, these data cannot distinguish whether their formation is suppressed in a Δ*rhp55* background or their resolution is improved in this background.

In complementary experiments the fate of chromosomes in cells following HU treatment was examined directly

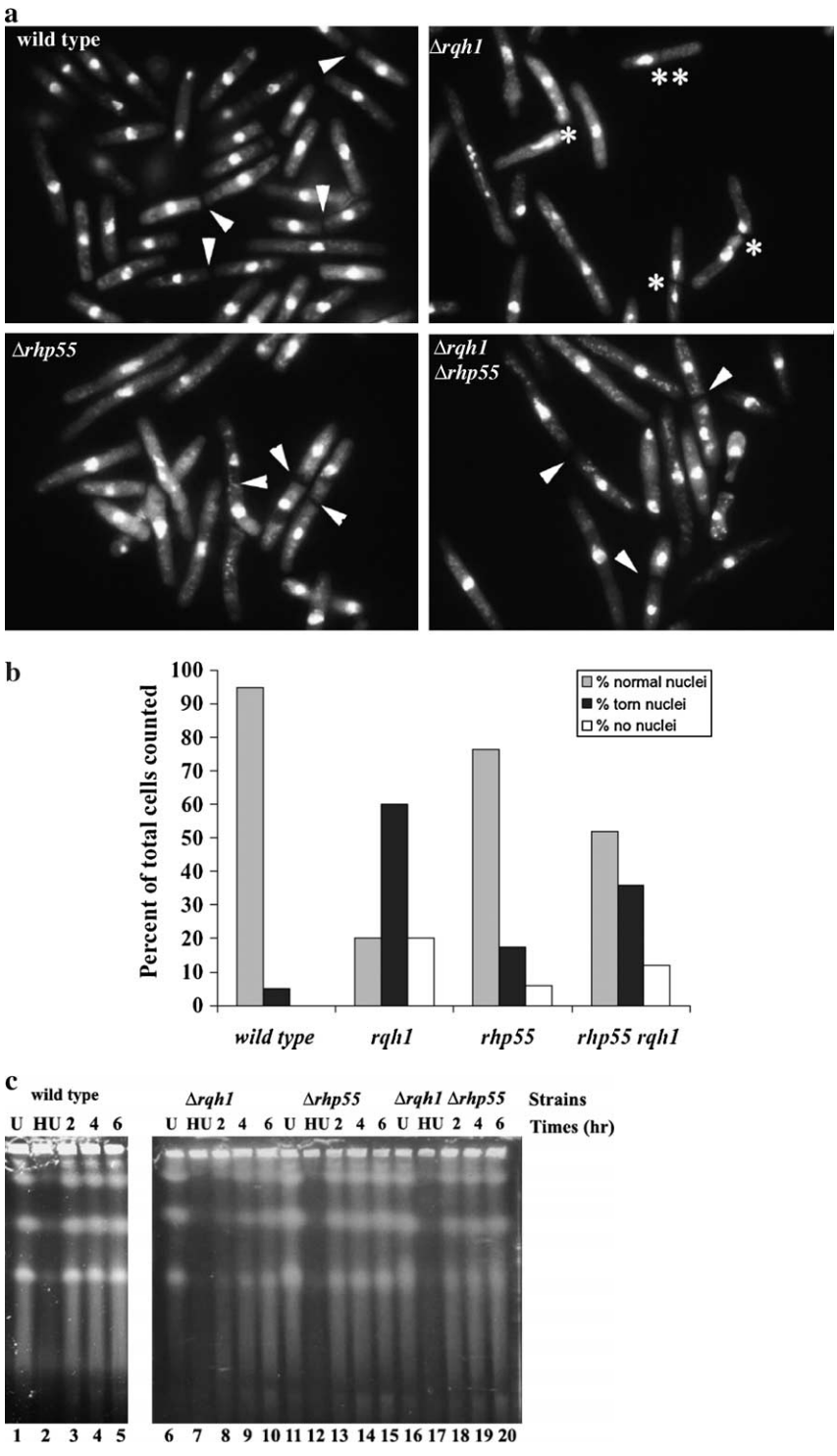


Figure 2.—Evidence that HR intermediates accumulate in HU-treated Δ*rqh1* cells that are suppressed by Δ*rhp55*. Previous studies had shown that aberrant mitosis occurs in Δ*rqh1* cells following replication arrest. We speculated that torn and unevenly distributed nuclear material was due to unresolved recombinant intermediates. We tested this hypothesis by determining if loss of $rhp55^{+}$ could suppress this phenotype. (a) Examples of DAPI-stained cells visualized by fluorescent microscopy are shown following HU treatment and a 3-hr recovery. Arrowheads point to septa of dividing cells. Asterisks indicate cut chromosomes. The double asterisk indicate cells where all DNA segregated into one daughter cell. (b) Quantitation of the number of cells with aberrant chromosomes visible following DAPI staining after a 3-hr release from replication block. (c) PFGE was used as another way of monitoring the fate of chromosomes following HU treatment. Replication fork structures and recombination intermediates are inhibited from exiting the well. We compare the chromosomes from Δ*rqh1* cells with those from wild type, Δ*rhp55*, and Δ*rqh1* Δ*rhp55*. Lanes 1, 6, 11, and 16, chromosomes from cycling cells; lanes 2, 7, 12, and 17, chromosomes from cells exposed to 15 mM HU for 5 hr; lanes 3, 8, 13, and 18, chromosomes from cells 2 hr after release; lanes 4, 9, 14, and 19, chromosomes from cells 4 hr after release; lanes 5, 10, 15, and 20, chromosomes from cells 6 hr after release.

by PFGE. Incompletely replicated DNA containing replication forks cannot migrate out of the wells of PFGs due to their branched structures (Cha and Kleckner 2002). Recombination intermediates presumably would behave likewise. In these studies, cells were collected at 2-hr time points following release from a 5-hr HU block. An example of one experiment is shown in Figure 2c. All three chromosomes are visible in the gel in samples prepared from unsynchronized cells (lanes 1, 6, 11, and 16). After 5 hr in HU all of the chromosomal material was found in the wells with no distinct chromosomes detected in the gel for any strain (lanes 2, 7, 12, and 17). By 2 hr after release, DNA synthesis appears to be complete in wild-type and Δ*rhp55* cells on the basis of the intensity of the chromosomal bands seen in the gel (lanes 3 and 13); compare with unsynchronized cells in lanes 1 and 11. Also no further increase in chromosome intensity is seen after 2 hr (compare lanes 3 and 13

to lanes 4 and 5 and lanes 14 and 15). By contrast chromosomal staining in the Δ*rqh1* cells is significantly less intense at 2 hr (lane 8). Even after 6 hr of recovery, the staining intensity of the chromosomes from Δ*rqh1* cells did not reach those of the unsynchronized cells (compare lane 10 to lane 6). Previous studies using FACS analysis showed that Δ*rqh1* cells are not delayed in completion of DNA synthesis following release from an HU block (Marchetti *et al.* 2002). This suggests that the DNA retained in the wells in the Δ*rqh1* cells is due to the presence of unresolved recombination intermediates. The intensity of chromosomal bands present in Δ*rqh1*Δ*rhp55* cells by 2 hr after release from HU (lane 18) is comparable with wild type or the Δ*rhp55* single mutant at this time point (lanes 3 and 13, respectively). Also the intensity of chromosome staining does not further intensify at later time points (compare lane 18 with lanes 19 and 20). We suggest that these results are further evidence that loss of *rhp55*$^+$ suppresses the accumulation of recombination intermediates in replication-arrested Δ*rqh1* cells but acknowledge that we cannot absolutely rule out the possibility that the retardation of chromosomal migration is due to residual replication intermediates.

Rhp51 and Rhp54 activities are required for the suppression of Δ*rqh1* HU sensitivity: We next asked if the suppression of Δ*rqh1* sensitivity to HU by Δ*rhp55*/Δ*rhp57* depends on the functions of Rhp51 and Rhp54. Figure 1, a and b, shows that both are critical in recovery of cells from replication arrest. To address this we created Δ*rqh1* Δ*rhp55* Δ*rhp51* and Δ*rqh1* Δ*rhp55* Δ*rhp54* triple mutants. We compared the HU sensitivities of these mutants to wild type and to single and double mutants. The Δ*rqh1* Δ*rhp55* Δ*rhp51* and Δ*rqh1* Δ*rhp55* Δ*rhp54* triple mutants are much more sensitive than the Δ*rqh1* Δ*rhp55* double mutant, showing that suppression by Δ*rhp55* is dependent on the presence of Rhp51 and Rhp54 (Figure 3, a and b). The growth of the Δ*rqh1* Δ*rhp55* Δ*rhp51* triple mutant on HU-containing plates did slightly improve over that of the Δ*rqh1* Δ*rhp51* double mutant. One interpretation of these findings is that two lines of suppression of Δ*rqh1* cells exist, one that is Rhp51 dependent and another that is Rhp51 independent. A comparable change is not seen in the Δ*rqh1* Δ*rhp55* Δ*rhp54* triple mutant where the sensitivity was the same as in the Δ*rqh1* Δ*rhp54* double mutant.

One note concerning these experiments is that the HU sensitivity of Δ*rhp51* cells appears to be less than that of Δ*rqh1* cells. This is in contrast to the results seen in Figure 1, where Δ*rqh1* and Δ*rhp51* mutants show similar sensitivities to HU. We have repeated both experiments multiple times, with identical results. Our only explanation is based on our observation that, in addition to forming a few visible colonies, Δ*rhp51* cells form microscopic colonies (~15–50 cells) on 3.6 mM HU plates after ≥5 days of incubation. By contrast Δ*rqh1* cells either die immediately, seen as single cells, or grow a very few generations, seen as colonies of 2–10 cells, when plated on 3.6 mM HU and incubated for 5 days. Thus, when Δ*rhp51* cells are spotted onto 3.6 mM HU plates, microcolonies form. These microcolonies are not visible individually but collectively form a visible spot when viewed in a spot test assay.

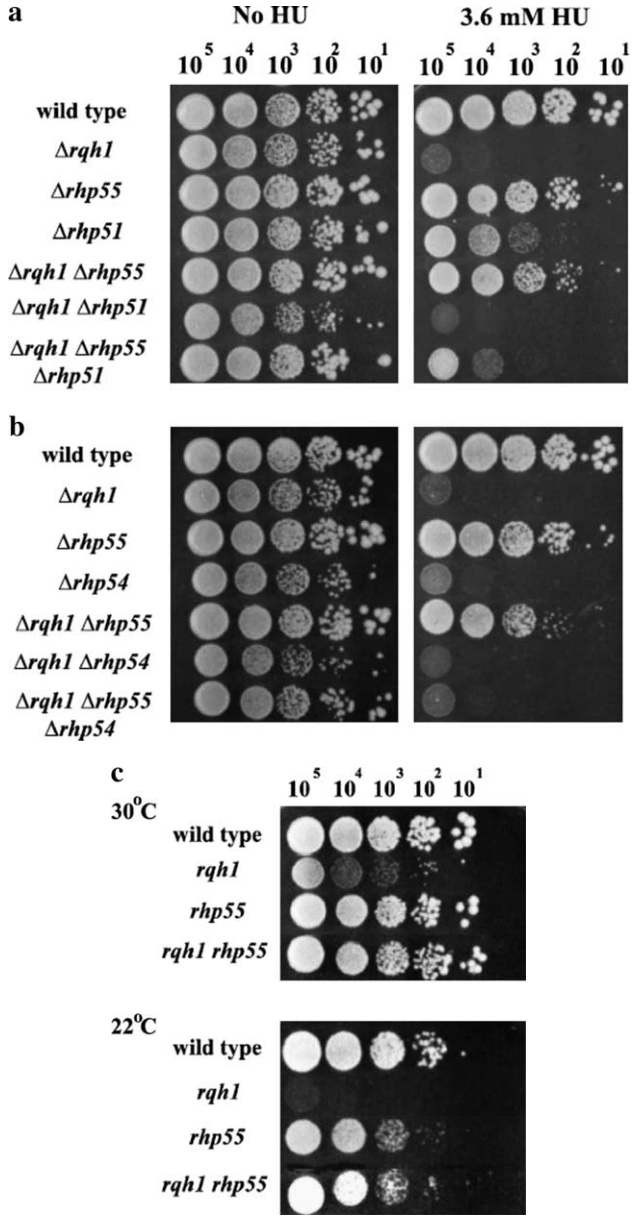


Figure 3.—Suppression of Δ*rqh1* HU sensitivity depends on the function of Δ*rhp51* and Δ*rhp54*. (a and b) Tenfold serial dilutions of each strain were spotted onto plates containing either 0 mM or 3.6 mM HU. Plates were incubated for 5 days at 30° and photographed. (c) At reduced temperatures Δ*rhp55* mutants show reduced resistance to HU but maintain their suppression of Δ*rhq1*. The plates contain 2.4 mM HU and were incubated at 30° for 5 days or 22° for 8 days.

The Rhp55/57 activity responsible for Δ*rqh1* sensitivity to HU treatment is independent of Rhp51 filament formation: In *S. cerevisiae* several studies have contributed to developing a profile of Rad55/57 functioning

in stimulating Rad51 filament formation. The evidence is threefold. First, while *rad55* and *rad57* are much less sensitive to IR damage at 30° compared to *rad51*, their sensitivities are much greater at lower temperatures (Lovett and Mortimer 1987; Johnson and Symington 1995). The argument for this phenomenon is that at lower temperatures the Rad51 filament is less stable and so depends more on rad55/57. Second, *in vitro* studies by P. Sung demonstrated that Rad51 filament formation on ssDNA is stimulated by the presence of Rad55/57 (Sung 1997). Finally, the IR sensitivity of *rad55/57* mutants was significantly reduced in strains overexpressing Rad51 or containing a Rad51 mutant with increased DNA binding capacity (Johnson and Symington 1995; Fortin and Symington 2002).

On the basis of these results, we sought to test whether the role of Rhp55/57 in nucleoprotein filament formation was separate from its role in suppressing the HU sensitivity in Δ*rqh1* cells. In *S. pombe,* *rhp55* and *rhp57* mutants also show cold-enhanced sensitivity; at 30° these mutants are much less sensitive to γ-ray damage than a Δ*rhp51* mutant, but at lower temperatures Δ*rhp55* and Δ*rhp57* mutants are as sensitive as a Δ*rhp51* mutant (Khasanov *et al.* 1999). We reasoned that if the Δ*rhp55/57* suppression of Δ*rqh1* sensitivity to replication arrest was lost at low temperatures it would be consistent with this suppression being associated with its role in Rhp51 nucleoprotein filament formation. If on the other hand we found that Δ*rhp55/57* suppression was maintained at lower temperatures this would support the conclusion that that suppression was due to loss of a function that is independent of filament formation. Figure 3c shows that when spotted onto plates containing 2.4 mM HU followed by incubation at 22°, the suppression of the HU sensitivity of Δ*rqh1* cells by Δ*rhp55* is maintained.

To further test for an Rhp55/57 activity independent of nucleoprotein filament formation, we cloned *rhp51*$^+$ into a series of thiamine-suppressible plasmids, pREP-3x, pREP-41x, and pREP-81x. These same plasmids were previously used to create Rhp51-overexpressing plasmids that were able to complement Δ*rhp51* in DNA damage assays (Kim *et al.* 2001). We confirmed that each plasmid was able to suppress the IR sensitivity of Δ*rhp51* (data not shown). We picked the plasmid that produced the lowest level of Rhp51 protein, pREP81x-Rhp51, for the remaining studies. Wild-type and Δ*rhp55* cells were transformed with pREP81X-Rhp51 and their sensitivity to IR was tested. Cells transformed with pREP81x-Rhp51 were incubated for 17 hr in the absence of thiamine to induce maximal Rhp51 expression. These cells were irradiated with varying doses of γ-rays, plated on YEA plus thiamine plates, and incubated at 30° for 5 days when colonies were counted. Control strains included Δ*rhp55* containing the vector alone and Δ*rhp55* with pREP81x-Rhp51 but grown with thiamine prior to irradiation. The results shown in Figure 4a demonstrate that overexpression of Rhp51 reduced the sensitivity of

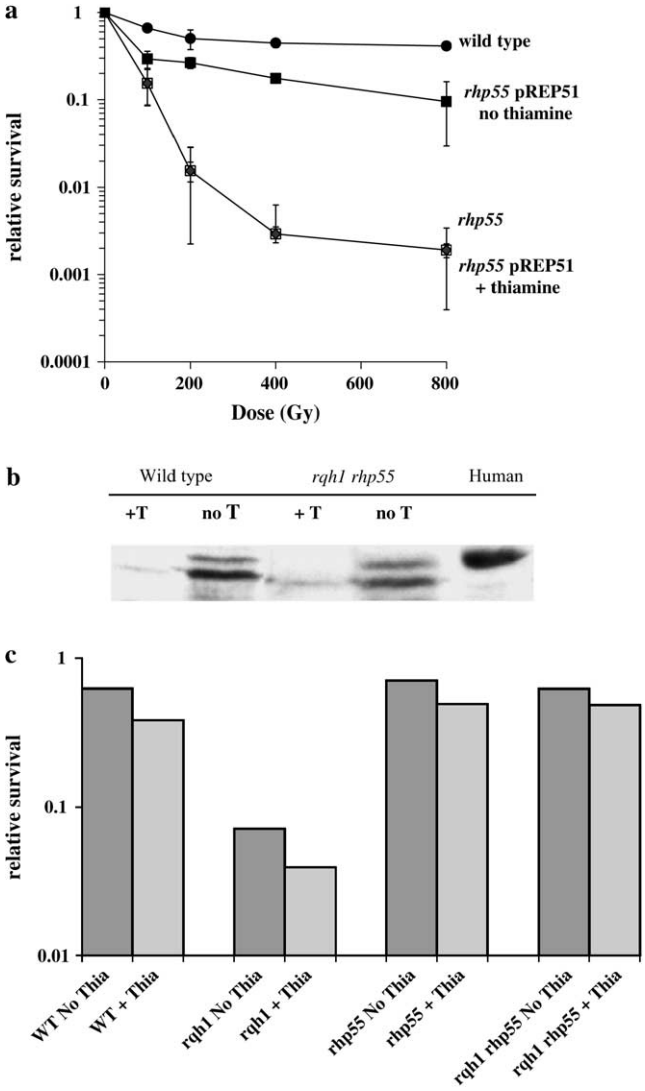


Figure 4.—Overexpression of Rhp51. Full-length *rhp51*$^+$ was cloned into the pREP81x vector (pREP 81x-Rhp51), transformed into various strains, and tested for HU sensitivity. (a) Wild-type and Δ*rhp55* cells containing pREP 81x-Rhp51 were grown to midlog in media either containing 8 mM thiamine or lacking thiamine. These cells were then irradiated with varying doses of γ-rays and subsequently plated onto YEA plates containing 8 mM thiamine. (b) Wild-type (WT), Δ*rhp55*, Δ*rqh1*, and Δ*rhp55* Δ*rqh1* strains were transformed with pREP 81x-Rhp51. Cells were grown to midlog in media either containing 8 mM thiamine or lacking thiamine. Then 15 mM HU was added to each culture and allowed to incubate for an additional 9 hr. Samples were then collected and plated onto YEA plates with 8 mM thiamine. The plates were incubated for 4 days and colonies were counted. (c) Extracts were prepared from wild-type and Δ*rhp55* Δ*rqh1* cells all containing pREP 81x-Rhp51 with cultures grown in the presence or absence of thiamine. A total of 150 μg of each extract was loaded onto a 12% PAGE SDS gel and the samples were separated by electrophoresis. As a control, 100 μg of nuclear extract prepared from HeLa cells was also loaded onto the gel. The gel was blotted and Rhp51 was detected using an antibody against human Rad51, which cross-reacts with *S. pombe Rhp51.* Antibody binding was detected by chemiluminescence.

Δ*rhp55* cells to near wild-type levels. These findings are consistent with Rhp55/57 playing an early role in nucleoprotein filament formation and, as seen in *S. cerevisiae*, overexpression of Rhp51 largely circumvents this need. This provides a mechanism of potentially separating the role of Rhp55/57 in nucleoprotein filament formation from other functions.

Δ*rqh1* and Δ*rqh1* Δ*rhp55* strains were transformed with pREP81x-Rhp51. We then tested whether inducing Rhp51 expression would influence the sensitivity of these strains to HU treatment. Cells were incubated for 17 hr in the presence or absence of thiamine. HU was then added to the cultures at a concentration of 15 mM. The cultures were incubated for 0, 3, 6, or 9 hr in HU before washing and plating onto YES plates. Plates were incubated for 5 days and colonies were counted. As seen in Figure 4b, the overexpression of Rhp51 did not reduce the Δ*rhp55* suppression of the HU sensitivity of Δ*rqh1* cells, arguing that the Rhp55/57 function responsible for this sensitivity is independent of nucleoprotein formation. For completion we confirmed that Rhp51 was overexpressed in these cells. Whole-cell extracts were prepared from wild type and Δ*rqh1* Δ*rhp55* cells grown in the presence or absence of thiamine for 17 hr. Western blot analysis (Figure 4c) shows that Rhp51 levels are significantly elevated in strains grown in the absence of thiamine.

The HU sensitivity of Δ*rqh1* cells can also be suppressed by Δ*swi5* and suppression by Δ*rhp55* is partially dependent on *swi5*$^+$: It has recently been reported that *S. pombe* has an Rhp55/57-independent recombination repair pathway that requires Rhp51 (Akamatsu *et al.* 2003). This pathway is defined by swi5$^+$, a gene originally identified in a screen for mating-type switching mutants (Egel *et al.* 1984). We considered the possibility that *swi5*$^+$ was required for the improved resistance of Δ*rqh1* Δ*rhp55* mutants. We first created a Δ*swi5* Δ*rhp55* double mutant that we tested for HU sensitivity. We found that while Δ*swi5* showed wild-type levels of resistance to HU the Δ*swi5* Δ*rhp55* double mutant was more sensitive than the Δ*rhp55* single mutant (Figure 5a). We found that the double mutant was not as sensitive to HU as a Δ*rhp51* mutant. This differs from the results reported for IR sensitivity of the double mutant, which was shown to be comparable to that of a Δ*rhp51* mutant, as we also found to be the case (Akamatsu *et al.* 2003; data not shown). We next examined the effect of Δ*swi5* on the HU sensitivity of Δ*rqh1*. We found that loss of *swi5*$^+$ partially suppressed the HU sensitivity of Δ*rqh1* cells although not back to the level of a Δ*swi5* single mutant (Figure 5b). Next we created a Δ*rqh1* Δ*rhp55* Δ*swi5* triple mutant and compared its HU sensitivity to that of the Δ*rqh1* Δ*rhp55* strain. The addition of the Δ*swi5* mutation to Δ*rqh1* Δ*rhp55* increased the HU sensitivity to an intermediate level between a Δ*rqh1* and a Δ*rqh1* Δ*rhp55* (Figure 5c). These data demonstrate that part of the suppression by Δ*rhp55* depends on a Swi5 function. However, the suppression of the HU sensitivity

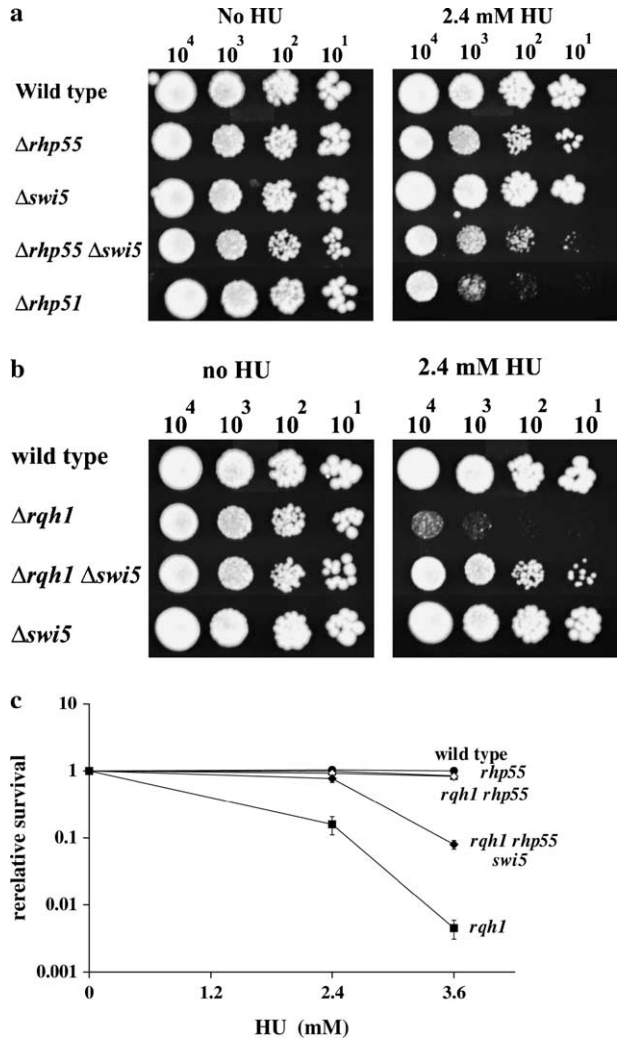


Figure 5.—Suppression of Δ*rqh1* HU sensitivity by Δ*rhp55* is partially dependent on Swi5. We investigated the possibility that Swi5 was necessary for the suppression of the HU sensitivity of Δ*rqh1* by Δ*rhp55*. (a) Serial dilutions of midlog cultures of wild-type, Δ*rhp55*, Δ*swi5*, Δ*rhp51*, and Δ*rhp55* Δ*swi5* cells were plated onto YEA or YEA containing 2.4 mM HU followed by incubation for 5 days. (b) Serial dilutions of midlog cultures of wild-type, Δ*rqh1*, Δ*swi5*, and Δ*rqh1* Δ*swi5* cells were plated onto YEA or YEA containing 2.4 mM HU followed by incubation for 5 days. (c) Midlog cultures of wild type, Δ*rhp55*, Δ*swi5*, Δ*rqh1*, Δ*rqh1* Δ*rhp55*, and Δ*rqh1* Δ*rhp55* Δ*swi5* were plated onto YEA plates containing varying concentrations of HU and incubated for 4–6 days before colonies were counted.

of Δ*rqh1* cells by Δ*swi5* shows that the situation is more complicated than Swi5 simply acting in an alternative pathway in the absence of Rhp55/57.

Rqh1 and HR share a common response to IR-induced DSBs: We also analyzed the sensitivity of our mutants to IR. IR creates DSBs that must be repaired by HR or NHEJ. The observation that Δ*rqh1* cells are sensitive to IR indicates that Rqh1 functions in the repair of DSBs (Figure 6a). Δ*rhp51* cells were the most γ-ray sensitive of the HR mutants tested (Figure 6b). The Δ*rqh1* Δ*rhp51* double mutant has sensitivity identical to that of the Δ*rhp51*

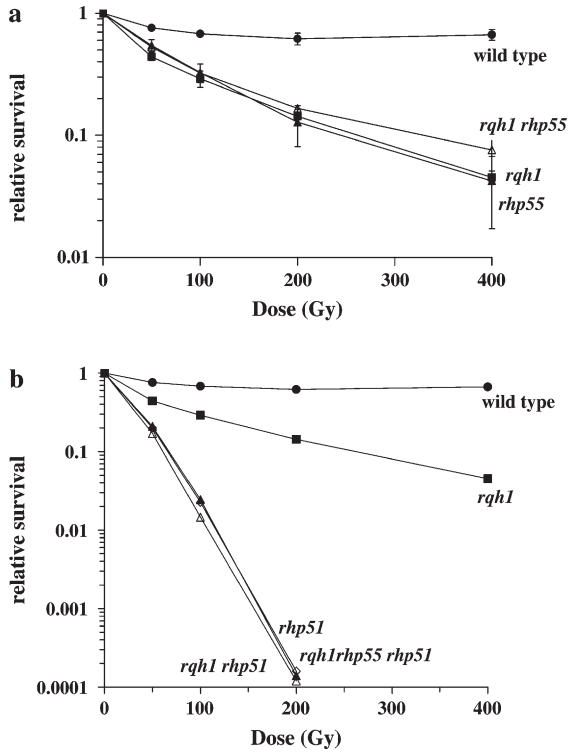


FIGURE 6.—The IR sensitivities of double mutants between Δ*rqh1* and HR genes show survival patterns similar to their HU sensitivities. The IR sensitivities of Δ*rhp55*, Δ*rhp51*, Δ*rqh1*, Δ*rqh1* Δ*rhp55*, and Δ*rqh1* Δ*rhp51* were tested. Cells from midlog cultures were plated onto YEA plates and irradiated with the indicated dose. Colonies were counted after 5 days. ●, wild type; ■, Δ*rqh1*. (a) ▲, Δ*rhp55*; △, Δ*rqh1* Δ*rhp55*. (b) ▲, Δ*rhp51*; △, Δ*rqh1* Δ*rhp51*; ◇, Δ*rqh1* Δ*rhp55* Δ*rhp51*.

single mutant, showing that these proteins are in the same epistasis group for repair of DSBs (Figure 6b; also see CASPARI *et al.* 2002). The Δ*rhp55* single mutant showed sensitivity to IR that was very similar to that of Δ*rqh1* (Figure 6a). Moreover, the Δ*rqh1* Δ*rhp55* double mutant was indistinguishable from either single mutant with regard to its IR sensitivity (Figure 6b). We also examined the γ-ray sensitivity of the Δ*rqh1* Δ*rhp55* Δ*rhp51* triple mutant to determine if the strong IR sensitivity of the Δ*rqh1* Δ*rhp51* mutant would be suppressed. Δ*rhp55* did not improve the IR resistance of a Δ*rqh1* Δ*rhp51* double mutant (Figure 6b). Comparable results were seen for Δ*rhp54* (data not shown). We did not see the mild improvement in survival of this triple mutant over the double mutant that we found with HU treatment (Figure 3a), showing that no Rhp51-independent repair of these DSBs takes place.

DISCUSSION

While Rqh1 plays an important role in recovery from replication arrest, it is unclear how it participates in this process. Several lines of evidence have suggested that it carries out its function with HR (MURRAY *et al.* 1997; STEWART *et al.* 1997; DAVEY *et al.* 1998). One possible role for Rqh1 helicase is to act at a late step in HR by unwinding the heteroduplex formed by strand invasion, although it has also been proposed to function in an earlier step of HR (CASPARI *et al.* 2002). In these studies we sought to investigate the role of Rqh1 in recovery from replication arrest.

Rhp51, Rhp54, and Rqh1 are critical in recovery from replication arrest: HU treatment leads to an S-phase arrest as replication is inhibited. Wild-type cells eventually recover from this arrest without loss of viability or obvious accumulation of mutations despite a dramatic increase in HR rates. Δ*rqh1* mutants show low survival and high rates of chromosomal loss following HU treatment (STEWART *et al.* 1997). In addition, Δ*rhp51* and Δ*rhp54* mutants are also quite sensitive to HU, demonstrating that HR plays a vital role in recovery from replication arrest. The need for HR in recovery from HU treatment can be explained in two ways: replication arrest ultimately leads to formation of DSBs, which would require HR or NHEJ for repair, or HR acts on a DNA structure other than a DSB, possibly protecting stalled forks from collapse and promoting replication restart. Support for the former explanation comes from data showing that replication arrest leads to formation of DSBs (MICHEL *et al.* 1997; LUNDIN *et al.* 2002), although this issue is far from resolved.

The relatively severe sensitivity of Δ*rqh1* cells to HU demonstrates that Rqh1 also plays an important role in replication arrest recovery. Δ*rhp55* and Δ*rhp57* mutants show mild sensitivity and would appear to play a minor role in this process. The interpretation of the role of Rhp55/57 is complicated by the reported backup role of Swi5 in repair of DSBs (see below) (AKAMATSU *et al.* 2003).

Loss of *rhp55*$^+$ or *rhp57*$^+$ suppresses the HU sensitivity of Δrqh1 cells: We found that double mutants between Δ*rqh1* and various HR genes showed very different sensitivities to HU. While Δ*rqh1* Δ*rhp51* and Δ*rqh1* Δ*rhp54* double mutants were more sensitive to HU than the single mutants, we found that the additional loss of either *rhp55*$^+$ or *rhp57*$^+$ suppressed the HU as well as the UV sensitivity of Δ*rqh1* cells. A recent article by DOE and WHITBY (2004) also reported that loss of *rhp55*$^+$ suppressed the HU and UV as well as MMS sensitivity of Δ*rqh1* mutants, although they did not describe studies beyond this point. They also stated that loss of *rhp51*$^+$ had a similar effect, which would be in conflict with our data. However, no data were shown for this statement, making it difficult to evaluate this claim. The suppression of the HU and UV sensitivity of Δ*rqh1* cells by Δ*rhp55* or Δ*rhp57* implies that, in response to these agents, Rhp55/57 functions in a process that is deleterious to cells lacking Rqh1. But what is this process? Previous studies in *S. cerevisiae* have characterized Rad55/57 as a mediator, aiding in the early step of HR by helping

to stabilize Rad51 loading onto single-stranded DNA (Symington 2002). Our results show that suppression of Δ*rqh1* by Δ*rhp55* largely depends on the presence of Rhp51 and Rhp54 (Figure 3, a and b). On the basis of the early roles of Rhp51 and Rhp54 in HR, these results suggest that this Rhp55/57 activity acts downstream of joint molecule formation.

To investigate this further we carried out two studies aimed at determining whether we could separate the role of Rhp55/57 in nucleoprotein filament formation from a late function in HR. First, *S. cerevisiae rad55/57* mutants are less sensitive to IR at 30° than are other members of the *RAD52* epistasis group. However, at lower temperatures (20°), they become more sensitive, approaching the level of *rad51* mutants (Lovett and Mortimer 1987; Johnson and Symington 1995). The same phenotype has been reported for Δ*rhp55/57* mutants in *S. pombe* (Khasanov *et al.* 1999). The explanation for this cold-enhanced sensitivity is that Rad55/57-independent Rad51 nucleation on DNA is inhibited at lower temperatures, increasing the requirement for Rad55/57 mediator function. We found that Δ*rhp55* is also more sensitive to HU at 22° than at 30°. However, even at 22° Δ*rhp55* suppressed the HU sensitivity of Δ*rqh1* (Figure 3c).

Second, in a separate experiment we overexpressed Rhp51 and showed that it suppressed the IR sensitivity of Δ*rhp55* mutants. This same result has been described in *S. cerevisiae* and is interpreted as further evidence of the role of Rad55/57 in helping to establish the Rhp51 nucleoprotein filament. The explanation is that having more Rhp51 on hand alters the kinetics of nucleoprotein filament formation, largely eliminating the need for Rhp55/57 in this process. We next tested whether overexpression of Rhp51 would alter the suppressor effect of Δ*rhp55* on the HU sensitivity of Δ*rqh1* cells. We reasoned that if the increase in resistance involved the role of Rhp55 in the nucleoprotein filament formation, then overexpressing Rhp51 should make a Δ*rqh1* Δ*rhp55* double mutant sensitive to HU. Overexpression of Rhp51 had no effect on the ability of Δ*rhp55* to suppress the HU sensitivity of Δ*rqh1* mutants. Together these results suggest that Rhp55/57 has a function that is independent of its role in Rhp51 nucleoprotein filament formation.

This raises the question of what is this second Rhp55/57 function. One clue may come from studies in human cells where Rad51 paralogs have been implicated in playing a late function in HR (Brenneman *et al.* 2002; Yokoyama *et al.* 2003; Liu *et al.* 2004). In one study it was shown that the human Rad51 paralog Rad51B binds to HJs (Yokoyama *et al.* 2003). Using cell free extracts, Liu *et al.* (2004) provided data suggesting that Rad51C and XRCC3 play a role in HJ resolution. And finally, Brenneman *et al.* (2002) carried out studies on XRCC3 and suggested that Rad51 paralogs were likely acting to stabilize the heteroduplex following strand invasion. Our data provide genetic evidence that Rhp55/57 is acting late in HR, likely downstream of joint molecule formation. If Rhp55/57 were acting to stabilize HJs, as suggested in human studies, then loss of $rhp55^+$ or $rhp57^+$ should destabilized HJs. How could this suppress the loss of Rqh1 activity? It is well established that RecQ helicases function to suppress recombination and crossovers. This could be accomplished either by blocking the initiation of HR or by eliminating recombination intermediates via a mechanism that yields noncrossover products (Ira *et al.* 2003; Wu and Hickson 2003). If it were the latter, then the likely role for RecQ helicases would be to resolve the joint molecule formed during HR. If this were the case then in the absence of RecQ, heteroduplex DNA would persist. If eliminating Rhp55/57 destabilized the HJ, this could lead to branch migration, leading to resolution of the joint molecule, minimizing the need for Rqh1. We recognize that any model proposed for RecQ helicases needs to include a role for Top3. We imagine that Top3 strand passage activity could act to allow the displaced strand to reform the original duplex DNA, although admittedly we have no direct evidence to support this. A study in *S. cerevisiae* proposed a model in which Sgs1 and Top3 function late in HR, but to resolve double HJ (Ira *et al.* 2003). An *in vitro* study using human Blm and Topo IIIα showed that these proteins could resolve a synthetic double HJ (Wu and Hickson 2003). Needless to say, the actual roles of RecQ and Top3 in HR remain uncertain.

Further evidence that Swi5 functions in a process similar to Rhp55/57: A recent article described results suggesting that Swi5 functions in a process parallel to Rhp55/57 that depends on Rhp51 (Akamatsu *et al.* 2003). On their own, Δ*swi5* mutants show little sensitivity to DNA damage, including IR, UV, or MMS treatment. However, when combined with Δ*rhp55*, the double mutant reaches a level of sensitivity to DNA damage that is comparable to the more sensitive Δ*rhp51* mutant. These data have been interpreted as showing that Swi5 acts as an alternative to Rhp55/57 (Akamatsu *et al.* 2003). We wanted to test whether Δ*rqh1* Δ*rhp55* mutants depended on Swi5 for recovery from HU. For this we created a Δ*rqh1* Δ*rhp55* Δ*swi5* triple mutant and compared its HU sensitivity to Δ*rqh1* Δ*rhp55*. As we suspected, the triple mutant showed increased sensitivity to replication arrest over the Δ*rqh1* Δ*rhp55* double mutant. The sensitivity is intermediate between Δ*rqh1* and Δ*rqh1* Δ*rhp55* mutants, implying that some of the recovery from arrest is dependent on a function of Swi5 in the absence of $rhp55^+$. However, the story is not simply that Swi5 acts in a parallel pathway in the absence of Rhp55/57. We also found that in an $rhp55^+$ background Δ*swi5* suppressed the HU sensitivity of Δ*rqh1*. This shows that Swi5 is functioning even in the presence of Rhp55/57. Further, we found that the Δ*rhp55* Δ*swi5* double mutant does not become nearly as sensitive to HU as a Δ*rhp51* mutant. These results suggest a slightly more complex func-

tion for Swi5 than simply acting in a parallel pathway to Rhp55/57 during recovery from replication arrest. The suppression of the HU sensitivity of Δ*rqh1* mutants by loss of *swi5*$^+$ is qualitatively different from the suppression by loss of *rhp55*$^+$ (compare the colony morphologies of Figures 3a and 5b and the level of suppression). This and the intermediate phenotype of the triple mutant imply that the mechanisms of suppression are different. Further experiments are necessary to better understand the roles that Rhp55 and Swi5 play in recovery from replication arrest.

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