The RecQ4 Orthologue Hrq1 Is Critical for DNA Interstrand Cross-Link Repair and Genome Stability in Fission Yeast

Lynda M. Grocock,^a John Prudden,^a J. Jefferson P. Perry,^{a,b} and Michael N. Boddy^a

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, USA,^a and School of Biotechnology, Amrita University, Kollam, Kerala, India^b

Of the five human RecQ family helicases, RecQ4, BLM, and WRN suppress distinct genome instability-linked diseases with severe phenotypes, often with indeterminate etiologies. Here, we functionally define Hrq1, a novel orthologue of RecQ4 from fission yeast. Biochemical analysis of Hrq1 reveals a DEAH box- and ATP-dependent 3'-5' helicase activity on various DNA substrates, including bubbles but not blunt duplexes, characteristic of the RecQ family. Cells lacking Hrq1 suffer spontaneous genomic instability and, consequently, require homologous recombination repair and the DNA damage checkpoint for viability. Hrq1 supports the nucleotide excision repair of DNA damage caused by the chemotherapeutic agent cisplatin and, in certain genetic contexts, UV light. Genetic epistasis analyses reveal that Hrq1 acts parallel to the PCNA/Ubc13/Mms2-dependent postreplication repair (PRR) pathway. Thus, in *hrq1Δ* cells, lesions are channeled through the PRR pathway, yielding hyper-recombinant and mutator phenotypes; analogous defects may underlie the genetic instability and diseases associated with RecQ4 dysfunction.

Faithful DNA repair mechanisms are critical for suppressing genetic changes and their potential sequelae, including cancer (18). Key mediators of genome stability include the RecQ family of DNA helicases, which are conserved from bacteria to humans (6, 11). Among their diverse DNA metabolic roles, RecQ helicases act at multiple, distinct steps within the homologous recombination (HR)-based DNA repair pathway (6, 11). For example, human BLM and budding yeast Sgs1 promote extensive resection of DNA ends for HR initiation. This is seemingly paradoxical to their roles of antagonizing HR by unwinding Rad51-mediated displacement loops (D loops) (37). At late stages of HR, RecQ helicases again play a critical role by promoting the dissolution of Holliday junctions (HJs; four-stranded junctions), which must be removed to allow chromosome segregation at mitosis (2). RecQ-mediated dissolution avoids chromosomal crossover and appears to be the dominant mode of HJ removal in mitosis (3). The Mus81-Eme1 nuclear resolvase provides an alternative pathway for HJ removal with the potential for producing crossovers (3, 7, 8). This accounts for the tumorigenic increased sister chromatid exchange observed in BLM patient cells (62). In addition to HR-mediated repair of DNA breaks, HJs may be produced at stalled replication forks via RecQ-mediated regression (11). Importantly, such regression, which involves the dissociation of nascent strands at the fork and their pairing to form an HJ, may provide the necessary space and time for the repair or bypass of DNA damage ahead of the fork.

In addition to BLM, there are four other RecQ helicases in humans. These include RecQ4, which is mutated in three syndromes, Rothmund-Thomson syndrome (RTS), Baller-Gerold syndrome (BGS), and Rapadilino syndrome (6, 11). These disorders are clinically distinct but show some overlap in their signature developmental abnormality, chromosome instability, and cancer predisposition phenotypes (24, 25, 56, 61). RecQ4 contains two functional domains that are conserved in higher eukaryotes. The N-terminal Sld2-like domain is essential for DNA replication initiation, and thus, the majority of disease-associated RecQ4 mutations are largely restricted to the helicase core (28, 36, 53, 64). RecQ4 dysfunction results in an increased rate of chromosome

aneuploidy and chromatid cohesion defects (14, 35, 38). Furthermore, based on colocalization and physical interaction with key DNA repair factors, such as Rad51 and XPA, RecQ4 likely has roles in DNA damage repair (15, 44). Consistent with this, some RTS patient cell lines exhibit sensitivity to genotoxic agents (20, 55, 57).

The presence of BLM homologues in budding yeast (Sgs1) and fission yeast (Rqh1) has greatly accelerated the determination of the roles of BLM in higher-eukaryotic genome stability. As yeasts are generally described as containing only a single RecQ helicase (6, 11), similar critical support for analyzing the other disease-associated RecQ helicases appears unavailable. However, sequence homology searches in fission yeast have revealed the presence of RecQ family members Tlh1 and Tlh2, which are encoded in the subtelomeric regions of chromosomes 1 and 2 (34). Furthermore, a recent bioinformatics analysis identified potential orthologues of RecQ4 in both plants and fungi, called the Hrq1 family (4). Thus, functional analysis of Hrq1 family members will uncover their key functions and may help to unravel the complexity of RecQ4-associated disorders.

Here, we have characterized fission yeast Hrq1 and determined that, as for other RecQ helicases, this protein plays fundamental roles in genome stability and DNA repair. Biochemical characterization of Hrq1 reveals that it exhibits 3'-5' DNA helicase activity, the same polarity as other RecQ helicases, including RecQ4 (52). Cells lacking Hrq1 exhibit spontaneous genome instability, constitutive activation of the DNA damage checkpoint, and hypersensitivity to the chemotherapeutic agent cisplatin. We show that Hrq1 facilitates the processing of a subset of nucleotide excision

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Address correspondence to Michael N. Boddy, nboddy@scripps.edu.

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repair (NER) substrates, including interstrand cross-links (ICLs), functioning parallel to the Ubc13/Mms2-activated postreplication repair (PRR) pathway. Genetic and biochemical analyses indicate that Hrq1 functions closely with the NER factor XPA, which has recently been shown to both functionally overlap and physically interact with human RecQ4 (15). Thus, Hrq1 represents a novel RecQ helicase in fission yeast that has intriguing functional parallels with human RecQ4.

MATERIALS AND METHODS

Strain construction, general yeast techniques, and drug treatments. Standard yeast techniques were used as described previously (39). Drugs were obtained from Sigma-Aldrich. An Hrq1-HD (DEAH domain D444A/E445A) mutant construct was made using QuikChange (Stratagene) and used to replace a *ura4⁺* marker inserted in the helicase domain of *hrq1* at its endogenous locus. The same process was used to generate Hrq1-CDTΔ (in which amino acids 50 to 250 were deleted) at the endogenous *hrq1* locus. Stable transformants were counterselected on 5-fluoroorotic acid (5-FOA) and sequenced. Generation of integrated *nmt*-driven expression constructs was performed by transformation of PCR products amplified from either an *nmt1*-FLAG:kanMX6 N-terminal tagging cassette (41) or pREP41:*nmt41*:GFP-Hrq1 (*mlu1* cut) and integration at the Hrq1 genomic locus or *ars1*, respectively. Cisplatin sensitivity was determined by incubating exponentially growing cells with the drug at 32°C. After 1 h, the drug was washed out and cells were plated onto nonselective media. Colonies were then counted after 2 to 3 days. A complete list of strains used in this study is provided in Table 1.

Western analysis of protein expression. Protein lysates were made in denaturing 8 M urea buffer as described in reference 45. Lysates were resolved using 4 to 20% (wt/vol) Tris-glycine gradient gel (Invitrogen). Levels of Hrq1-tandem affinity purification protein (Hrq1-TAP) were assessed by immunoblotting with peroxidase-antiperoxidase antibody (Sigma) and chemiluminescence detection. Tubulin levels were probed with GTU-88 (Sigma), and GFP-Hrq1 was probed with anti-green fluorescent protein (anti-GFP) (Roche). Membranes were then probed with IRDye800 secondary antibody (LI-COR Biosciences), and fluorescence was detected using an Odyssey infrared imaging system (LI-COR Biosciences).

Protein purification. 3XFLAG::Hrq1/HD/CDTΔ was expressed from the *nmt1* promoter at the endogenous locus in essential minimal medium (EMM) lacking thiamine for 30 h. Cells were harvested in 20 mM HEPES (pH 7.6), frozen at −80°C, and then lysed using a grinder mill (Retsch). Ground cell powder was thawed in lysis buffer (50 mM HEPES, pH 7.6, 75 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 0.1% [vol/vol] Triton X-100, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 5% [vol/vol] glycerol, protease inhibitor cocktail) (Roche). Cell lysate was incubated with anti-Flag M2 affinity gel (Sigma) for 1 h at 4°C. The gel was washed with 50× bead volume wash buffer (50 mM HEPES, pH 7.6, 75 mM KCl, 2 mM MgCl₂, 5% [vol/vol] glycerol) and then incubated with 100 μg ml^{−1} FLAG peptide. Eluate was snap-frozen in liquid nitrogen and stored at −80°C. Coimmunoprecipitation was performed following the same protocol as described above in a strain background containing Rhp14-myc. Normalized amounts of both inputs and immunoprecipitate were analyzed by Western blotting and probed using peroxidase-antiperoxidase antibody and monoclonal antibody 9E10.

Oligonucleotide substrates. 5' IR700-labeled and unlabeled PAGE-purified oligonucleotides were purchased from IDT. Oligonucleotide substrates were synthesized as described previously (7). 3' overhang, 5' overhang, bubble, and duplex structures were made by annealing one of the following oligonucleotides with 5' IR700-labeled X1 (5' GACGCTG CCGAATTCTGGCTTGCTAGGACATCTTTGCCACGTTGACCCG): 3' O/H (5' AAAGATGTCCTAGCAAGCCAGAATTCGGCAGCGTC), 5' O/H (5' CGGGTCAACGTGGGCAAGATGTCCTAGCAAGCCAG), bubble (5' CGGGTCAACGTGGGCAAGTCTATGCTTACGTCGACGCAAG ATTCCGCGACGCTC), and duplex (5' CGGGTCAACGTGGGCAAGAA

TABLE 1 *S. pombe* strains used during this study

Strain	Genotype ^a
NBY128	<i>mus81::kanMX6</i>
NBY780	<i>h⁺</i>
NBY871	<i>nse6::natMX6</i>
NBY940	<i>his3-D1 arg3-D4 ade6-M375 int::pUC8/his3⁺/RTS1 site A orientation 1/ade6-L469</i>
NBY941	<i>his3-D1 arg3-D4 ade6-M375 int::pUC8/his3⁺/RTS1 site A orientation 2/ade6-L469</i>
NBY2027	<i>rhp51::ura4⁺</i>
NBY2047	<i>rad60-E380R:kanMX6</i>
NBY2077	<i>rql1::ura4⁺</i>
NBY2112	<i>ubc13::kanMX6</i>
NBY2135	<i>rad13::ura4⁺</i>
NBY2141	<i>rhp14::kanMX6</i>
NBY2159	<i>hrq1-TAP:kanMX6</i>
NBY2173	<i>rhp14-myc:kanMX6</i>
NBY2189	<i>rad22-YFP:kanMX6</i>
NBY2922	<i>ura4⁺</i>
NBY3034	<i>mms2::kanMX6 hrq1::hphMX6</i>
NBY3037	<i>ubc13::kanMX6 hrq1::hphMX6</i>
NBY3039	<i>hrq1::hphMX6</i>
NBY3045	<i>hrq1::hphMX6 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3⁺/RTS1 site A orientation 2/ade6-L469</i>
NBY3046	<i>hrq1::hphMX6 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3⁺/RTS1 site A orientation 1/ade6-L469</i>
NBY3069	<i>hrq1::hphMX6 chk1::ura4⁺</i>
NBY3075	<i>pcn1-K164R:ura4⁺</i>
NBY3084	<i>nse6::natMX6 hrq1::hphMX6</i>
NBY3119	<i>pcn1-K164R:ura4⁺ hrq1::hphMX6</i>
NBY3124	<i>hphMX6:nmt1:3XFLAG-Hrq1</i>
NBY3382	<i>rad16::kanMX6</i>
NBY3439	<i>hrq1-HD</i>
NBY3464	<i>hrq1-CDTΔ</i>
NBY3469	<i>hphMX6:nmt1:3XFLAG-Hrq1-HD</i>
NBY3597	<i>hphMX6:nmt1:3XFLAG-Hrq1-CDTΔ</i>
NBY3598	<i>rad22-YFP:kanMX6:hrq1-CDTΔ</i>
NBY3599	<i>rad22-YFP:kanMX6:hrq1-HD</i>
NBY3639	<i>pREP41:nmt41:GFP-hrq1:LEU2</i>
NBY3737	<i>rad13::ura4⁺</i>
NBY3738	<i>rad16::kanMX6 hrq1::hphMX6</i>
NBY3739	<i>ubc13::kanMX6 rhp14::kanMX6 hrq1::hphMX6</i>
NBY3741	<i>hrq1-CDTΔ-TAP:kanMX6</i>
NBY3742	<i>rad22-YFP:kanMX6 hrq1::hphMX6</i>
NBY3746	<i>pREP41:nmt41:GFP-hrq1-CDTΔ:LEU2</i>
NBY3756	<i>ura4⁺ hrq1::hphMX6</i>
NBY3860	<i>pREP41:nmt41:GFP-hrq1-HD:LEU2</i>
NBY3891	<i>hrq1-HD-TAP:kanMX6</i>
NBY3952	<i>rev3::kanMX6</i>
NBY3953	<i>rev1::kanMX6</i>
NBY3978	<i>rev3::kanMX6 hrq1::hphMX6</i>
NBY3979	<i>rev1::kanMX6 hrq1::hphMX6</i>
NBY4023	<i>rhp14-Myc:kanMX6 hphMX6:nmt1:3XFLAG-Hrq1</i>
NBY4076	<i>rql1::ura4⁺ hrq1::hphMX6</i>

^a Unless otherwise indicated, strains are *ura4-D18 leu1-32*.

TGTCCTAGCAAGCCAGAATTCGGCAGCGTC). Labeled DNA substrates were purified on a nondenaturing 10% Tris-borate-EDTA gel and stored in TMS buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA).

Helicase assay. Various amounts of purified FLAG-Hrq1 or FLAG-tagged Hrq1 mutants were incubated with 12.5 pmol of DNA substrate in helicase buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 3.5 mM DTT, 0.25% [vol/vol] Tween 20, 5 mM MgCl₂, 100 μg ml^{−1} bovine serum albumin [BSA], 25 μM complementary unlabeled oligonucleotide) in a

FIG 1 Analysis of Hrq1 mutant phenotypes. (A) Domain organization of *Schizosaccharomyces pombe* Hrq1 compared to that of human RecQ family proteins. The Hrq1 helicase-dead mutations are indicated by an asterisk. EXO, exonuclease domain; DUF, domain of unknown function 1998; RQC, RecQ carboxy-terminal domain; HRDC, helicase-and-RNase-D C-terminal domain; NLS, nuclear localization signal; aa, amino acids. (B) Representative live-cell images of the indicated strains, grown on nonselective media. Bar, 10 μm. (C) Quantitation of the strains with cells shown in panel B. The average cell length relative to that of the wild type was measured over three independent experiments. *P* values compared to wild-type cell length were determined using unpaired Student's *t* test. Values of <0.05 were deemed statistically significant. (D) Tetrad dissection of a cross between the *hrq1Δ* and *chk1Δ* mutants, showing a slow-growth phenotype in the double mutant. (E) Western blot analysis demonstrating that the expression levels of both TAP-tagged Hrq1-HD and Hrq1-CDTΔ mutants are similar to that of wild-type Hrq1-TAP. Whole-cell lysates were immunoblotted with peroxidase-antiperoxidase (PAP) or antitubulin as a loading control.

final volume of 20 μl. ATP (5 mM) was added as indicated in the figure legends. The reaction mixture was incubated at 30°C for 30 min. Protein-DNA complexes were disrupted by 1 μg ml⁻¹ proteinase K digestion for 15 min at 50°C. Heat-denatured substrates were generated by incubation at 95°C for 5 min. Reaction products were separated on a 6.5% nondenaturing tris-borate-EDTA acrylamide gel, and the fluorescent signal was quantified using an Odyssey infrared imaging system (LI-COR Biosciences).

Microscopy. Cells were photographed using a Nikon Eclipse E800 microscope equipped with a Photometrics Quantix charge-coupled-device (CCD) camera. Unsynchronized cells were treated with 50 μg ml⁻¹ cisplatin or an equivalent volume of dimethyl sulfoxide (DMSO). After 2 h, drug was washed out and cells were imaged. Quantification was performed by scoring 500 or more nuclei from at least three independent experiments.

Recombination assay. Recombination assays were performed as described previously (42). Following overnight growth in selective media, *ade⁻/his⁺* strains were streaked onto nonselective plates and incubated for 4 days. Colonies were then suspended in distilled water (dH₂O) and plated onto media selective for adenine. Strains containing the RTS replication fork block were plated at a density of ~10⁴ per plate, whereas strains containing the inactive RTS site were plated at ~10⁵ per plate. Recombination rates were measured for nine independent isolates from each strain over three independent experiments.

Mutator analysis. The mutator analysis assay was performed as previously described (21). Strains preselected in minimal media lacking uracil were grown overnight. Exponentially grown cultures were then incubated with cisplatin for 1 h. The drug was then washed out, and cells were

incubated for 16 h in media containing uracil. Cells were plated onto minimal media containing 5-fluoroorotic acid (5-FOA) at a density of ~10⁵ cells per plate, and colonies were counted after 4 days. This assay was performed for three independent isolates from each strain, and the results were averaged from three independent experiments.

RESULTS

Novel RecQ family helicase critical for genome stability in fission yeast. We observed that the uncharacterized Hrq1 DEAH box helicases of both fission and budding yeast share sequence similarity to the helicase cores of the established BLM RecQ family helicases of each organism, called Rqh1 and Sgs1, respectively. Also, a recent bioinformatics study identified Hrq1 family proteins as orthologues of the human disease-associated RecQ4 helicase (4). Hrq1 exhibits a modular structure, with functional domains flanking the conserved superfamily II helicase core, in keeping with other RecQ family members (Fig. 1A) (6, 11). To probe Hrq1 function, we constructed various mutant alleles that included a full gene deletion, a deletion of the amino-terminal Cdt1-related winged-helix domain, and point mutations within the conserved DEAH motif of the helicase core (Fig. 1A). *hrq1Δ* cells exhibit heterogeneity in cell length, being on average ~15% longer than wild-type cells (Fig. 1B and C). Similar but more pronounced cell elongation was observed for helicase-dead Hrq1 (Hrq1-HD) and Hrq1-CDTΔ, which lacks the Cdt1 homology domain (Fig. 1B and C). An elongated cell phenotype in fission

yeast often indicates that genome aberrations have activated the DNA structure checkpoints and delayed cell cycle progression (48). Consistently, deletion of the G₂ DNA damage checkpoint kinase Chk1 in *hrq1Δ* cells both abolishes the cell elongation phenotype and results in poor cell viability as judged by tetrad analysis (Fig. 1B, C, and D). The Hrql-HD and wild-type Hrql proteins are equally expressed, as determined by Western blotting, whereas Hrql-CDTΔ is more abundant (Fig. 1E). Thus, Hrql mutant phenotypes were not caused by underexpression. These data demonstrate that both the helicase activity and the Cdt1 homology domain of Hrql are critical to suppress spontaneous genome instability.

Hrql is a nuclear protein critical for survival of cisplatin-induced DNA interstrand cross-links. We determined the genotoxin sensitivity profile of Hrql mutant cells to better define the role(s) of Hrql in genome maintenance. Compared to selected nucleotide excision repair (NER; *rhp14Δ*, HuXPA), postreplicative repair (PRR; *ubc13Δ*), and homologous recombination (HR; *rhp51Δ*, HuRad51) pathway mutants, *hrq1Δ* cells showed negligible sensitivity to UV irradiation, replication inhibition by hydroxyurea (HU), or alkylating agent methyl methanesulfonate (MMS) (Fig. 2A). In addition, Hrql mutants were not sensitive to oxidative damage induced by hydrogen peroxide (data not shown). Strikingly, however, *hrq1Δ*, Hrql-HD, and Hrql-CDTΔ cells were hypersensitive to DNA interstrand cross-linking (ICL) agents mitomycin C (MMC) and cisplatin, in response to both chronic and acute exposures (Fig. 2B and C). Hrql-HD cells were more sensitive to cisplatin than cells lacking Hrql altogether, suggesting that the helicase-dead form of Hrql dominantly interferes with other repair processes (Fig. 2C). Supporting a dominant negative function, overexpression of Hrql-HD reduces the population-doubling rate and causes DNA damage and checkpoint-mediated growth arrest, whereas overexpression of Hrql wild type or Hrql-CDTΔ is relatively well tolerated (Fig. 2D and data not shown). Given the exquisite sensitivity of HR mutants to cisplatin (Fig. 2B) and the requirement for HR in the absence of Hrql (described later), we also monitored the abundance of Rad22 (HuRad52)-yellow fluorescent protein (YFP) foci in Hrql wild-type or mutant cells. While Rad22-YFP levels were similar for all of the untreated strains, cisplatin treatment induced a significantly higher level of Rad22-YFP foci in Hrql mutants than in wild-type cells (Fig. 2E). Again, consistent with a dominant negative effect of Hrql-HD, the highest levels of cisplatin-induced foci were observed in Hrql-HD cells (Fig. 2E). Overall, these data indicate that Hrql is a novel and critical mediator of cellular resistance to cisplatin.

We next determined the subcellular localization of Hrql and whether cisplatin treatment alters its distribution. We were unable to detect GFP-tagged Hrql expressed at its endogenous locus, likely indicating that Hrql abundance is limited. Therefore, we expressed GFP epitope-tagged wild-type, Hrql-HD, and Hrql-CDTΔ from the *nmt41* promoter of an integrated vector. Whereas GFP-Hrql wild type and GFP-Hrql-HD expressed similar levels of protein, Hrql lacking its Cdt1-like domain was more abundant (Fig. 2F). Consistent with its key role in genome stability, GFP-Hrql was present in the nuclei of cells (Fig. 2G). In addition, wild-type Hrql foci were induced following cisplatin treatment (Fig. 2G). Interestingly, GFP-Hrql-HD formed numerous nuclear foci, even in the absence of exogenous DNA damage. This suggests that Hrql-HD is recruited to spontaneously occurring

lesions or sites of action but is stalled there due to lack of helicase activity, thus providing a potential mechanistic basis for the observed dominant negative phenotype. Hrql lacking the Cdt1-like domain displays a pan-cellular localization and, unlike the wild type, did not form cisplatin-induced foci (data not shown). Therefore, the Cdt1-like domain may retain Hrql in the nucleus and may promote recruitment to sites of action.

Hrql acts in NER-initiated ICL repair. To determine the pathway(s) of ICL repair facilitated by Hrql, we undertook genetic analyses of the *hrq1Δ* and ICL repair factor mutants. Compared to those in mammalian cells and budding yeast, little is known about ICL repair pathways in fission yeast. Therefore, we tested the cisplatin sensitivity of fission yeast lacking homologues of key ICL repair factors identified in other eukaryotes, including the NER factors XPF and XPA (19, 31, 63). Consistent with observations in other organisms, we found that cells lacking Rad16 (HuXPF) are exquisitely sensitive to cisplatin (Fig. 3A). Although cisplatin sensitive, Rhp14 (HuXPA) mutant cells were more resistant than *rad16Δ* cells (Fig. 3B) (63). This suggests that Rad16-Swi10 performs NER-independent functions in “unhooking” ICLs, as observed in mammals. Notably, we determined that the cisplatin sensitivity of *hrq1Δ rhp14Δ* and *hrq1Δ rad16Δ* double mutant cells was equivalent to that of the *rhp14Δ* and *rad16Δ* single mutants, respectively (Fig. 3A and B). These data indicate a role for Hrql within a pathway(s) that involves lesion processing by Rad16-Swi10 and Rhp14.

Dissecting the repair pathways for cisplatin-induced lesions is complicated by the fact that >95% of such lesions are intrastrand and not interstrand cross-links (23). Intrastrand cross-links do not form an impasse like interstrand cross-links and can be removed by NER or bypassed by PRR-mediated damage tolerance. In budding yeast, the Ubc13/Mms2 complex polyubiquitinates Pcn1(PCNA)-K164 to facilitate damage tolerance by template switching and/or fork regression (16, 54). In fission yeast, however, Pcn1(PCNA)-K164 polyubiquitination by Ubc13/Mms2 stimulates both recruitment of translesion polymerases (e.g., Rev1/3) and template switching (12). We found that *ubc13Δ* cells, like those lacking NER components, are hypersensitive to cisplatin (Fig. 2B and 3C). However, unlike the epistasis observed between *hrq1Δ* and NER mutants, *ubc13Δ hrq1Δ*, *rev1Δ hrq1Δ*, and *rev3Δ hrq1Δ* double mutants were synergistically more sensitive to cisplatin than either single mutant (Fig. 3C and D). This was also found to be true for *rhp14Δ ubc13Δ* double mutants, with no further sensitization conferred by additional deletion of Hrql (Fig. 3B). Interestingly, we found that *hrq1Δ ubc13Δ* double mutants were also synergistically sensitive to UV and that an *hrq1Δ ubc13Δ rhp14Δ* triple mutant behaved like the *ubc13Δ rhp14Δ* double mutant (Fig. 3E and F). Thus, Hrql functions in parallel to Ubc13/Mms2-mediated PRR and within the NER pathway. A role for Hrql in NER was further supported by the detection of a weak but specific physical interaction between FLAG-Hrql and Rhp14-myc (Fig. 3G), which is consistent with the observed physical interaction between mammalian RecQ4 and XPA (15).

Hrql suppresses spontaneous genome instability. Our epistasis analyses of the *hrq1Δ*, *ubc13Δ*, *rhp14Δ*, and *rad16Δ* mutants indicate that Hrql acts parallel to Ubc13-mediated PRR and with NER factors in response to UV and cisplatin. Furthermore, even in the absence of exogenous genotoxins, we observed synergistic growth defects of cells with *hrq1Δ* in combination with *ubc13Δ* and other mutants in the PRR pathway, including Ubc13 cofactor

FIG 2 Genotoxin sensitivity profile of cells lacking the nuclear protein Hrq1. (A and B) Comparison of the DNA damage sensitivity profiles of the indicated Hrq1 strains with those of the NER (*rhp14Δ*), HRR (*rhp51Δ*), and PRR (*ubc13Δ*) mutants demonstrates that Hrq1 sensitivity is limited to chronic treatment with the ICL agents cisplatin and mitomycin C (MMC). Fivefold serial dilutions of the indicated strains were either plated onto nonselective media with no treatment (Untreated) or UV irradiated (200 J m⁻²). For chronic treatments, cells were plated onto media containing the indicated doses of genotoxin. Plates were then incubated for 3 days at 30°C. (C) Comparison of the survival rates of wild-type cells and the indicated Hrq1 mutants following acute treatment of increasing concentrations of cisplatin. (D) Analysis of the population-doubling rate of the indicated Hrq1 mutants shows growth arrest of cells overexpressing the Hrql-HD mutant. The plot shown is a representative image from three independent experiments. (E) Quantitation of Rad22-YFP focus formation in the absence of or immediately following cisplatin (50 μg ml⁻¹) treatment in the indicated strains. *P* values compared to wild-type focus number in the presence and absence of drug treatment were determined using unpaired Student's *t* test. Values of <0.05 were deemed statistically significant. (F) Western blot analysis of protein levels from *nmt41*-driven expression of GFP-tagged Hrq1, Hrql-HD, and Hrql-CDTΔ. Whole-cell lysates were immunoblotted with antisera for GFP (anti-GFP) or antitubulin as a loading control. (G) Representative images and quantitation of nuclear focus formation of wild-type GFP-Hrq1 and GFP-tagged Hrql-HD in the absence (No Drug) and presence of cisplatin treatment. *P* values compared to wild-type focus number in the presence and absence of drug were determined using unpaired Student's *t* test.

FIG 3 Epistasis analyses of the *hrq1Δ* mutant and NER and PRR repair mutants. (A to F) Cell survival assays of the *hrq1Δ* mutant in combination with *rad16Δ*, *rhp14Δ*, *ubc13Δ*, *rev1Δ*, and *rev3Δ* mutants following cisplatin treatment (A to D) or UV exposure (E and F). Representative images from at least three independent experiments are shown. (G) Western blot showing immunoprecipitation (IP) of FLAG-Hrq1 and the coprecipitation of Rhp14-myc.

Mms2 and PCNA mutants (Fig. 4A) (30). No synergistic growth defects were observed when *hrq1Δ* was combined with various NER-defective mutations, including *rhp14Δ* (HuXPA), *rad13Δ* (HuXPG), and *rad16Δ* (HuXPF) (Fig. 4B). Thus, in response to spontaneous DNA lesions, Hrq1 also appears to act in an NER-dependent, but PRR-independent, repair pathway.

The recently completed high throughput genetic interaction maps for fission yeast (50) provide both robust support for our

genetic analyses and additional insight on Hrq1 function. A powerful tool provided by the Krogan laboratory (<http://interactome-cmp.ucsf.edu/>) enables pathway analysis for a large number of genes (50). Querying the database for genetic interaction spectra most related to that of *hrq1Δ* revealed that *rhp14Δ* has a near synonymous profile (correlation coefficient of 0.92). Furthermore, the high throughput data set indicated that *hrq1Δ* cells are dependent on certain PRR factors, the DNA damage checkpoint

FIG 4 Genetic relationship of the *hrq1Δ* mutant and DNA damage repair mutants. (A and B) Synthetic sickness results when *hrq1Δ* is combined with postreplicative repair mutants or an *nse6Δ* mutant, while an epistatic relationship exists between *hrq1Δ* and NER mutants. Fivefold serial dilutions of the

(49), and HR factors, including Rad51 and Eme1 (18). Our direct analyses agree well with and extend this high throughput data set.

Tetrad analysis of a cross between *hrq1Δ* cells and cells lacking the Eme1 heterodimeric partner Mus81 (*mus81Δ*) (7) revealed that the double mutant was inviable (Fig. 4C). In addition, crosses between *hrq1Δ* and *rhp51Δ* (HuRad51) cells failed to yield double mutants (data not shown), underscoring the importance of HR in the absence of Hrq1. The Hrq1-HD allele was also lethal with both *mus81Δ* and *rhp51Δ*, indicating that the helicase activity of Hrq1 is critical for function (Fig. 4C and data not shown). Interestingly, whereas *hrq1Δ* is synthetic sick with *ubc13Δ*, the Hrq1-HD *ubc13Δ* double mutant is inviable (Fig. 4C and data not shown). Hrq1-HD is also lethal in combination with the Smc5-Smc6 complex subunit *nse6Δ* mutant (43), whereas *hrq1Δ* exhibits a synthetic sick interaction with *nse6Δ* (Fig. 4C). Therefore, consistent with its inhibition of cell growth when overexpressed and enhanced sensitivity to cisplatin, Hrq1-HD likely dominantly interferes with other repair pathways. Finally, *hrq1Δ* is synthetic lethal with *rad60^{E380R}*, a hypomorphic allele of the Smc5-Smc6 cofactor Rad60, which plays major roles in replication stress tolerance and HR (Fig. 4C) (9, 46, 47).

Parallel functions of RecQ family helicases Hrq1 and Rqh1.

We were interested to determine whether Hrq1 had shared functions with the only other constitutively expressed RecQ helicase in fission yeast, Rqh1 (human BLM homologue [11]). An *hrq1Δ* *rqh1Δ* double mutant was viable but exhibited synthetic sickness, and therefore, we compared the DNA damage sensitivity profiles of the *rqh1Δ*, *hrq1Δ*, and *hrq1Δ* *rqh1Δ* mutants. As previously shown, the *rqh1Δ* mutant was sensitive to UV damage (58), replication fork collapse induced by the topoisomerase I poison camptothecin (CPT) (13), and ICL-inducing agents (27) (Fig. 4D and E). The *hrq1Δ* *rqh1Δ* double mutant was synergistically sensitive to CPT and both acute and chronic cisplatin treatments (Fig. 4D and E), indicating independent roles for these helicases in DNA repair.

Hyperrecombination and mutator phenotypes of Hrq1 mutant cells. The dependency of *hrq1Δ* cells on Rhp51, Mus81-Eme1, Rad60, and Nse6 suggests that HR levels are elevated in Hrq1 mutants. To test this, we employed a system that allows scoring of recombination events at a polar site-specific replication fork block, *RTS1* (1). This system revealed an approximately 15-fold increase in recombination events over results seen with the wild type when replication forks were stalled at *RTS1* in cells lacking the RecQ helicase Rqh1 (1). Interestingly, a significant increase in recombination events was detected in *hrq1Δ* cells, but the ratio of wild-type to *hrq1Δ* cells was independent of *RTS1* orientation (Fig. 5A). Thus, Hrq1 suppresses spontaneous HR but, unlike Rqh1, does not specifically contribute to replication fork stability at the active *RTS1* fork pause site.

We also analyzed mutation frequency in *hrq1Δ* cells by using a forward assay based on the resistance of *ura4* mutants to

indicated strains were plated onto nonselective media and grown for 3 days at 30°C. (C) Tetrad dissection of mutant crosses showing severe synthetic sickness or lethality in combination with *hrq1Δ* or Hrq1-HD. The key depicts the genotypes present within the tetrads, which are denoted by the different-shaped white-outlined symbols. (D and E) Genotoxin sensitivity of *hrq1Δ* in combination with *rqh1Δ*; both acute and chronic cisplatin treatments show synthetic sickness. Representative images from at least three independent experiments are shown.

FIG 5 Hyperrecombination and mutator phenotypes of *hrq1Δ* cells. (A) Generation of *ade⁺* from adjacent *ade6⁻* heteroalleles as a result of the RTS-mediated stalled replication fork and subsequent HRR was measured in wild-type and *hrq1Δ* backgrounds. *P* values compared to wild-type values were determined using unpaired Student's *t* test. Values of <0.05 were deemed statistically significant. (B and C) Cisplatin-induced mutation rates per generation were determined for both wild-type and *hrq1Δ* strains. Error bars represent standard deviations (SD) from three independent experiments.

5-fluoroorotic acid (5-FOA). Compared to the wild type, *hrq1Δ* cells exhibited a significantly higher basal mutation rate at the *ura4* locus, which was further exacerbated by cisplatin treatment (Fig. 5B and C). Increases in cisplatin dosage caused an elevated mutation rate that was significantly more pronounced in *hrq1Δ* cells (Fig. 5B and C). Events at the *ura4* locus were predominantly point mutations and not gross deletions (data not shown), suggesting the engagement of elevated error-prone translesion synthesis (TLS) in the absence of Hrql (i.e., Rev1/3) (Fig. 3D).

Hrql exhibits DEAH motif-dependent 3'-5' helicase activity. To define Hrql activity *in vitro*, we purified equivalent amounts of wild-type, helicase-dead, and Cdt1 domain-deleted versions from fission yeast by affinity chromatography (Fig. 6A). As for other RecQ helicases (17, 22), Hrql exhibits a 3'-5' polarity, unwinding substrates that contain a 3' overhang but not a 5' overhang or duplex DNA (Fig. 6B and C). The Hrql-HD allele does not unwind the substrate, demonstrating that the helicase activity we are measuring is intrinsic to Hrql and is not derived from a low-level contaminant (Fig. 6B, D, and F). Interestingly, Hrql is also active on bubble substrates, which may reflect roles for Hrql in melting such structures during the NER of certain lesions (Fig. 6D). We also tested a potential role for the winged-helix Cdt1-like domain in helicase function and found that the Hrql-CDTΔ mutant exhibits robust helicase activity (Fig. 6B and F). Therefore, Hrql-CDTΔ phenotypes are not the result of impaired helicase function; rather, the Cdt1-like domain likely promotes correct subcellular/subnuclear localization.

DISCUSSION

Our analyses of Hrql from fission yeast revealed enzymatic and functional characteristics that are congruent with its bioinformatic assignment as a RecQ4 orthologue (4). Hrql, like other

RecQ helicases, suppresses spontaneous genome instability and does so in a manner dependent on its 3'-5' helicase activity. Furthermore, like RecQ4 (15), Hrql acts in an NER-dependent pathway for DNA lesion repair. Our data do not exclude a role in base excision repair as has been proposed for RecQ4 (55); however, *hrq1Δ* insensitivity to oxidative stress suggests that functions within this repair pathway are not the primary role of Hrql. Nevertheless, further defining Hrql pathways and mechanisms of action will likely provide a valuable framework for determining key conserved RecQ4 functions in disease suppression.

Hrql mutant cells are hypersensitive to cisplatin, a chemotherapeutic agent that produces various DNA lesions, of which the ICL is the most toxic. By tethering the two strands of a DNA duplex, ICLs block the essential processes of transcription and replication, which require strand separation. There has been only a limited analysis of fission yeast pathways that mediate cisplatin resistance, although initial studies have suggested primary roles for NER- and HR-mediated repair pathways that are evolutionarily conserved in higher eukaryotes (27, 63). Our data underscore the importance of NER in cisplatin resistance and further demonstrate that, as in mammals, the XPF-ERCC1 homologues play a predominant role. We also determined that the Ubc13/Mms2 subpathway of PRR plays a critical role parallel to that of Hrql and NER in cisplatin resistance. ICLs cannot be bypassed via PRR without initial endonucleolytic processing. Thus, the predominant role of Ubc13/Mms2, in the absence of NER and Hrql, is likely in the tolerance of intra- rather than interstrand cross-links, which represent over 95% of cisplatin-induced lesions. The fact that the UV sensitivity of *ubc13Δ* cells is strongly exacerbated by deletion of either Hrql or Rhp14 (XPA) further supports a role for Hrql in intrastrand lesion processing by NER. Overall, these data indicate that Hrql

FIG 6 Helicase activity of purified Hrq1. (A) Coomassie staining showing normalized levels of wild-type FLAG-Hrq1 and FLAG-tagged Hrq1 mutant protein purified from *S. pombe*. (B) Hrq1 helicase activity was detected on a 3' overhang DNA substrate over a range from 0.25 μ M to 1 μ M. Activity was measured through the appearance of single-stranded DNA (ssDNA) as indicated by heat denaturation (lane 1). Helicase activity was not observed in the absence of ATP (lane 2) or in the presence of Hrq1-HD (lanes 9 and 10). Proteinase K was not added to lanes 8, 10, and 12. (C) Helicase activity was not observed on 5' overhang and flush duplex DNA substrates. Denatured substrate is indicated in lane 1. Proteinase K was not added to lanes 4. (D) Helicase activity was also tested against duplex DNA containing a 12-bp noncomplementary bubble. Activity was detected over a range from 0.5 μ M to 1 μ M. Denatured substrate is shown in lane 1. Helicase activity was not detected in the absence of ATP (lane 2) or the presence of Hrq1-HD (lane 7). (E and F) Enrichment of unwound ssDNA from the 3' overhang substrate shown in panel B was quantified for wild-type Hrq1 and Hrq1 mutants. Representative images from at least three independent experiments are shown. Unless otherwise stated, 1 μ M protein was used in all helicase assays. The asterisk denotes labeled oligonucleotide.

promotes NER-dependent lesion processing when PRR is compromised (Fig. 7). As noted above, prior to endonucleolytic processing, ICLs cannot be bypassed using the Ubc13/Mms2-dependent branch of PRR. Thus, ICLs effectively nullify damage tolerance by PRR and render cells highly dependent on NER and Hrq1 for survival. This model may explain why *hrq1Δ* cells are sensitive to cisplatin but not other genotoxins.

Hrq1 and NER mutants exhibit an epistatic relationship, with Hrq1 mutant cells being less cisplatin sensitive than those lacking NER factors. We also detected a weak interaction between the NER factor Rhp14 and Hrq1, as observed for RecQ4 and XPA, further supporting a role in NER. Despite this physical interaction, spontaneous or cisplatin-induced GFP-Hrq1 focus formation is Rhp14 independent (data not shown). We propose that Hrq1 facilitates processing of a subset of lesions by NER, and we envisage nonexclusive roles in this regard. As with other RecQ helicases, Hrq1 may facilitate replication fork regression at some lesions to provide space and time for the NER process to be completed, thereby preventing fork collapse. Another potential role is in directly promoting the NER process, which, due to the unique

constraints of an ICL on strand separation, may benefit from local unwinding to produce the favored substrate of the NER nucleases (i.e., duplex single-strand junctions [63]). The bubble unwinding activity we demonstrated for Hrq1 may be relevant in this scenario. Alternatively, following ICL "unhooking," Hrq1 may promote repair synthesis by displacing the incised strand, providing access for polymerases. The repair mechanism(s) of ICLs and the specific roles for many of the experimentally identified factors remain enigmatic. However, elegant studies have indicated that replication forks converging on an ICL can promote repair via NER and recombination, without a clear requirement for helicase activities (26, 33). Thus, future studies are required to precisely define the mechanism of Hrq1 in cisplatin resistance.

Notably, despite insensitivity to most genotoxins tested, *hrq1Δ* and Hrq1-HD cells require the Chk1-dependent G₂ DNA damage checkpoint for viability. Therefore, ICLs generated by endogenously occurring processes, such as lipid peroxidation, appear to be a frequent lesion. Alternatively, Hrq1 may be involved in another aspect of DNA metabolism, which, when defective, activates the DNA damage checkpoint. For example, hypomorphic muta-

FIG 7 Model depicting the potential roles of Hrq1 in NER-based lesion processing. Cisplatin- and UV-induced intrastrand lesions are processed by one of two primary pathways. In PRR, Ubc13-dependent polyubiquitination of PCNA initiates either template switching at the lesion, which proceeds via HR, or translesion synthesis. Alternatively, global genome (GG) or transcription-coupled (TC) branches of NER excise the lesion and resynthesize the missing nucleotides across the resultant gap, processes that, in the absence of PRR, require the helicase activity of Hrq1. Interstrand lesions that cannot be bypassed by PRR are also repaired by an NER-dependent excision process, in a currently enigmatic repair pathway facilitated by Hrq1.

tions in essential DNA replication initiation factors render fission yeast dependent on Chk1 for viability (32, 65). Here, the bubble melting function of Hrq1 may also be relevant to a replication initiation role. In this regard, it is noteworthy that metazoan RecQ4 is essential for DNA replication initiation, via the Sld2-homologous domain in its amino terminus (53). Hrq1 lacks the Sld2-like domain but instead possesses an amino domain sharing sequence homology to the replication initiation factor Cdt1 winged-helix domain. A separate gene encoding Cdt1 is present in yeast, which perhaps renders Hrq1 largely redundant in replication initiation (29, 40). Nevertheless, the Hrq1 Cdt1 homology domain may play a role in recruiting Hrq1 to stalled replication forks or perhaps to certain origins. Based on our subcellular-localization analysis, the Cdt1 homology domain is indeed required for normal localization and function of Hrq1 in genome stability. The Cdt1-like domain is not required for folding or *in vitro* helicase activity of Hrq1, further supporting a specific function for the domain in enzyme targeting.

In addition to protecting against cisplatin-induced DNA damage, Hrq1 suppresses spontaneous genome instability. Besides the synthetic growth defects of *hrq1Δ* with PRR mutants, including *pcn1-K164R*, *ubc13Δ*, and *mms2Δ* mutants, cells lacking Hrq1 require the Rad51 recombinase and the HJ resolvase Mus81-Eme1 for viability. Synthetic lethality with *mus81Δ* indicates that recombination processes involving HJs, excluding DNA double-strand break (DSB) repair, are elevated in the absence of Hrq1 (7, 8, 51). We demonstrated that in the absence of Hrq1, spontaneous lesion repair is funneled into the Ubc13/Mms2-based PRR pathway. Thus, the most parsimonious explanation for the reliance of

hrq1Δ cells on Mus81-Eme1 and Rad51 is engagement of the PRR template-switching damage avoidance mechanism (59). Engagement of this HR-dependent pathway may also explain the synthetic cisplatin sensitivity of *rqh1Δ* and *hrq1Δ* mutants, where Rqh1 is predicted to function within the latter stages of the PRR pathway, perhaps during hemicatenane removal (10). Damage avoidance involves use of the undamaged nascent strand of the sister chromatid as a template to faithfully copy information past the lesion and requires HR and HJ processing (59). Thus, the outputs of PRR, including damage avoidance and error-prone TLS (12), can account for the elevated recombination and mutation rates detected in *hrq1Δ* cells. Increased Rad51-dependent recombination is further supported by the synthetic sick phenotype of *hrq1Δ* with *nse6Δ*. Cells lacking the Smc5-Smc6 complex subunit Nse6 cannot tolerate UV-induced Rad51-dependent recombination structures, which likely represent HJs, as *nse6Δ* mutant UV sensitivity is partially suppressed by expression of the bacterial resolvase RusA (43). Finally, it is also possible that Hrq1 couples NER lesion processing with replication fork progress, thereby preventing collision with incised repair intermediates that could otherwise cause replication fork collapse. Again, in this scenario Mus81-Eme1 and Rhp51 would be crucial, as they are essential for replication restart and viability of cells with just a single collapsed fork (51).

We have defined critical genome stability roles of a novel RecQ family helicase in fission yeast, which potentially has major ramifications for unraveling the enigmatic functions of RecQ4 in human disease. Several lines of evidence indicate that Hrq1 represents a new RecQ family helicase in fission yeast, which is generally

described as having only a single RecQ helicase, Rqh1. First, bioinformatics analysis recently identified a group of fungal and plant proteins as likely orthologues of the human disease-associated RecQ4 helicase (4). Second, Hrq1 possesses RecQ polarity DEAH box-dependent 3'-5' DNA helicase activity. Third, as with other RecQ helicases, Hrq1 maintains genome stability in the face of endogenous and exogenous genotoxins. Intriguingly, our data reveal clear parallels between Hrq1 and RecQ4 function, with both helicases operating in NER-based processes; in each case, this is supported by both genetic and biochemical evidence. If RecQ4 also acts parallel to the PRR pathway, then this could explain some of the RecQ4-related disease phenotypes. For example, a subset of RTS patients exhibits photosensitivity and predisposition to skin cancer, consistent with NER deficiency (5, 60). Furthermore, RTS cells have delayed NER kinetics, which can be complemented by expression of RecQ4 (15). However, the literature regarding genotoxin sensitivity of RTS patient cells is heterogeneous; based on our Hrq1 data, we suggest that this could be related to differing efficiency of PRR in these patients. That is, fission yeasts lacking Hrq1 are not UV sensitive unless PRR is also compromised. However, they do exhibit a hyperrecombination and mutator phenotype, likely due to the increased use of PRR. Thus, if PRR were inefficient in certain RTS patients due to secondary mutations, they might also be genotoxin sensitive. Ongoing studies of Hrq1 and RecQ4 will test these hypotheses and may provide important answers to the enigmatic roles of RecQ4 in disease.

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