Characterization of the interaction between the *Saccharomyces cerevisiae* Rad51 recombinase and the DNA translocase Rdh54*

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*Running title: Functional interaction between Rdh54 and Rad51

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Background: Rdh54 is a motor protein able to dissociate Rad51 recombinase from chromatin.

Results: The Rad51 binding domain of Rdh54 resides within its N terminus and is required for Rad51 removal from DNA.

Conclusions: Rdh54-Rad51 interaction is important for Rad51 dissociation from chromatin but not for DNA repair.

Significance: Rdh54 inhibits accumulation of toxic Rad51–DNA complexes.

SUMMARY

The *Saccharomyces cerevisiae* Rdh54 protein is a member of the Swi2/Snf2 family of DNA translocases required for meiotic and mitotic recombination and DNA repair. Rdh54 interacts with the general recombines Rad51 and Dmc1, and promotes D-loop formation with either recombinase. Rdh54 also mediates the removal of Rad51 from undamaged chromatin in mitotic cells, which prevents formation of nonrecombinogenic complexes that can otherwise become toxic for cell growth. In order to determine which of the mitotic roles of Rdh54 are dependent on Rad51 complex formation, we finely mapped the Rad51 interaction domain in Rdh54, generated N-terminal truncation variants, and characterized their attributes biochemically and in cells. Here we provide evidence suggesting that the N-terminal region of Rdh54 is not necessary for the response to the DNA damaging agent methyl methanesulfonate (MMS). However, truncation variants missing 75 to 200 residues at the N terminus are sensitive to Rad51 overexpression. Interestingly, a hybrid protein containing the N-terminal region of Rad54, responsible for Rad51 interaction, fused to the Swi2/Snf2 core of Rdh54 is able to effectively complement the sensitivity to both MMS and excess Rad51 in *rdh54* null cells. Altogether, these results reveal a distinction between damage sensitivity and Rad51 removal as regards to Rdh54 interaction with Rad51.

Homologous recombination (HR) mediated by the recombinase Rad51 and its meiotic homolog Dmc1 (1) is essential for genome stability and meiotic chromosome segregation. Double-stranded DNA breaks (DSBs) (2,3) can occur spontaneously from stalled and reversed replication forks, from trapped topoisomerase complexes, and from replication transcription collisions. Exogenous DSBs arise from exposure to high energy radiation and chemicals, and programmed breaks also occur, especially during meiosis. Accordingly, HR defects have been correlated with human cancer-prone diseases as well as various forms of cancer (4).

During DSB repair by HR, Rad51 first binds to single-stranded DNA (ssDNA) derived from the
processing of a primary lesion to form a nucleoprotein filament. Once assembled, the filament is capable of conducting a search for a homologous target in dsDNA and catalyzing invasion of the target to form a DNA joint called the displacement loop (D-loop). Subsequent steps entail extension of the 3' end of the invading strand by DNA synthesis followed by resolution of DNA intermediates. However, Rad51 by itself is inefficient in D-loop formation and requires the activity of other proteins to facilitate this process. Rad54 and Rdh54, members of the Swi2/Snf2 family of DNA translocases, have been shown to enhance the efficiency of Rad51-mediated D-loop formation. Importantly, these translocases are also capable of displacing Rad51 from dsDNA, an attribute that is likely important for clearing Rad51 from the nascent D-loop to facilitate repair DNA synthesis and also for preventing the non-specific accumulation of Rad51 on bulk chromatin (5,6). Moreover, in vitro, both Rad54 and Rdh54 are able to remodel chromatin via nucleosome mobilization in an ATP hydrolysis-dependent manner (7,8).

Despite their biochemical similarities, there are important differences as to the biological functions of Rad54 versus Rdh54. Whereas Rad54 has been proposed to participate in mitotic DSB repair and intrachromosomal recombination, Rdh54 seems to work primarily in interhomologue recombination in both mitotic and meiotic cells. Cytological studies have shown that Rdh54 is normally found at kinetochores and exposure to DNA damage promotes its recruitment to repair foci in a Rad51 and Rad52-dependent manner (9). Since Rdh54 has a role in resuming cell growth to DSB-induced checkpoint arrest (i.e., adaptation), it has been suggested that Rdh54 facilitates communication between DNA repair and checkpoint control (10).

Yeast two-hybrid and biochemical analyses have shown that both Rad54 and Rdh54 physically and functionally interact with the Rad51 recombinase. Rdh54 has also been found to interact with the meiosis-specific recombinase Dmc1 (1). Although its role in meiosis is not fully understood, Rdh54 is thought to promote colocalization of Rad51 and Dmc1 and to have a role in the dissolution of sister chromatid cohesion to facilitate appropriate interhomologue recombination and chromosome segregation (11-13). Importantly, the translocase function of Rdh54 acts to promote dissociation of Dmc1 from nonrecombinogenic sites on chromatin to allow Dmc1 to work in meiotic recombination (14). Recent studies suggest that Rdh54 has a similar role to prevent sequestration of Rad51 at non-DSB sites in mitotic cells. Indeed, biochemical and cytological analyses have shown that Rdh54 removes Rad51 from dsDNA and chromatin, to promote the intracellular recycling of Rad51 and freeing the primer end in the nascent D-loop structure to facilitate DNA synthesis initiation (15,16). Further in vitro studies have shown that the Rad51-binding domain of Rdh54 resides within its N terminus and suggested that complex formation is required for promoting efficient D-loop reaction, for its chromatin remodeling activity, and for Rad51 removal from dsDNA (8,15).

To better understand the biological significance of the interaction between Rdh54 and Rad51, we have generated yeast mutants expressing N-terminal truncations of Rdh54. By genetic analysis, we provide evidence that the N-terminal region of Rdh54 is required for overcoming the toxic effect of Rad51 overexpression but dispensable for the repair of DNA damaged by MMS. Furthermore, we demonstrate that the N-terminal domain of Rdh54 is necessary for Rad51 binding in vitro and required for the removal of Rad51 from dsDNA. Interestingly, a chimeric protein harboring the Rad54 N-terminal region that interacts with Rad51 and the Swi2/Snf2 core domain of Rdh54 is fully active in vitro and in cells. The functional significance of these results is discussed.

**EXPERIMENTAL PROCEDURES**

*Yeast strains –* All the yeast strains used in the genetic experiments are isogenic to the W303 RAD5 background (ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1).

*Plasmid constructs –* DNA plasmids for bacterial expression and protein purification were generated by PCR amplification and cloning of wild type RDH54, rdh54 truncation alleles, and RAD54-RDH54 hybrid sequence into the NeoI and Xhol sites of the pET32a vector (Novagen) to add thioredoxin and His6 tags to the N terminus of each protein. The DNA templates for these PCR reactions were plasmid pHK489 (full-length
RDH54 with its endogenous promoter into vector pRS314) and plasmid pHK471 (N-terminal 600bp of RAD54 fused to C-terminal RDH54 with an NdeI linker in between and the endogenous RDH54 promoter into vector pRS414). It should be noted that the RDH54 coding sequence has two apparent ATG start sites, 34 amino acid residues apart. Although the first ATG site was initially thought to be the in vivo start site, it has become apparent that the downstream ATG is used in vivo. Thus, old nomenclature should be corrected by -34 aa residues. Here we use the downstream ATG as residue 1. This becomes important for comparison and discussion of the N-terminal region and its conservation with the hRad54B sequence.

The forward PCR oligonucleotides used to generate the constructs were as follow: 5'-CTATAGGGGAGAGCCACCATGGCACAAGATA CCQAATATAGAG-3' for full-length wild type Rdh54; 5'-CATGCCATGGCAAAACCAACCGCCACTGC AGTCACA-3' for the rdh54Δ25 truncation mutant; 5'-CATGCCATGGCAAAAGTTACTGTCAACTTG AAAAGA-3' for the rdh54Δ40 truncation mutant; 5'-CATGCCATGGCACAAAGTTACTGTCAACTTGA AATCTT-3' for the rdh54Δ50 truncation mutant; 5'-CATGCCATGGCAATCATGTACAGGAAGCC TACTACC-3' for the rdh54Δ75 truncation mutant; 5'-CATGCCATGGCAATCATGTACAGGAAGCC TACTACC-3' for the rdh54Δ75 truncation mutant; 5'-CATGCCATGGCATTCAAACTGAACCAAAGAAAAGA-3' for the rdh54Δ75 truncation mutant; and 5'-CATGCCATGGCAAAAGTTACTGTCAACTTGA AATCTT-3' for the rad54-Rdh54 hybrid. The reverse PCR primer used for all the expression constructs was: 5'-GATCCGCTCGAGTTATCATTGTTCTCTGAGACATAT CTC-3'. The PCR products (RDH54 promoter and ORF sequences) were then ligated and cloned into pRS314 as XhoI-NcoI-SacII. To confirm that the internal NcoI site did not affect in vivo function, we generated wild type RDH54 and RAD54 constructs containing an NcoI site in between amino acid residues 200 and 201. These constructs complemented activity efficiently and produced protein to the same levels than wild type (data not shown). All the plasmid constructs described here were sequenced to verify that no unwanted mutations were introduced during cloning.

Complementation of DNA damage sensitivity – Cells carrying RDH54 pRS314-based constructs (CEN TRP1) were grown in SC-TRP liquid medium at 30°C. Cells were then counted and 10-fold serial dilutions prepared in sterile water. Serial dilutions were plated onto SC-TRP solid medium containing different doses of the alkylating agent methyl methanesulfonate (MMS) or the DNA crosslinking agent cisplatin. Additionally, SC-TRP plates were irradiated with ultraviolet (UV) light or gamma ionizing radiation (IR) using a Gammacell-220 60Co irradiator. Pictures were taken after 3 days of incubation at 30°C.
Rad51 overexpression assay – To determine the sensitivities of yeast cells to Rad51 or rad51 K191A overexpression, strains were freshly transformed with pYES-GAL-RAD51, pYES-GAL-rad51 K191A, or the empty vector pYES2 (2µ URA3) together with the RDH54 pRS314-based constructs. Cells were then grown into liquid SC-URA-TRP (glucose) overnight. The cells were then serially diluted and each dilution spotted onto fresh solid plates SC-URA-TRP (glucose) and SC-URA-TRP (galactose). Cells were grown at 30°C and pictures taken after 3 days.

Expression and purification of N-terminal truncation variants of Rdh54 – Rdh54, Rdh54-Rdh54 hybrid, and rdh54 Δ50 and Δ75 were expressed and purified from E. coli Rosetta cells (Novagen) as described previously (15). The wild type Rdh54 protein in this study has the 34 additional aa residues that originated from 102 nucleotides upstream of the RDH54 gene in the Saccharomyces genome database (SGD). The extra residues do not affect the biochemical activities of Rdh54 presented in this paper (15).

ATPase assay - Rdh54 and its variants (75 nM) were incubated with 1 mM ATP and 0.5 µCi of [γ-32P] ATP at 30°C in 10 µl buffer A (35 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl2, and 100 mM KCl) containing 100 ng/µl bovine serum albumin and pBluescript II SK DNA (23 µM base pairs, linearized with HindIII). Aliquots (2 µl) of the reactions were withdrawn at the indicated times and mixed with 2 µl 0.5M EDTA to halt the reaction. The level of ATP hydrolysis was determined by thin layer chromatography and phosphorimaging analysis (5).

In vitro pulldown of Rad51 – His6-Rdh54 and its N-terminal variants (4 µg) were incubated with Rad51 (4 µg) in 20 µl of buffer B (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT) containing 10 mM Imidazole for 30 min on ice. 10 µl Ni-NTA agarose beads (Qiagen) were added to the mixture and incubated for 1 hr at 4°C. Then, the beads were separated from the supernatant by centrifugation. After washing three times with 40 µl of buffer B containing 10 mM Imidazole, the beads were treated with 30 µl of 2% SDS to elute bound proteins. The supernatant, wash, and eluate (8 µl each) were analyzed by SDS-PAGE and Coomassie blue staining.

Rad51 removal from dsDNA – Rad51 (2.3 µM) was incubated with magnetic beads containing 600-bp biotinylated dsDNA (100 µM base pairs) (15) in 18 µl of buffer A containing 2 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 15 µg/ml creatine kinase) for 10 min at 30°C. Next, the indicated amounts of Rdh54 and its variants were added to the mixture with a 3-min incubation at 30°C, followed by addition of ΦX174 ssDNA (150 µM nucleotides) and 10-min incubation to trap dissociated Rad51. The beads and supernatant were separated using the Magnetic Particle Separator (Boehringer Mannheim). Bound proteins were eluted from the beads with 20 µl of 2% SDS. The supernatant and SDS eluate (8 µl each) were analyzed by SDS-PAGE and Coomassie blue staining.

RESULTS

Generation of rdh54 N-terminal truncation mutant constructs– Biochemical studies have shown that Rdh54 interacts with the Rad51 recombinase and that the binding domain resides within the N terminus (15) (Figure 1). To obtain genetic evidence that complex formation is required for the roles of Rdh54 in vivo, we cloned N-terminal truncation mutants of RDH54 into a CEN TRP1 vector such that protein expression is driven by the native RDH54 promoter. These mutant alleles encode truncation variants missing from 25 up to 200 amino acid residues (Figure 1).

In vitro pulldown of Rad51 – His6-Rdh54 and its N-terminal variants (4 µg) were incubated with Rad51 (4 µg) in 20 µl of buffer B (25 mM Tris-HCl, pH 7.5, 150 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT) containing 10 mM Imidazole for 30 min on ice. 10 µl Ni-NTA agarose beads (Qiagen) were added to the mixture and incubated for 1 hr at 4°C. Then, the beads were separated from the supernatant by centrifugation. After washing three times with 40 µl of buffer B containing 10 mM Imidazole, the beads were treated with 30 µl of 2% SDS to elute bound proteins. The supernatant, wash, and eluate (8 µl each) were subjected to SDS-PAGE and Coomassie blue staining.
cisplatin but saw no sensitivity to any of these agents (Figure 2A). We also replaced the chromosomal RDH54 locus with mutant alleles containing these truncations and obtained the same results with regards to MMS sensitivity (data not shown). Interestingly, none of these truncation mutants was able to interact with Rad51 by yeast two-hybrid assay (data not shown) and, consistent with this observation, in vitro pulldown experiments revealed that some of the truncated rdh54 proteins were defective for interaction with Rad51 (Figure 5C). Moreover, these truncated rdh54 proteins do not enhance the damage sensitivity of a rad54Δ strain (Figure 2B).

The N-terminal domain of Rdh54 is required for resistance to Rad51 overexpression – Overexpression of Rad51 is detrimental to cell growth in the absence of Rdh54, as Rad51 can associate with undamaged chromatin and form cytotoxic complexes (16) (Figure 3A). In addition, biochemical studies have shown that an rdh54 protein variant missing the first 99 amino acid residues has a reduced efficiency for Rad51 removal from dsDNA (15). In order to determine whether Rdh54-Rad51 complex formation is necessary for removal of excess Rad51 bound to chromatin in vivo, we introduced the plasmids that encode the rdh54 truncation mutants into rdh54 null cells together with a GAL1-RAD51 plasmid for Rad51 overexpression. We also overexpressed the ATPase-defective rad51 mutant, rad51 K191A, which possesses reduced DNA binding affinity and thus is unable to bind chromatin effectively and less able to induce growth defects. In our experimental setting, overexpression of Rad51 and rad51 K191A only occurs in medium containing galactose. As shown in Figure 3B, functional interaction between Rdh54 and Rad51 is necessary for the response to Rad51 overexpression. The first 50 amino acid residues of Rdh54 do not seem to be required for this activity, as cells carrying mutant alleles encoding truncation variants lacking 50 or fewer residues do not show cell growth defects upon Rad51 overexpression. On the other hand, rdh54 mutant alleles encoding truncations ranging from 75 to 200 residues of Rdh54 show the same growth defect as the null mutant (Figure 3B). Sensitivity to Rad51 overexpression correlates with a defect in interaction with Rad51 (Figure 5C).

The N-terminal domain of Rad54 confers resistance in rdh54 mutant cells upon Rad51 overexpression – In order to better understand the role of the Rad51 binding domain of Rdh54 in the response to Rad51 overexpression, we generated a hybrid construct by replacing the first 200 amino acid residues of Rdh54 with the N-terminal domain of Rad54 (Figure 1B). Previous studies have shown that similar to Rdh54, the N-terminal domain of Rad54 is responsible for the interaction with Rad51 (17,18). The Rad54-Rdh54 hybrid protein is functional, as a CEN TRP1 construct that expresses it is able to complement the MMS sensitivity of rdh54 null cells (Figure 4A). Increased copy of RAD54 (CEN RAD54) did not alter rdh54Δ resistance to MMS. Importantly, the hybrid construct is also able to complement the growth defect observed in the rdh54 truncation mutants after Rad51 overexpression in a manner similar to wild type RDH54 (Figure 4B). We have obtained similar results by replacing the chromosomal RDH54 gene with the hybrid gene (data not shown).

In vitro properties of truncated and chimeric Rdh54 proteins – We decided to further study the truncation variants missing 50 or 75 amino acid residues from the N terminus of Rdh54, as these two mutants showed a difference in the ability to counteract high Rad51 levels. We also examined the Rad54-Rdh54 chimeric protein as it behaves as functional Rdh54 in Rad51 overexpression studies. The three variant proteins were expressed in an N-terminally thioredoxin-His6 tagged form in E. coli, and purified to near homogeneity (Figure 5A) using the multi-step purification procedure that has been previously described (15).

As all three variants possess the intact seven Swi2/Snf2 motifs and ATPase core domain of Rdh54 (Figure 1), we expected them to have a wild type level of ATPase activity. In congruence with this premise, the three rdh54 variants exhibited no significant difference in ATP hydrolysis activity compared to wild type protein (Figure 5B). Next, the capacity of the variant proteins to interact with Rad51 was examined by in vitro affinity pulldown. The His6-tagged rdh54 variants were first incubated with Rad51 and then with Ni-NTA agarose beads. Examination of the Rad51 content eluted from the Ni-NTA resin (Figure 5C) showed that deletion of either 50 or 75 residues reduces Rad51 interaction, with the latter
mutant being more strongly impaired in this regard, while Rad54-Rdh54 interacts with Rad51 as avidly as full-length Rdh54.

Previously, we have shown that Rdh54 is able to disrupt Rad51-dsDNA nucleoprotein filaments. ATP hydrolysis by Rdh54 and a strong interaction with Rad51 are required for maximal reaction efficiency (15). Here, we asked whether variations in the N-terminal domain of Rdh54 lead to changes in the robustness of Rad51 removal. We conducted the Rad51 removal assay as described before (15). Briefly, Rad51-dsDNA filaments were assembled on biotin-dsDNA-streptavidin magnetic beads, followed by incubation with Rdh54 or one of its variants, and addition of excess of ssDNA that can sequester dissociated Rad51 molecules in the supernatant (Figure 5D). Thus, the amount of Rad51 transferred to the supernatant provided an indication of Rad51 removal from dsDNA. The results showed that wild type Rdh54, rdh54Δ50, and Rad54-Rdh54 removed Rad51 from the dsDNA-magnetic beads with a similar efficiency (Figure 5D). Interestingly, the rdh54Δ75 variant has a significantly reduced activity in this regard. Taken together, the results revealed that deletion of 75 residues from the N terminus of Rdh54 severely reduces the interaction between Rdh54 and Rad51 and impairs the ability of Rdh54 to dissociate the Rad51-dsDNA nucleoprotein filament.

**DISCUSSION**

Previous studies have shown a role for Rdh54 in interhomologue recombination and in the regulation of the Dmc1 recombinase during meiosis (1,11,19,20). Similar to other members of the Swi2/Snf2 family of chromatin remodeling proteins, Rdh54 possesses a DNA translocase activity that is fueled by ATP hydrolysis (21). The meiotic roles of Rdh54 are consistent with its ability to interact with Dmc1 and to translocate on DNA to prevent the accumulation of Dmc1 on chromatin, thus allowing its intracellular recycling for meiotic recombination processes (14). Moreover, Rdh54 promotes Dmc1-mediated DNA strand invasion (22). The translocase activity of Rdh54 is also required to counteract Rad51 that accumulates on chromatin, either from overexpression of Rad51 or from loss of two other translocases, namely, Rad54 and Uls1 (16).

Additionally, Rdh54 is required for the adaptation to DSB-induced checkpoint arrest (10). Rdh54 phosphorylation is induced by persistent DNA damage and is recruited to a DSB site and eventually is able to reverse the checkpoint arrest (23).

The above activities are dependent on the interaction of Rdh54 with Rad51. Biochemical studies have shown that the Rad51 interaction domain of Rdh54 resides within its N terminus, and that complex formation is indispensible for its D-loop promoting activity (8,15). Maximal efficiency of Rad51 removal from dsDNA is reliant on this N-terminal domain, as a truncation variant of Rdh54 missing the first 99 amino acid residues is significantly impaired for this functional attribute (15). Here, we have defined the Rad51 interaction domain further and asked which activities are dependent on this domain. For this purpose, we generated yeast mutant strains expressing N-terminal truncation variants of Rdh54 of up to 200 residues and tested them in DNA damage repair and the response to Rad51 overexpression. We have provided evidence that the N-terminal region of Rdh54 is required for an appropriate response to excess Rad51 but actually dispensable for the response to MMS damage. As the deletion mutant and the ATPase-defective mutant are sensitive to MMS damage while the N-terminal truncation mutants are not sensitive, this suggests that ATPase activity is necessary for a full response to MMS damage. This conclusion correlates with our measures of ATPase activity, which are unchanged in the N-terminal truncation mutants (Figure 5B).

Mutant rdh54 proteins with up to 50 of the N-terminal residues deleted retain significant Rad51 interaction in vitro and are able to counteract the deleterious effects of Rad51 overexpression and the formation of Rad51-dsDNA complexes. Deletions removing 75 or more residues of the N-terminal region render cells sensitive to Rad51 overexpression and impair the ability of Rdh54 to dissociate Rad51-dsDNA complexes. The interaction with Rad51 mediated via the N-terminal region of Rdh54 is thus crucial for the ability of Rdh54 to remove Rad51 from undamaged chromatin. It remains to be established whether these truncation mutants are also unable to bind Dmc1.
Previously, we purified and characterized two truncation mutants, rdh54Δ68 and rdh54Δ99 (15). The results showed that the rdh54Δ68 variant is slightly impaired for Rad51 interaction and the promotion of D-loop formation but proficient in Rad51 removal from dsDNA. The rdh54Δ99 variant, on the other hand, is quite defective in Rad51 interaction and D-loop reaction and partially impaired in Rad51 removal (15). These observations are consistent with the results we report here. Regarding the role of Rdh54 in DNA damage repair, it is possible that the interaction region for Rad51 bound to dsDNA in a recombination intermediate is distinct from the interaction domain of Rad51 bound to chromatin, or that the role of Rdh54 in damage repair is focused more on interaction with different proteins such as stalled replication machinery or DNA damage checkpoint factors (23). The fact that the Rdh54 N terminus is required for D-loop formation with chromatinized DNA (8) yet is dispensable for repair of MMS damage further reinforces the idea that the Rdh54 function in MMS damage repair is not directly through homologous recombination.

Rad54 has also been shown to physically interact with Rad51 and to promote D-loop formation via its N-terminal domain (18). As Rad54 and Rdh54 share no homology at their N termini, the substitution of the Rad54 N-terminal region in place of the Rdh54 N terminus can give clues as to the function of this domain in vivo. This is particularly interesting as this domain appears to be conserved among Rdh54 orthologues (24). Our data show that a RAD54-RDH54 hybrid construct is able to complement the sensitivity to MMS and Rad51 overexpression of an rdh54Δ mutant strain. The in vitro data presented here also indicate effective targeting of the Rdh54 translocase domain by the Rad54 N-terminal domain in the dissociation of Rad51-dsDNA complexes.

In conclusion, the results from our study have unexpectedly revealed a differential requirement for Rad51 interaction in the abilities of Rdh54 to function as a Rad51-dsDNA dissociative motor and in chromosome damage repair. Moreover, these results show that a functional Rdh54 protein can be constructed by fusing the Rad54 N terminus to the Swi2/Snf2 core of Rdh54.

REFERENCES

Functional interaction between Rdh54 and Rad51


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**FOOTNOTES**

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¶ The abbreviations used are: HR, homologous recombination; MMS, methyl methanesulfonate; DSB, double strand break; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; bp, base pair.

**FIGURE LEGENDS**

**Figure 1. Domain architecture of Rdh54 and rdh54 truncation mutants.** (A) Schematic overview depicting the conserved regions of Rdh54, a 924 amino acid DNA translocase with a Rad51 binding domain of approximately 100 amino acid residues at the N terminus (15). The boxed regions represent the seven helicase-like Swi2/Snf2 motifs in Rdh54. The N-terminal truncations of Rdh54 used in these studies are represented below. (B) Domain architecture of the Rad54-Rdh54 hybrid construct. The arrow represents an Ncol site used to fuse the Rad54 N-terminal domain with the C terminus of Rdh54.

**Figure 2. DNA damage sensitivity of wild type and rdh54 truncation mutants.** (A) Sensitivity to the DNA alkylating agent MMS and to other damaging agents was examined by plating 10-fold serial dilutions of freshly grown *rdh54Δ* cells and (B) *rad54Δ rdh54Δ* cells carrying *CEN TRP1* constructs containing wild type *RDH54*, *rdh54 truncation mutants* and the ATPase defective *rdh54 K318R* mutant onto SC-TRP plates with different doses of MMS, UV light, gamma IR and cisplatin. The plates were photographed after 72 hours of incubation at 30°C.

**Figure 3. Sensitivity of wild type *RDH54* and *rdh54* truncation variants to *RAD51* overexpression.** Sensitivity to *RAD51* overexpression was examined by plating 10-fold serial dilutions of freshly grown *rdh54Δ* cells carrying *CEN TRP1* and GAL 2µ *URA3* constructs onto glucose (Glu) or galactose (GAL) SC-URA-TRP plates. (A) Wild type *RDH54* and (B) *rdh54* mutant constructs were introduced into an *rdh54* null strain and tested for their ability to complement sensitivity to high Rad51 expression on galactose containing medium. Plates were incubated at 30°C and photographed after 72 hours.

**Figure 4. Sensitivity of a *RAD54-RDH54* hybrid mutant to MMS and *RAD51* overexpression.** (A) *CEN TRP1* constructs containing *RDH54*, *RAD54* and the *RAD54-RDH54* hybrid were introduced into an *rdh54Δ* strain and tested for sensitivity to MMS and (B) to *RAD51* overexpression. 10-fold serial dilutions were prepared from freshly grown cells and plated onto SC-TRP plates with MMS and onto SC-URA-TRP plates containing glucose and galactose, respectively. Plates were incubated at 30°C and photographed after 72 hours.

**Figure 5. Rad51 binding and Rad51 removal activities of purified *rdh54* variants.** (A) Purified wild type Rdh54, *rdh54 Δ50*, *rdh54 Δ75*, and *Rad54-Rdh54* (R-rdh54), 1 µg each, were analyzed by SDS-PAGE and Coomassie blue staining. (B) ATP hydrolysis by Rdh54 and the *rdh54* variants was examined. (C) Pulldown assays to examine the interaction between *rdh54* variants and Rad51. After mixing Rad51 with His6-tagged Rdh54 or its variants, protein complexes were captured with Ni-NTA agarose. The supernatant (S), wash (W), and SDS eluate (E) were analyzed by SDS-PAGE and Coomassie blue staining. % Rad51 indicates the percentage of bound Rad51. (D) (i) the schematic of the reaction to examine Rad51 removal from dsDNA is shown. (ii) Rad51-dsDNA magnetic beads were incubated with 30, 60, 120 nM of wild type Rdh54 (lanes 2-4), *rdh54 Δ50* (lanes 5-7), *rdh54 Δ75* (lanes 8-10), and *Rad54-Rdh54* (R-rdh54) (lanes 11-13), and the supernatant and bead fractions were analyzed by SDS-
PAGE and Coomassie blue staining. The results were quantified and plotted. The amount of Rad51 that was spontaneously released from the beads (lane 1) had been subtracted from lanes 2-13. CK, creatine kinase used in the reaction buffer as part of the ATP regenerating system.
Figure 2

A

rdh54Δ +
CEN empty vector
CEN RDH54
CEN rdh54 Δ25
CEN rdh54 Δ40
CEN rdh54 Δ50
CEN rdh54 Δ75
CEN rdh54 Δ93
CEN rdh54 Δ200
CEN rdh54 K318R

B

rad54Δ rdh54Δ +
CEN empty vector
CEN RDH54
CEN rdh54 Δ25
CEN rdh54 Δ40
CEN rdh54 Δ50
CEN rdh54 Δ75
CEN rdh54 Δ93
CEN rdh54 Δ200
CEN rdh54 K318R

[Images of plates with various treatments: SC-TRP, 0.02% MMS, 100 J/m² UV, 100Gy IR, 0.1 mM cisplatin]
Figure 3

A

rdh54Δ +
CEN vector + GAL vector  
CEN vector + GAL-RAD51  
CEN vector + GAL-RAD51  
CEN vector + GAL-rad51 KA  
RDH54 + GAL vector  
RDH54 + GAL-RAD51  
RDH54 + GAL-RAD51  
RDH54 + GAL-rad51 KA

B

rdh54Δ +
rdbh54 Δ25 + GAL vector  
rdbh54 Δ25 + GAL-RAD51  
rdbh54 Δ25 + GAL-RAD51  
rdbh54 Δ25 + GAL-rad51 KA  
rdbh54 Δ40 + GAL vector  
rdbh54 Δ40 + GAL-RAD51  
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rdbh54 Δ40 + GAL-rad51 KA  
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rdbh54 Δ75 + GAL-rad51 KA  
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rdbh54 Δ93 + GAL-RAD51  
rdbh54 Δ93 + GAL-rad51 KA  
rdbh54 Δ200 + GAL vector  
rdbh54 Δ200 + GAL-RAD51  
rdbh54 Δ200 + GAL-RAD51  
rdbh54 Δ200 + GAL-rad51 KA
Figure 4

A

rdh54Δ +

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<th>SC-TRP</th>
<th>0.02% MMS</th>
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B

rdh54Δ +

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<tr>
<th>CEN RAD54-RDH54 + GAL vector</th>
<th>SC-URA-TRP (Glu)</th>
<th>SC-URA-TRP (GAL)</th>
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<tr>
<td>CEN RAD54-RDH54 + GAL-RAD51</td>
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<tr>
<td>CEN RAD54-RDH54 + GAL-RAD51</td>
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<tr>
<td>CEN RAD54-RDH54 + GAL-rad51 KA</td>
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Characterization of the interaction between the *Saccharomyces cerevisiae* Rad51 recombinase and the DNA translocase Rdh54
Sergio R. Santa Maria, YoungHo Kwon, Patrick Sung and Hannah L. Klein

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