The Role of MRN in the S-Phase DNA Damage Checkpoint Is Independent of Its Ctp1-dependent Roles in Double-Strand Break Repair and Checkpoint Signaling

Mary E. Porter-Goff and Nicholas Rhind

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605

Submitted October 1, 2008; Revised January 28, 2009; Accepted January 30, 2009

Monitoring Editor: Daniel J. Lew

The Mre11-Rad50-Nbs1 (MRN) complex has many biological functions: processing of double-strand breaks in meiosis, homologous recombination, telomere maintenance, S-phase checkpoint, and genome stability during replication. In the S-phase DNA damage checkpoint, MRN acts both in activation of checkpoint signaling and downstream of the checkpoint kinases to slow DNA replication. Mechanistically, MRN, along with its cofactor Ctp1, is involved in 5' resection to create single-stranded DNA that is required for both signaling and homologous recombination. However, it is unclear whether resection is essential for all of the cellular functions of MRN. To dissect the various roles of MRN, we performed a structure–function analysis of nuclease dead alleles and potential separation-of-function alleles analogous to those found in the human disease ataxia telangiectasia-like disorder, which is caused by mutations in Mre11. We find that several alleles of *rad32* (the fission yeast homologue of *mre11*), along with *ctp1Δ*, are defective in double-strand break repair and most other functions of the complex, but they maintain an intact S phase DNA damage checkpoint. Thus, the MRN S-phase checkpoint role is separate from its Ctp1- and resection-dependent role in double-strand break repair. This observation leads us to conclude that other functions of MRN, possibly its role in replication fork metabolism, are required for S-phase DNA damage checkpoint function.

INTRODUCTION

Mre11-Rad50-Nbs1 (MRN) is a heterotrimeric complex composed of Mre11, Rad50, and Nbs1 (Carney *et al.*, 1998; Haber, 1998; D'Amours and Jackson, 2002; Williams *et al.*, 2007). Originally identified in budding yeast as recombinational repair proteins, Rad50 and Mre11 are conserved from humans to bacteria and even some bacteriophages. The third MRN subunit found in eukaryotes, Nbs1, is thought to play a regulatory role (Lee *et al.*, 2003). The degree of conservation of the MRN complex between species suggests a critical role in DNA transactions. One of its conserved roles is in the S-phase DNA damage checkpoint, in which it is involved in slowing replication in response to DNA damage in both humans and fission yeast (Young and Painter, 1989; Lavin and Shiloh, 1997; Stewart *et al.*, 1999; Chahwan *et al.*, 2003).

The MRN complex has many roles in cellular metabolism; it functions not only in the S-phase DNA damage checkpoint but also in recombinational repair, telomere maintenance, processing of double-strand breaks in meiosis and another, as yet undefined, role in normal replication (Carney *et al.*, 1998; Haber, 1998; D'Amours and Jackson, 2002). Its role in normal replication prevents the accumulation of broken replication forks possibly through homologous recombination (Costanzo *et al.*, 2001; Maser *et al.*, 2001; Chahwan *et al.*, 2003; Trenz *et al.*, 2006; Wen *et al.*, 2008). In vertebrates, the deletion of any member of the MRN complex results in cell

lethality (Xiao and Weaver, 1997; Luo *et al.*, 1999; Zhu *et al.*, 2001; Dumon-Jones *et al.*, 2003; Difilippantonio *et al.*, 2005). The deletion of MRN subunits in budding and fission yeast is characterized by slow growth, S-phase accumulation, and increased chromosome instability. These phenotypes are probably due to defects in repairing spontaneous fork collapses (Hartsuiker *et al.*, 2001; Myung and Kolodner, 2002). Hypomorphic mutations in Mre11 and Nbs1 lead to two diseases in humans, ataxia-telangiectasia-like disease (ATLD) and Nijmegen breakage syndrome (NBS) (Petrini, 2000). Both of these diseases are phenotypically related to ataxia-telangiectasia (AT), which is caused by mutations in the checkpoint kinase AT-mutated (ATM) (Painter and Young, 1980; Stewart *et al.*, 1999). In addition to neuronal and immunological phenotypes, AT patients display predisposition to cancer, hypersensitivity to double-strand break-inducing agents, chromosome instability, and radioresistant DNA synthesis (RDS). RDS occurs when cells are unable to induce the S-phase DNA damage checkpoint and therefore are unable to slow replication. Cells from AT patients show no slowing of replication in response to ionizing radiation and radiomimetic agents (Lavin and Shiloh, 1997). Cells of ATLD and NBS patients show an intermediate overall slowing of replication (Young and Painter, 1989; Falck *et al.*, 2002). These hypomorphic mutations must maintain some checkpoint-independent function of the MRN complex because the cells are able to survive (Carney *et al.*, 1998; Stewart *et al.*, 1999). Thus, it has been suggested that they display separation of checkpoint-dependent and -independent functions of MRN.

In humans and fission yeast, MRN plays roles both upstream and downstream of the checkpoint kinases to mediate the DNA damage response (Lavin, 2004; Willis and Rhind, unpublished data). The complex is one of the first

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-09-0986>) on February 11, 2009.

Address correspondence to: Nicholas Rhind (nick.rhind@umassmed.edu).

sets of proteins recruited to sites of damage both in humans and in *Saccharomyces cerevisiae* (Nelms *et al.*, 1998; Lisby *et al.*, 2004). MRN directly activates ATM, although partial activation still occurs without the MRN (Lee and Paull, 2004). In addition, MRN is phosphorylated by ATM on Nbs1 (Stewart *et al.*, 1999; Gatei *et al.*, 2000; Lim *et al.*, 2000; Zhao *et al.*, 2000). In addition to its role in the activation of checkpoint signaling, MRN is required downstream of the checkpoint kinases to slow replication in response to DNA damage (Falck *et al.*, 2002; Willis and Rhind, unpublished data). The downstream checkpoint-dependent role of MRN is not well understood, but it may involve recombinational repair of stalled forks (Rhind and Russell, 2000; Willis and Rhind, 2009).

In *Schizosaccharomyces pombe*, S-phase checkpoint signaling requires Rad3, the fission yeast homologue of ATR (ATM- and Rad3-related kinase); the ATM homologue Tel1 is not required for the S-phase DNA damage checkpoint (Bentley *et al.*, 1996; Nakamura *et al.*, 2002; Willis and Rhind, unpublished data). Nonetheless, MRN is required for full activation of checkpoint signaling during S-phase (Willis and Rhind, unpublished data).

MRN possesses several biochemical activities, including double-strand and single-strand DNA binding, DNA tethering, and ATPase, adenylate kinase, and endo- and exonuclease activities (Williams *et al.*, 2007). Mre11 is the nuclease subunit of MRN. It interacts with both Rad50 and Nbs1 independently and can itself form homodimers and homomultimers (D'Amours and Jackson, 2002). Mre11 contains DNA binding domains as well as phosphoesterase motifs responsible for the nuclease activities of the complex (Furuse *et al.*, 1998; Usui *et al.*, 1998; Moreau *et al.*, 1999; Figure 1). The nuclease activity of the MRN complex plays a critical role in 5' resection of double-strand breaks to create single-strand intermediates for repair by homologous recombination (Lewis *et al.*, 2004; Jazayeri *et al.*, 2006; Myers and Cortez, 2006; Chen *et al.*, 2008). The resection activity and double-strand break repair function of MRN require a cofactor: Ctp1 in fission yeast, Sae2 in budding yeast, and CtIP in humans (Chen *et al.*, 2007; Limbo *et al.*, 2007; Sartori *et al.*, 2007).

With all the roles that the MRN plays in cellular metabolism, dissecting its role in the S-phase DNA damage checkpoint is difficult. For example, it is not known whether MRN has one major biochemical activity that is responsible for all of its DNA repair roles or whether it has multiple separable biochemical activities that are independently required for different roles. Mutations that separate its role in the checkpoint from its other roles would directly address this question. In particular, analysis of MRN separation-of-function alleles might elucidate which of the various biochemical activities of MRN are required for checkpoint-dependent slowing of replication in response to DNA damage. Mutations in *S. cerevisiae* Mre11 show separation of function between its meiotic roles and its mitotic roles (Nairz and Klein, 1997; Symington, 2002). Furthermore, several nuclease dead mutations in budding yeast maintain many mitotic MRN functions, yet they are defective in meiotic function and are sensitive to DNA damage (Bressan *et al.*, 1998; Moreau *et al.*, 1999; Chen *et al.*, 2001; Yazdi *et al.*, 2002; Krogh *et al.*, 2005). Mutations that separate the checkpoint-dependent from checkpoint-independent roles in fission yeast have not been reported, but based on characterization of human ATLD and NBS phenotypes, which seem to affect checkpoint-dependent but not constitutive functions of MRN, we hypothesized that they would exist. Given the precedent of the ATLD alleles in humans and the nuclease dead alleles in budding yeast, we decided to focus on the fission yeast homologue of Mre11, Rad32. We have phenotypically characterized various alleles of *rad32* and found that the role of MRN in checkpoint-dependent slowing of replication is, in fact, genetically separable from its roles in checkpoint signaling and homologous recombination.

Figure 1. (A) Schematic of Rad32 and the sites of mutations. (B) Wild-type (yFS467), *rad32Δ* (yFS450), *rad32-H134N* (yFS468), *rad32-D65N* (yFS469), *rad32-D25A* (yFS470), *rad32-H134L/D135V* (yFS471), *rad32-N122S* (yFS472), *rad32-W215C* (yFS473), and *rad32ΔC* (yFS466) cells in log phase were imaged in 100 $\times$ magnification. Five of the seven mutants seem similar to the *rad32Δ* strain. The other two mutants have a wild-type appearance. The morphological defect is quantified below each figure with the percentage of cell in each culture displaying normal morphology ($n = 500$ for each).

MATERIALS AND METHODS

Yeast were grown in yeast extract plus supplements (YES) at 30°C and manipulated by standard methods (Forsburg and Rhind, 2006). Temperature-sensitive strains were grown at 25°C unless otherwise stated. Strains used for this study are listed in Table 1.

Strain Construction

Rad32 C-terminal deletion strain was made using a polymerase chain reaction (PCR) cassette insertion to replace residues 528–649 with a 13Myc tag (Bahler *et al.*, 1998). The sequences of the oligonucleotides are listed in Supplemental Table S1. *rad32::kanMX4*, *rad3::ura4*, *rad2::ura4* have been described previously (Bentley *et al.*, 1996; Morishita *et al.*, 2002; Chahwan *et al.*, 2003).

Site-directed alleles of *rad32* were made by oligomediated mutagenesis. Primers were designed to change the desired amino acids by four or less nucleotide changes and also create unique restriction sites. Primer sequences containing mutations and the appropriate restriction site changes are in Supplemental Table S1. The 3' end of *rad32* was cloned into pFA6a-13Myc::natMX6 (Sato *et al.*, 2005) by using MPG50 and MPG51 with HindIII and PacI, creating pFS305. A second 3' piece was cloned into that plasmid by using MPG52 and MPG53 cut with SacI and SacII, creating pFS306. Finally, 5' mutants were cloned into pFS306 by using MPG3 and MPG6 with NdeI and HindIII as the product of a two-step PCR; each mutant primer was paired with either MPG3 or MPG6 for the first step and then each mutant product pair was used as template for PCR with MPG3 and MPG6 to create each of the six mutants. The resulting plasmids (pFS307–pFS313) were cut with NdeI and SacII; the *rad32* open reading frame fragment was transformed into wild-type (wt) cells (yFS105). Integrants were selected on nourseothricin. Accurate integration was confirmed by PCR and sequencing.

X-Ray, UV Irradiation, Methyl Methane Sulfonate (MMS), and Hydroxyurea (HU) Survival Assays

Cells were grown to mid-log phase in liquid YES and fivefold serially diluted. Aliquots of each dilution were plated on solid medium containing 0.01% MMS,

Table 1. Yeast strains used in this study

Strain	Genotype	Source
NYSPG2	<i>h+ leu1-32 ura4-D18 ade6-704 rad2::ura4</i>	Tsutsui <i>et al.</i> (2005)
OL4121	<i>h- leu1-32 ura4-D18 ctp1::kanMX6</i>	Limbo <i>et al.</i> (2007)
yFS447	<i>h+ leu1-32 ura4-D18 ade6-216 his3-D1 pku70::his3</i>	Manolis <i>et al.</i> (2001)
yFS105	<i>h- leu1-32 ura4-D18</i>	Lab stock
yFS189	<i>h- leu1-32 ura4-? ade6-704 rad3::ura4</i>	Lab stock
yFS231	<i>h- leu1-32 ura4-D18 ade6-M210 his3-D1 trt1::his3</i>	Lab stock
yFS249	<i>h- leu1-32 ura4-D18 nbs1::kanMX6</i>	Lab stock
yFS260	<i>h- leu1-32 ura4-D18 rad3::ura4 cdc10-M17</i>	Lab stock
yFS262	<i>h+ leu1-32 ura4-D18 ade6-210 his3-D1 cdc10-M17 rad32::kanMX4</i>	Lab stock
yFS450	<i>h- leu1-32 ura4-D18 his3-D1 rad32::kanMX4</i>	Lab stock
yFS466	<i>h- leu1-32 ura4-D18 rad32ΔC-13Myc:kanMX6</i>	This study
yFS467	<i>h- leu1-32 ura4-D18 rad32-13Myc:natMX6</i>	This study
yFS468	<i>h- leu1-32 ura4-D18 rad32-H134N-13Myc:natMX6</i>	This study
yFS469	<i>h- leu1-32 ura4-D18 rad32-D65N-13Myc:natMX6</i>	This study
yFS470	<i>h- leu1-32 ura4-D18 rad32-D25A-13Myc:natMX6</i>	This study
yFS471	<i>h- leu1-32 ura4-D18 rad32-H134L/D135V-13Myc:natMX6</i>	This study
yFS472	<i>h- leu1-32 ura4-D18 rad32-N122S-13Myc:natMX6</i>	This study
yFS473	<i>h- leu1-32 ura4-D18 rad32-W215C-13Myc:natMX6</i>	This study
yFS474	<i>h+ leu1-32 ura4-D18 ade6-M210 his3-D1 cdc10-M17 rad32-13Myc:kanMX4</i>	This study
yFS475	<i>h+ leu1-32 ura4-D18 ade6-M210 cdc10-M17 rad32-H134N-13Myc:natMX6</i>	This study
yFS476	<i>h+ leu1-32 ura4-D18 ade6-M210 cdc10-M17 rad32-N122S-13Myc:natMX6</i>	This study
yFS477	<i>h- leu1-32 ura4-D18 ade6-M210 cdc10-M17 rad32-W215C-13Myc:natMX6</i>	This study
yFS478	<i>h+ leu1-32 ura4-D18 cdc10-M17 rad32-H134L/D135V-13Myc:natMX6</i>	This study
yFS479	<i>h+ leu1-32 ura4-D18 ade6- M210 cdc10-M17 rad32-D25A-13Myc:natMX6</i>	This study
yFS481	<i>h+ leu1-32 ura4-D18 ade6-M210 cdc10-M17 rad32-D65N-13Myc:natMX6</i>	This study
yFS483	<i>h- leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32-13Myc:natMX6</i>	This study
yFS484	<i>h+ leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32-N122S-13Myc:natMX6</i>	This study
yFS485	<i>h- leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32-D65N-13Myc:natMX6</i>	This study
yFS486	<i>h+ leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32-D25A-13Myc:natMX6</i>	This study
yFS487	<i>h+ leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32-H134L/D135V-13Myc:natMX6</i>	This study
yFS488	<i>h+ leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32-N122S-13Myc:natMX6</i>	This study
yFS489	<i>h- leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32-W215C-13Myc:natMX6</i>	This study
yFS490	<i>h- leu1-32 ura4-D18 nbs1-HA-TAP:kanMX6 rad32ΔC-13Myc:kanMX6</i>	This study
yFS611	<i>h+ leu1-32 ura4-D18 ade6-M210 cdc10-M17 rad32ΔC-13Myc:kanMX6</i>	This study
yFS618	<i>h+ leu1-32 ura4-? rad32-N122S-13Myc:natMX6 rad3::ura4</i>	This study
yFS619	<i>h+ leu1-32 ura4-? rad32-W215C-13Myc:natMX6 rad3::ura4</i>	This study
yFS650	<i>h+ leu1-32 ura4-D18 cdc10-M17 ctp1::kanMX6</i>	This study
yFS651	<i>h+ leu1-32 ura4-D18 rad32-13Myc:natMX6</i>	This study
yFS652	<i>h+ leu1-32 ura4-D18 rad32-H134N-13Myc:natMX6</i>	This study
yFS653	<i>h+ leu1-32 ura4-D18 rad32-D65N-13Myc:natMX6</i>	This study
yFS654	<i>h+ leu1-32 ura4-D18 his3-D1 rad32::kanMX4</i>	This study
yFS655	<i>h+ leu1-32 ura4-D18 rad32ΔC-13Myc:kanMX6</i>	This study
yFS656	<i>h+ leu1-32 ura4-D18 rad32-D25A-13Myc:natMX6</i>	This study
yFS657	<i>h+ leu1-32 ura4-D18 rad32-H134L/D135V-13Myc:natMX6</i>	This study
yFS658	<i>h+ leu1-32 ura4-D18 rad32-N122S-13Myc:natMX6</i>	This study
yFS659	<i>h+ leu1-32 ura4-D18 rad32-W215C-13Myc:natMX6</i>	This study
yFS660	<i>h+ leu1-32 ura4-D18 ctp1::kanMX6</i>	This study
yFS670	<i>h- leu1-32 ura4-? rad32-D65N-13Myc:natMX6 rad3::ura4</i>	This study
yFS671	<i>h+ leu1-32 ura4-? ade6-704 rad32-H134N-13Myc:natMX6 rad3::ura4</i>	This study
yFS672	<i>h- leu1-32 ura4-? rad32ΔC-13Myc:kanMX6 rad3::ura4</i>	This study
yFS673	<i>h+ leu1-32 ura4-? ade6-704 rad50::kanMX6 rad3::ura4</i>	This study
yFS674	<i>h+ leu1-32 ura4-? rad32-D25A-13Myc:natMX6 rad3::ura4</i>	This study
yFS675	<i>h+ leu1-32 ura4-? nbs1::kanMX6 rad3::ura4</i>	This study
yFS676	<i>h- leu1-32 ura4-? his3-D1 rad32::kanMX6 rad3::ura4</i>	This study
yFS677	<i>h- leu1-32 ura4-? ctp1::kanMX6 rad3::ura4</i>	This study
yFS678	<i>h? leu1-32 ura4-? rad32-H134L/D135V-13Myc:natMX6 rad3::ura4</i>	This study

0.03% MMS, 1 mM HU, or 3 mM HU and then grown for 4 d at 30°C. For acute UV exposure, mid-log liquid YES cultures were counted, plated on YES solid media, irradiated in a UV Stratalinker 2400 (Stratagene, La Jolla, CA), and grown for 30°C for 3 or 4 d. X-ray irradiation was done in a Faxitron x-ray RX-650 at 130 kVp at 5 mA (10 Gy/min) at room temperature with cells suspended in liquid YES; cells were immediately plated in triplicate on YES solid media, incubated for 3 or 4 d, and counted.

Telomere Southern

Genomic DNA was isolated from each strain. DNA was digested overnight with EcoRI and run on a 2% agarose gel. Ethidium bromide staining confirmed

equal loading. The gel was transferred by neutral transfer to Hybond N+ (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) and probed with ³²P-labeled telomere associated-1 (TAS1) (Nakamura *et al.*, 2002).

Rad2 Synthetic Lethality

rad32 mutant strains were crossed to *rad2::ura4*, and spores were digested with glusulase and plated on YES. The resulting colonies were replica plated to Edinburgh minimal media 2 supplemented with leucine, adenine, and histidine (EMM2 LAH) and either nourseothricin (Werner BioAgents, Jena, Germany) or Geneticin (G418; Invitrogen, Carlsbad, CA) as appropriate. Surviving colonies were counted. Random spore analysis was used because of

the poor spore viability of the *rad32* mutants. To correct for the low spore viability of *rad32* mutants, expected viability (E) was calculated by normalizing the number of *rad32* mutant (–) *rad2Δ* colonies to the number of *rad32–rad2+* and *rad32+ rad2Δ* colonies by using the formula $E = A \times (B/A) \times (C/A)$, where A is the number of *rad32+ rad2+* colonies, B is the number of *rad32–rad2+* colonies, and C is the number of *rad32+ rad2Δ* colonies.

Nonhomologous End Joining (NHEJ) Assays

The NHEJ plasmid assay was performed essentially as described previously (Manolis *et al.*, 2001). The plasmid pUR19 (Barbet *et al.*, 1992) was linearized with *Sma*I. Logarithmically growing cells were transformed with equal amounts of undigested or linear DNA by using the lithium acetate transformation method (Okazaki *et al.*, 1990). Cells were plated on EMM2 LAH plates in triplicate. *ura+* colonies were counted, and NHEJ frequency was calculated by the number of colonies from linear DNA over those transformed with undigested DNA. Cells grown in nitrogen-free media were first grown to log phase in YES and then transferred to EMM2-N media (United States Biological, Swampscott, MA) for 36 h before transformation (Ferreira and Cooper, 2004).

Coimmunoprecipitations

Cells were grown to mid-log phase in liquid YES, and then 40 OD of cells were collected, washed with water, frozen in liquid nitrogen, and stored at –80°C. Extracts were prepared as described previously (Boddy *et al.*, 1998). Cells were lysed with 200 μ l of lysis buffer. Cleared cell extracts were incubated with rabbit immunoglobulin G (IgG)-Agarose beads (Sigma-Aldrich, St. Louis, MO). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted, and visualized with anti-HA antibody directly conjugated to horseradish peroxidase (HRP; Roche Diagnostics, Indianapolis, IN). HRP was quenched with 0.10% sodium azide, the blot was washed, and proteins were visualized with anti-Myc antibody directly conjugated to HRP.

Cds1 Kinase Assay

Cells were grown in liquid YES to mid-log phase and treated with 0.03% MMS for 4 h. Then, 10 OD pellets were collected for the kinase assay. In vitro kinase assays were performed as described previously (Kai *et al.*, 2001; Dutta *et al.*, 2008; Willis and Rhind, 2008). Immunopurified Cds1 was incubated with myelin basic protein and [γ -³²P]ATP at 30°C for 15 min. Proteins were separated by SDS-PAGE and visualized by autoradiography.

S-Phase Progression

Synchronization, flow cytometry of isolated nuclei, and S-phase progression analysis was performed as described previously (Kommajosyula and Rhind, 2006; Willis and Rhind, 2008).

RESULTS

In a search for alleles that would separate the checkpoint functions of MRN from its checkpoint-independent functions, we constructed seven site-directed alleles analogous to nuclease dead alleles in *S. cerevisiae* (*rad32-D25A*, *rad32-D65N*, *rad32-H134N*, and *rad32-H134L/D135V*) or to ATLD alleles in humans (*rad32-N122S*, *rad32-W215C*, and *rad32ΔC*) (Figure 1A; Bressan *et al.*, 1998; Moreau *et al.*, 1999; Stewart *et al.*, 1999; Chen *et al.*, 2001; Yazdi *et al.*, 2002; Fernet *et al.*, 2005). These alleles were used to replace the wild-type *rad32* allele at its endogenous locus. All of the alleles are tagged with 13 copies of the Myc epitope, so our analyses use *rad32-13Myc* for the wild-type control. In a variety of phenotypic assays, we found that cells carrying two of our alleles, the class I mutants (*rad32-N122S* and *rad32-W215C*), seem similar to wild type; cells carrying three alleles, the class II mutants (*rad32-D25A*, *rad32-D65N*, and *rad32-H134N*), seem similar to *rad32Δ* cells; cells carrying two other alleles, the class III mutants (*rad32-H134L/D135V* and *rad32ΔC*) resemble *rad32Δ* cells in all assays except the checkpoint assay, in which they display checkpoint proficiency (Table 3). The details of these phenotypic characterizations follow.

The simplest phenotypic assays for *rad32* alleles are growth rate and cell morphology. *rad32Δ* cells accumulate DNA damage, which leads to cell cycle arrest and death in a significant fraction of cells and reduces the growth rate of the culture (Hartsuiker *et al.*, 2001; Myung and Kolodner, 2002; Chahwan *et al.*, 2003). The class II and III mutants resemble *rad32Δ* cells in both growth rate and heterogeneity

of cell morphology; the class I mutants exhibit wild-type morphology and growth rate (Figure 1B). These results suggest that the class I mutants retain the Rad32 constitutive repair and genome stability functions, functions that seem to be missing in the class II and III mutants.

Sensitivity to DNA Damage and Replication Arrest

To more carefully investigate the repair and genome stability phenotypes of our mutants, we used a panel of assays. We first analyzed the DNA damage sensitivity of our mutants. We tested the survival of cells treated with UV irradiation, x-ray irradiation, and the alkylating agent MMS. In response to all three treatments, our mutants fall into two broad categories, with the class I mutants being similar to wild-type cells in resistance to DNA damage and the class II and III mutants being similar to *rad32Δ* cells in sensitivity (Figure 2, A–C). However, within those categories there is some heterogeneity. For example, both of the class I mutants are slightly more sensitive to MMS than wild type. In addition, one of the class II mutants, *rad32-D65N*, is slightly more resistant to all three DNA-damaging agents than *rad32Δ* whereas both of the class III mutants are slightly more resistant to UV and one of them, *rad32ΔC*, is slightly more resistant to MMS.

We also tested our mutants for sensitivity to HU, which blocks replication by inhibiting deoxyribonucleotide synthesis. Again, the class I mutants are similar to wild-type cells, and the class II and III mutants are similar to *rad32Δ* cells, with some heterogeneity. In this case, several of the class II and III mutants, *rad32-H134N*, *rad32-D65N*, and *rad32ΔC*, show intermediate resistance to HU between that of wild-type and *rad32Δ* cells.

Telomere Maintenance

rad32Δ strains have shortened telomeres compared with wild type, and *rad32Δ rad3Δ* strains suffer catastrophic telomere loss and are only able to survive by circularizing their chromosomes (Wilson *et al.*, 1999; Nakamura *et al.*, 2002). We tested our mutant strains for telomere loss in a *rad3Δ* background. *rad3Δ* double mutants were constructed and genomic Southern blots were probed with a telomere-associated sequence. *rad32-D25A*, *rad32-H134L/D135V*, and *rad32ΔC* lost their telomeres, whereas all other mutants have maintained their telomeres (Figure 3).

Synthetic Lethality of *rad32* and *rad2* Double Mutants

As an independent assay for the role of MRN in DNA repair, we examined the viability of our alleles in combination with *rad2Δ*. Rad2 is the fission yeast homologue of Fen1, a flap endonuclease involved in the maturation of Okazaki fragments in lagging-strand DNA synthesis (Murray *et al.*, 1994; Symington, 1998; Moreau *et al.*, 1999). Without Rad2, cells use other mechanisms to mature the RNA-containing flap (Symington, 1998). *rad32Δ* is synthetically lethal with *rad2Δ*, apparently because MRN-dependent homologous recombination is required after replication in the absence of Rad2 (Tavassoli *et al.*, 1995; Symington, 1998). Because of the low viability of spores from diploids heterozygous for our *rad32* alleles, we did our assay using random spore analysis. The class I alleles are not synthetically lethal with *rad2Δ*; all of the class II and III alleles are lethal (Table 2).

Nonhomologous End Joining

In *S. cerevisiae*, the MRN complex is required for NHEJ (Zhang and Paull, 2005). Previously, it was shown that the MRN is not required for NHEJ in asynchronous fission yeast (Manolis *et al.*, 2001). However, because *S. pombe* spends most of its time in G2, these experiments did not address the requirement in G1, when NHEJ is most active (Ferreira and

Figure 2. DNA-damaging agent sensitivity of wild-type (yFS467), *rad32Δ* (yFS450), *rad32-H134N* (yFS468), *rad32-D65N* (yFS469), *rad32-D25A* (yFS470), *rad32-H134L/D135V* (yFS471), *rad32-N122S* (yFS472), *rad32-W215C* (yFS473), and *rad32ΔC* (yFS466). (A) MMS and HU sensitivity. Cells were diluted in a threefold serial dilution and spotted onto YES plates containing 1 or 3 mM HU or 0.01 or 0.03% MMS as indicated. (B) UV irradiation sensitivity. The curves are average of at least five independent experiments. Error bars represent the SE of the mean. (C) x-ray sensitivity. The curves represent the average of triplicate plating of a single experiment $\pm$ SE of the mean.

Cooper, 2004). In fact, there is a requirement for some proteins in G1 that are not required in G2 (Ferreira and Cooper, 2004). To look at the role of the MRN in NHEJ in G1, we used a plasmid religation assay in cells arrest in G1 by nitrogen starvation. Figure 4 confirms that the MRN is not required for NHEJ in fission yeast, even in G1.

Meiosis

MRN is required for the processing of double-strand breaks in meiosis and thus for spore viability (Tavassoli *et al.*, 1995; Wilson *et al.*, 1998; Symington, 2002). To test the function of our alleles in meiosis, we measured spore viability by random spore analysis. We tested the viability of spores from zygotes homozygous for our alleles. Class I mutants have spore viabilities similar to that of wild-type cells. Class II and class III mutants show a substantial reduction in spore viability, with all but one showing the same lack of spore viability as *rad32Δ* zygotes; one class III allele, *rad32ΔC*, retains some residual spore viability (Table 2).

MRN Complex Formation

Observations in *S. cerevisiae* indicate that some of the nuclease motif mutations disrupt MRN complex formation (Krogh *et al.*, 2005). To assay for complex formation in our strains, we tested the interaction of Rad32 with Nbs1. We built doubly tagged strains carrying both a mutant *rad32* allele carboxy-terminally tagged with 13 copies of the Myc epitope and a carboxy-terminal HA and protein A affinity (HA-TAP)-tagged *nbs1* allele. Cells expressing both tagged proteins were lysed and Nbs1 captured on IgG agarose beads. After washing the beads, bound proteins were separated by SDS-PAGE, blotted, and probed with anti-HA to

Figure 3. Telomere function. Southern blot analysis of EcoRI-digested genomic DNA from *rad32Δ* (yFS450), wild type (yFS467), *rad3Δ* (yFS189), *ctp1Δ* (OL4121), *trt1Δ* (yFS231), *rad32Δ rad3Δ* (yFS676), *nbs1 rad3Δ* (yFS675), *rad50Δ rad3Δ* (yFS673), *ctp1Δ rad3Δ* (yFS677), *rad32-D25A rad3Δ* (yFS674), *rad32-D65N rad3Δ* (yFS670), *rad32-H134N rad3Δ* (yFS671), *rad32ΔC rad3Δ* (yFS672), *rad32-N122S rad3Δ* (yFS618), and *rad32-W215C rad3Δ* (yFS619), probed with the TAS1 probe. *trt1Δ*, telomerase reverse transcriptase, was used as a control for telomere loss (Nakamura *et al.*, 1998). *rad32-H134L/D135V rad3Δ* (yFS678) loses telomeres (data not shown).

Table 2. Spore viability and Rad2 synthetic lethality

Genotype	<i>rad32</i> % spore viability ^b	<i>rad32 rad2</i> spore viability ^a				Expected viability ^c
		<i>rad32+</i> <i>rad2+</i>	<i>rad32-</i> <i>rad2+</i>	<i>rad32+</i> <i>rad2Δ</i>	<i>rad32-</i> <i>rad2Δ</i>	
<i>rad32+</i>	56.6 ± 17.7	183	101	84	52	46
<i>N122S</i>	40.0 ± 19.3	271	107	53	27	21
<i>W215C</i>	36.7 ± 18.1	310	156	135	69	68
<i>rad32Δ</i>	0.04 ± 0.02	40	9	14	0	3
<i>H134N</i>	0.63 ± 0.58	340	104	65	0	20
<i>D65N</i>	0.16 ± 0.14	352	102	92	0	27
<i>D25A</i>	0.03 ± 0.02	79	30	29	0	11
<i>H134L/D135V</i>	0.06 ± 0.05	126	42	51	0	17
ΔC	6.58 ± 2.68	872	341	172	0	67

^a Total number of colonies.^b Mean ± SEM (n = 3).^c Expected number of *rad32-* *rad2Δ* colonies, normalized for *rad32-* spore viability.

visualize Nbs1 (Figure 5). The blot was reprobed with anti-Myc to visualize Rad32. Two of the class II protein, Rad32-H134N and Rad32-D65N, as well as one of the class III proteins, Rad32- ΔC , expressed well and bound to the beads at wild-type levels. Both of the class I proteins and one of the class III proteins, Rad32-H134L/D135V, expressed at lower levels than wild type. In addition, these proteins bound the beads with variable efficiency; in some preps they showed significant binding, albeit less than wild type, and in others they showed only background levels of binding. These mutations are believed to be disrupted the Nbs1 binding domain and inconsistent Nbs1 binding has been reported for the human homologues of the class I proteins (Stewart *et al.*, 1999; Lee *et al.*, 2003; Fernet *et al.*, 2005). Finally, one class II protein, Rad32-D25A, reproducibly expressed at lower levels and did not bind to the beads above background levels, consistent with its failure to bind Rad50 (Tomita *et al.*, 2003). *rad32-D25A* cells also failed to express Nbs1 at detectable levels in some preparations while expressing at very low levels in others.

S-Phase DNA Damage Checkpoint

We next examined the S-phase checkpoint response to damage of our *rad32* mutants. MMS, which produces DNA dam-

age in the form of base adducts, was used to induce the checkpoint. MMS-induced damage is recognized during replication and therefore is a good activator of the S-phase DNA damage checkpoint. Cells were synchronized in G1 by a combination of elutriation and a *cdc10-ts* block and release, treated with 0.03% MMS and followed through replication by flow cytometry (Figure 6, B–D). Untreated cells replicated between 40 and 80 min after release from G1. MMS-treated wild-type cells fail to complete replication by 180 min. However, checkpoint defective strains, such as *rad3Δ*, continue replication despite the presence of DNA damage. *rad32Δ* cells present a partial checkpoint response as they slow their S-phase progression more than *rad3Δ* but do not slow to the same extent as wild-type cells (Willis and Rhind, unpublished data). The class I mutants, which are wild type in other assays, had intact checkpoints and the class II mutants, which act as nulls in other assays, had defective checkpoints. However, the class III mutants, which act as nulls in other assays, have intact checkpoints and thus display a separation-of-function phenotype.

Cds1 Kinase Activity

To test whether the checkpoint phenotypes correlate with defects in the role of MRN in S-phase DNA damage checkpoint signaling, we checked to see whether the Cds1 kinase activity is compromised in our mutants. MRN mutants in human cells display a partial defect in ATM checkpoint signaling (Lee and Paull, 2004). Similarly, we have seen a partial defect in Cds1 S-phase checkpoint kinase activity in *nbs1Δ* cells (Willis and Rhind, unpublished data). We found that the class I alleles have wild-type levels of kinase activity, whereas class II and III alleles have significantly lower kinase activity levels (Figure 7). One class II allele, *rad32-D25A*, is as defective for signaling as *rad32Δ*. Another class II allele, *rad32-H134N*, and both class III alleles are severely defective but show some residual signaling. The third class II allele, *rad32-D65N*, shows intermediate signaling.

Ctp1 Is Not Required for the S-Phase DNA Damage Checkpoint

To test the requirement of 5' resection in the various functions of MRN, we examined the phenotypes of *ctp1Δ* cells. Ctp1 has recently been shown to function with the MRN complex in 5' resection (Limbo *et al.*, 2007; Sartori *et al.*, 2007; Takeda *et al.*, 2007). In our panel of phenotypic assays, *ctp1Δ* behaves as a class III separation of function allele (Table 3).

Figure 4. The MRN is not required for nonhomologous end joining. Analysis of wild-type (yFS467), *rad32Δ* (yFS450), and *pku70Δ* (yFS447) NHEJ levels by using a plasmid religation assay. *pku70Δ*, which is required for NHEJ in G1, was used as a control (Manolis *et al.*, 2001; Ferreira and Cooper, 2004).

Figure 5. Association of Rad32 mutants with Nbs1. Strains containing *nbs1*-HA-TAP and *rad32*+ (yFS483), *rad32*+ (but not *nbs1*-TAP) (yFS467), *rad32*-H134N (yFS475), *rad32*-D65N (yFS485), *rad32*-D25A (yFS486), *rad32*-H134L/D135V (yFS487), *rad32*-N122S (yFS488), *rad32*-W215C (yFS489), and *rad32*ΔC (yFS490) tagged with 13 Myc epitopes were grown to mid-log phase. TAP-tagged protein was precipitated with IgG agarose beads from soluble cell lysate. Extract lanes (E) contain 1.5% of immunoprecipitated input. The Western was probed with anti-HA directly conjugated to HRP. The HA-linked HRP was inactivated with sodium azide, and the blot was probed with anti-Myc directly conjugated to HRP. The asterisk indicates the residual anti-HA signal after sodium azide treatment.

In most assays, deletion of *ctp1* causes a similar phenotype to those of *rad32*Δ (Limbo *et al.*, 2007; Akamatsu *et al.*, 2008, Figure 6A; data not shown). In particular, *ctp1*Δ cells display a significant defect in activation of the Cds1 checkpoint kinase in response to MMS-induced DNA damage during S phase, although not as strong a defect as *rad32*Δ cells (Figure 7). However, when tested for their ability to slow replication in response to DNA damage, *ctp1*Δ cells display a fully functional S-phase DNA damage checkpoint (Figure 8B). Therefore, *ctp1*Δ and the class III separation-of-function mutants define a resection- and signaling-independent role for MRN in the S-phase DNA damage checkpoint.

DISCUSSION

To dissect the role of the MRN complex in the S-phase DNA damage checkpoint, we created site-directed mutations in the Rad32 (Mre11) subunit that disrupt nuclease function or mimic human ATLD mutations. As a result, we have defined a function for MRN in S-phase checkpoint role that is independent of its Ctp1-dependent roles in double-strand break resection, constitutive repair, and checkpoint signaling. Specifically, we identified three classes of mutants: class I mutants are similar to wild type in all assays, class II mutants are similar to *rad32* null mutants, and class III mutants behave like null mutants in all assays except the S-phase DNA damage checkpoint assay. In this respect, the class III mutants phenocopy *ctp1* null mutants and genetically separate the Ctp1-dependent functions of MRN from its S-phase checkpoint function. This genetic separation of function demonstrates that at least one of the defining roles of MRN is independent of its well-studied involvement in DNA damage recognition and checkpoint signaling. Table 3 summarizes our results.

The class I alleles *rad32*-N122S and *rad32*-W215C were both designed to recapitulate human ATLD alleles (Stewart *et al.*, 1999; Fernet *et al.*, 2005). The sites mutated are conserved residues in conserved regions of the protein. However, the fission yeast alleles do not show the strong checkpoint phenotypes characteristic of the human alleles. Nonetheless, the fission yeast mutants do display mild HU sensitivity and mild spore viability defects, indicating that they somewhat impair MRN function (Figure 2). Furthermore, they display reduced MRN complex formation (Figure 5), consistent with

the fact that they are thought to compromise Nbs1 binding in humans (Hopfner *et al.*, 2001; Lee *et al.*, 2003).

The class II alleles *rad32*-D25A, *rad32*-D65N, and *rad32*-H134N affect conserved nuclease residues and disrupt *in vitro* nuclease activity in the budding yeast protein (Furuse *et al.*, 1998; Usui *et al.*, 1998; Moreau *et al.*, 1999). They are phenotypically similar to the null allele. However, two of them, *rad32*-D65N and *rad32*-H134N, are slightly more resistant to DNA damage than the null, suggesting they retain some residual MRN function (Figure 2). Furthermore, these alleles are functional in telomere maintenance (Figure 3). This apparent nuclease-independent function at the telomere is consistent with the recent demonstration that MRN nuclease activity is not required for ATM(Tel1) activation in mice, the presumptive function of MRN in telomere maintenance (Buis *et al.*, 2008). The third allele, *rad32*-D25A, is indistinguishable for *rad32*Δ. Furthermore, it expresses poorly and fails to form MRN complexes (Figure 5), suggesting that the D25A mutation disrupts overall protein structure, consistent with behavior of the budding yeast protein (Krogh *et al.*, 2005). The fact that the D25A mutation leads to significant reduction in Nbs1 expression suggests that lack of Mre11 binding destabilizes Nbs1, in agreement with reduced Xrs2 expression in budding yeast D25A cells (Krogh *et al.*, 2005). The fact that our stable nuclease-dead alleles behave mostly as nulls contrasts with the behavior of the analogous alleles in budding yeast, which retain significant resistance to DNA damage (Krogh *et al.*, 2005). This result highlights differences between the roles of fission yeast MRN and the budding yeast MRX in DNA damage metabolism, which are mirrored by differences in their roles in replication slowing (Chahwan *et al.*, 2003; Andrews and Clarke, 2005).

The class III alleles display a separation-of-function phenotype; they disrupt all of the functions of MRN except for its checkpoint-dependent role in slowing replication in response to DNA damage. One of the class III alleles, *rad32*ΔC, is analogous to a human ATLD allele (Stewart *et al.*, 1999). *Rad32*ΔC deletes the C-terminal DNA binding domains of Rad32. A plausible explanation for the phenotype of *rad32*ΔC cells is that *Rad32*ΔC compromises Ctp1 recruitment to sites of MRN function (see below). The phenotypes of the other class III allele, *rad32*-H134L/D135V, are harder to explain. This allele, originally described as *mre11*-3 in bud-

Figure 6. S-phase checkpoint. (A) Wild-type (yFS474), *rad3Δ* (yFS260), *rad32Δ* (yFS262), *rad32-H134N* (yFS475), *rad32-W215C* (yFS477), *rad32-H134L/D135V* (yFS478), and *rad32ΔC* (yFS611) cells were synchronized in G1 by a combination of a *cdc10-M17ts* block and release and centrifugal elutriation and followed through replication by flow cytometry. (B) Quantification of control strains. Each curve represents the average of at least three experiments, and error bars represent SE of the mean. (C) Quantification of *rad32-H134L/D135V* (yFS478), *rad32-N122S* (yFS476), and *rad32ΔC* (yFS611) mutant strains with functional checkpoints, which slow their progression through S phase in response to MMS. (D) Quantification of *rad32-D25A* (yFS479) and *rad32-D65N* (yFS481) mutant strains with defective checkpoints, which do not slow their progression through S phase in response to MMS.

ding yeast, affects two conserved nuclease residues and has been characterized as nuclease dead in the context of the human protein (Arthur *et al.*, 2004). Nonetheless, it retains DNA damage recognition functions in human cells and replication slowing functions in fission yeast (Arthur *et al.*, 2004; Figure 5). In particular, it behaves differently from the other nuclease-dead alleles in fission yeast, including one allele, *rad32-H134N*, that affects one of the same residues. One possibility is that there is sufficient binding of manganese in the Rad32-H134L/D135V active site to retain resid-

ual nuclease function that would support the S-phase DNA damage checkpoint role, but not other roles, even though that level of nuclease activity would be immeasurable in an *in vitro* assay (Lewis *et al.*, 2004). In a similar vein, Rad32-H134L/D135V may disrupt only a subset of the various endo- and exonuclease activities of MRN, perhaps disrupting its 3' exonuclease activity, the activity that has been assayed, but not its endonuclease activity (Williams *et al.*, 2008). Alternatively, it is possible that Rad32-H134L/D135V disrupts all nuclease activity, but the Rad32-H134N and the

Figure 7. Cds1 kinase activity. Asynchronous cultures of wild type (yFS467), *rad32-N122S* (yFS472), *rad32-W215C* (yFS473), *rad3Δ* (yFS189), *nbs1Δ* (yFS249), *rad32Δ* (yFS450), *rad32-H134N* (yFS468), *rad32-D65N* (yFS469), *rad32-D25A* (yFS470), *rad32-H134L/D135V* (yFS471), *rad32ΔC* (yFS466), and *ctp1Δ* (OL4121) cells were grown to log phase and treated with 0.03% MMS for 4 h. Cds1 kinase activity was measured by phosphorylation of MBP. (A) Autoradiograph of Cds1 kinase activity and Coomassie-stained gel. MBP and the IgG heavy chain are indicated. (B) Quantification of Cds1 kinase activity relative to untreated wild-type kinase activity. Each represents an average of three independent experiments and error bars represent the SE of the mean.

other nuclease-dead mutations disrupt additional functions, such as ATP binding or DNA binding, required for replication slowing. Recently, the Russell laboratory has made a *rad32-H134S* mutant, which is not highly sensitive to DNA-damaging agents (Williams *et al.*, 2008). They suggested that the nuclease active site in this mutant still forms Rad32/Mre11 dimers and that dimerization of Mre11 is critical for MRN function. It is possible that *rad32-H134L/D135V* is able to form Rad32 dimers, whereas *rad32-H134N* cannot.

The class III alleles phenocopy *ctp1Δ*. Ctp1, the homologue of budding yeast Sae2 and human CtIP, is an MRN cofactor that is recruited to sites of DNA damage by MRN

Table 3. Summary of phenotypes

	<i>S. cerevisiae</i>		Mammalian phenotype	Doubling time (h)	% normal cell morphology	DNA damage resistance				HU resistance	<i>rad2</i> synthetic lethality	Spore viability	Telomere function	Replication slowing	Complex formation	Cds1 kinase activity	Summary	Class
	biochemical phenotype	phenotype				IR	UV	MMS										
<i>rad32+</i>	wt	wt	wt	2.5	99	+++	+++	+++	+++	+++	No	Yes	Yes	Yes	+	+++	wt	I
<i>N122S</i>	NA	ATLD	ATLD	3	75	+++	+++	+++	+++	+++	No	Yes	Yes	Yes	+	+++	~wt	I
<i>W215C</i>	NA	ATLD	ATLD	3.1	82	+++	+++	+++	+++	+++	No	Yes	Yes	Yes	+	+++	~wt	I
<i>rad32Δ</i>	null	inviable	inviable	4.3	32	-	-	-	-	-	Yes	No	No	partial	-	+/-	null	II
<i>D25A</i>	NucD	NA	NA	4.1	26	-	-	-	-	-	Yes	No	No	partial	-	+/-	null	II
<i>H134N</i>	NucD	NA	NA	3.9	23	+/	+	+	+	+	Yes	No	Yes	partial	+	+	~null	II
<i>D65N</i>	NucD	NA	NA	4.5	23	+	+	+	+	+	Yes	No	Yes	partial	+	+	~null	II
<i>H134L/D135V</i>	NucD	NA	NA	4.1	31	-	+	-	-	-	Yes	No	No	Yes	- ^a	+	SoF	III
<i>ΔC</i>	NA	ATLD	ATLD	3.7	27	+/	+	+	+	+	Yes	No	No	Yes	+	+	SoF	III
<i>ctp1Δ</i>	NA	NA	NA	3.3	41	- ^b	- ^b	- ^c	- ^c	- ^c	Yes ^d	No ^b	Yes	Yes	+	+	SoF	III

NA, not applicable; NucD, nuclease dead.

^a These proteins show variable Nbs1 binding. In some experiments it is weak, in others it is not above background.

^b Previously published (Limbo *et al.*, 2007).

^c Data not shown.

^d Previously published (Akamatsu *et al.*, 2008).

Figure 8. (A) Cellular morphology of log phase *ctp1Δ* (OL4121) cells imaged in 100 $\times$ magnification. *ctp1Δ* morphology is similar to that of the *rad32Δ* strain. (B) Quantification of flow cytometry analysis of *ctp1Δ* (yFS650) S-phase DNA damage checkpoint assayed as described in Figure 4.

and is required for MRN-dependent resection at double-strand breaks (Clerici *et al.*, 2005; Limbo *et al.*, 2007; Sartori *et al.*, 2007; Akamatsu *et al.*, 2008). The similarity between *ctp1* and the class III alleles suggests that these alleles interfere specifically with the Ctp1-dependent functions of MRN. Ctp1 and MRN have not been shown to directly interact, but MRN is required for Ctp1 to be recruited to a double-strand break, leading to the suggestion that the two may interact on DNA (Limbo *et al.*, 2007). That Rad32 Δ C lacks the C-terminal DNA binding domain suggests that this region may be involved in Ctp1 interaction. However, that the *rad32Δ*C mutant has a less severe meiotic phenotype but more severe signaling phenotype than *ctp1Δ*, and that both class III alleles lack a telomere function that *ctp1Δ* retains, suggests that the class III alleles effects other functions of MRN, beyond its Ctp1-dependent functions.

The alleles modeled on the human ATLD alleles behave differently in our assays, with two alleles, *rad32-N122S* and *rad32-W215C*, being class I wild-type alleles and one allele, *rad32Δ*C, being a class III separation-of-function allele. However, the class I alleles do show some defects, particularly in complex formation, and the class III allele does show some resistance to DNA damage and HU. These results suggest that the ATLD alleles are not qualitative separation-of-function alleles but rather hypomorphs of various strengths that disrupt some MRN functions but not others due to threshold effects. In vitro biochemical analyses of these alleles have lead to a similar conclusion (Paull, personal communication). It is worth noting that the inferred separation-of-function phenotype of the human ATLD alleles—checkpoint defective but constitutive repair competent—is the opposite of the separation-of-function phenotype we observe for our class III alleles.

Phenotypic analysis of our *rad32* alleles provides a basis for speculation on the mechanistic role of MRN in the S-phase DNA damage checkpoint. Our first conclusion is that the role of MRN in replication slowing is independent from its role in checkpoint signaling. We draw this conclusion from the fact

that the class III separation-of-function mutants have strong signaling defects and yet display fully functional replication slowing. Furthermore, one of the class II mutants, *rad32-D65N*, is as defective as *ctp1Δ* for signaling, but it fails to slow, showing that there is no correlation between signaling and slowing. We have recently reached the same conclusion based on the constitutive activation of checkpoint signaling not suppressing the slowing defect in *nbs1Δ* cells (Willis and Rhind, unpublished data). This conclusion is also consistent with two observations from human NBS cells. First, these cells display slowing defects in conditions in which they seem to activate ATM normally (Difilippantonio *et al.*, 2005; Kanu and Behrens, 2007). Second, they degrade Cdc25A, a downstream target of the checkpoint, demonstrating that signaling to that branch of the S-phase DNA damage checkpoint is intact (Yazdi *et al.*, 2002).

Our second conclusion is that the role of MRN in replication slowing is independent from its resection activity and its role homologous recombination. The conclusion that MRN does not need to resect DNA to fulfill its role in replication slowing is supported by the observation that Ctp1, which is required for resection, is not required for replication slowing (Clerici *et al.*, 2005; Limbo *et al.*, 2007; Sartori *et al.*, 2007; Akamatsu *et al.*, 2008; Figure 6B). The conclusion that homologous recombination is not required for replication slowing is supported by several lines of evidence. First, our class III separation-of-function alleles are synthetically lethal with *rad2Δ* (Table 2). Synthetic lethality with *rad2Δ* is characteristic of mutations that disrupt homologous recombination (Symington, 1998). In addition, Ctp1 is also required for homologous recombination (Limbo *et al.*, 2007). Finally, we have recently shown that Rad51-dependent homologous recombination is not required for checkpoint-dependent replication slowing in fission yeast (Willis and Rhind, 2009).

Our third conclusion is that the role of MRN in replication slowing may be independent from its nuclease activity. Conclusions regarding the role of nuclease activity of MRN in replication slowing are complicated by the fact that three of the nuclease-dead mutants, *rad32-D25A*, *rad32-D65N*, and *rad32-H134N*, are class II alleles and fail to slow replication, but a fourth nuclease-dead mutants, *rad32-H134L/D135V*, is a class III allele and is proficient for slowing. As described above, these results indicate that there are biochemical differences between the class II nuclease-dead alleles and *rad32-H134L/D135V*, but whether the later allele has some residual nuclease activity or the former alleles disrupt functions in addition to nuclease activity awaits a thorough biochemical characterization of all four proteins.

What role does this leave for MRN in checkpoint-dependent slowing of replication? It has been suggested that the slowing of replication is a consequence of DNA repair (Rhind and Russell, 2000). Our results support this conclusion, in that the role of MRN is downstream of signaling, but the suggest that this repair is some MRN repair function other than homologous recombination. We have recently shown that Mus81 and Rqh1 are both required downstream of checkpoint signaling in the S phase DNA damage checkpoint (Willis and Rhind, 2009). Both of these proteins are implicated in replication fork metabolism: Mus81 as an endonuclease that can cleave stalled forks and promote sister recombination and Rqh1 as a helicase involved in the regulation of fork regression (Murray *et al.*, 1997; Stewart *et al.*, 1997; Roseaulin *et al.*, 2008). MRN can tether DNA and has been suggested to be involved in coordinating recombinational repair at stalled forks (Lisby and Rothstein, 2004; Lisby *et al.*, 2004). Because stalled forks do not present double-strand ends, the role of MRN at stalled forks may not involve its resection activity and thus may be independent

of Ctp1. In such a role, it may support the functions of Mus81 and Rqh1 in checkpoint-dependent slowing of replication. However, once forks collapse, double-strand breaks are produced, and the resection activity of MRN may be required for homologous-recombinational restart mechanisms (Trenz *et al.*, 2006). This distinction between stalled forks that are stabilized by the checkpoint and collapsed forks that need to be restarted by homologous recombination may explain why the Ctp1-dependent resection activity of MRN is dispensable for checkpoint-dependent replication slowing but required for checkpoint-independent repair of spontaneous S-phase damage (Trenz *et al.*, 2006; Wen *et al.*, 2008).

Regardless of the mechanism of the role of MRN in the S-phase DNA damage checkpoint, our results demonstrate that MRN does more in the checkpoint than simply recognize DNA damage and activate checkpoint signaling. Furthermore, they demonstrate that the checkpoint role of MRN is independent from its resection and homologous recombination activities. Therefore, MRN has at least two biochemically distinct repair activities. Understanding these diverse activities will be essential for understanding the role MRN plays in protecting the genome from both exogenous and endogenous DNA damage and in preventing cancer.

ACKNOWLEDGMENTS

We thank P. Russell (The Scripps Research Institute), P. Jeggo (University of Sussex), and Y. Tsutsui (Osaka University) for providing strains used in this work, T. Wang (Stanford University) for the anti-Cds1 antibody, T. Nakamura (University of Illinois, Chicago) for the Tas1 plasmid; and members of the Rhind laboratory, especially Prasanta Patel, for helpful discussions and experimental assistance. This work was funded by National Institutes of Health grant GM-069957 (to N. R.).

REFERENCES

- Akamatsu, Y., Murayama, Y., Yamada, T., Nakazaki, T., Tsutsui, Y., Ohta, K., and Iwasaki, H. (2008). Molecular characterization of the role of the *Schizosaccharomyces pombe* nip1+/ctp1+ gene in DNA double-strand break repair in association with the Mre11-Rad50-Nbs1 complex. *Mol. Cell. Biol.* 28, 3639–3651.
- Andrews, C. A., and Clarke, D. J. (2005). MRX (Mre11/Rad50/Xrs2) mutants reveal dual intra-S-phase checkpoint systems in budding yeast. *Cell Cycle* 4, 1073–1077.
- Arthur, L. M., Gustavsson, K., Hopfner, K. P., Carson, C. T., Stracker, T. H., Karcher, A., Felton, D., Weitzman, M. D., Tainer, J., and Carney, J. P. (2004). Structural and functional analysis of Mre11-3. *Nucleic Acids Res.* 32, 1886–1893.
- Bahler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A. r., Steever, A. B., Wach, A., Philippsen, P., and Pringle, J. R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* 14, 943–951.
- Barbet, N., Muriel, W. J., and Carr, A. M. (1992). Versatile shuttle vectors and genomic libraries for use with *Schizosaccharomyces pombe*. *Gene* 114, 59–66.
- Bentley, N. J., Holtzman, D. A., Flaggs, G., Keegan, K. S., DeMaggio, A., Ford, J. C., Hoekstra, M., and Carr, A. M. (1996). The *Schizosaccharomyces pombe* rad3 checkpoint gene. *EMBO J.* 15, 6641–6651.
- Boddy, M. N., Furnari, B., Mondesert, O., and Russell, P. (1998). Replication checkpoint enforced by kinases Cds1 and Chk1. *Science* 280, 909–912.
- Bressan, D. A., Olivares, H. A., Nelms, B. E., and Petrini, J. H. (1998). Alteration of N-terminal phosphoesterase signature motifs inactivates *Saccharomyces cerevisiae* Mre11. *Genetics* 150, 591–600.
- Buis, J., Wu, Y., Deng, Y., Leddon, J., Westfield, G., Eckersdorff, M., Sekiguchi, J. M., Chang, S., and Ferguson, D. O. (2008). Mre11 nuclease activity has essential roles in DNA repair and genomic stability distinct from ATM activation. *Cell* 135, 85–96.
- Carney, J. P., Maser, R. S., Olivares, H., Davis, E. M., Le Beau, M., Yates, R., Jr., Hays, L., Morgan, W. F., and Petrini, J. H. (1998). The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell* 93, 477–486.
- Chahwan, C., Nakamura, T. M., Sivakumar, S., Russell, P., and Rhind, N. (2003). The fission yeast Rad32 (Mre11)-Rad50-Nbs1 complex is required for the S-phase DNA damage checkpoint. *Mol. Cell. Biol.* 23, 6564–6573.
- Chen, L., Nievera, C., Lee, A. Y., and Wu, X. (2008). Cell cycle-dependent complex formation of BRCA1/CtIP/MRN is important for DNA double-strand break repair. *J. Biol. Chem.* 283, 7713–7720.
- Chen, L., Trujillo, K., Ramos, W., Sung, P., and Tomkinson, A. E. (2001). Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. *Mol. Cell* 8, 1105–1115.
- Chen, X., Zhao, R., Glick, G. G., and Cortez, D. (2007). Function of the ATR N-terminal domain revealed by an ATM/ATR chimera. *Exp. Cell Res.* 313, 1667–1674.
- Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P. (2005). The *Saccharomyces cerevisiae* Sae2 protein promotes resection and bridging of double strand break ends. *J. Biol. Chem.* 280, 38631–38638.
- Costanzo, V., Robertson, K., Bibikova, M., Kim, E., Grieco, D., Gottesman, M., Carroll, D., and Gautier, J. (2001). Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication. *Mol. Cell* 8, 137–147.
- D'Amours, D., and Jackson, S. P. (2002). The Mre11 complex: at the crossroads of DNA repair and checkpoint signalling. *Nat. Rev. Mol. Cell Biol.* 3, 317–327.
- Difilippantonio, S., *et al.* (2005). Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. *Nat. Cell Biol.* 7, 675–685.
- Dumon-Jones, V., Frappart, P. O., Tong, W. M., Sajithlal, G., Hulla, W., Schmid, G., Herceg, Z., Digweed, M., and Wang, Z. Q. (2003). Nbn heterozygosity renders mice susceptible to tumor formation and ionizing radiation-induced tumorigenesis. *Cancer Res* 63, 7263–7269.
- Dutta, C., Patel, P. K., Rosebrock, A., Oliva, A., Leatherwood, J., and Rhind, N. (2008). The DNA replication checkpoint directly regulates MBF-dependent G1/S transcription. *Mol. Cell. Biol.* 28, 5977–5985.
- Falck, J., Petrini, J. H., Williams, B. R., Lukas, J., and Bartek, J. (2002). The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. *Nat. Genet.* 30, 290–294.
- Fernet, M., Gribaa, M., Salih, M. A., Seidahmed, M. Z., Hall, J., and Koenig, M. (2005). Identification and functional consequences of a novel MRE11 mutation affecting 10 Saudi Arabian patients with the ataxia telangiectasia-like disorder. *Hum. Mol. Genet.* 14, 307–318.
- Ferreira, M. G., and Cooper, J. P. (2004). Two modes of DNA double-strand break repair are reciprocally regulated through the fission yeast cell cycle. *Genes Dev.* 18, 2249–2254.
- Forsburg, S. L., and Rhind, N. (2006). Basic methods for fission yeast. *Yeast* 23, 173–183.
- Furuse, M., Nagase, Y., Tsubouchi, H., Murakami-Murofushi, K., Shibata, T., and Ohta, K. (1998). Distinct roles of two separable in vitro activities of yeast Mre11 in mitotic and meiotic recombination. *EMBO J.* 17, 6412–6425.
- Gatei, M., Young, D., Cerosaletti, K. M., Desai-Mehta, A., Spring, K., Kozlov, S., Lavin, M. F., Gatti, R. A., Concannon, P., and Khanna, K. (2000). ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nat. Genet.* 25, 115–119.
- Haber, J. E. (1998). The many interfaces of Mre11. *Cell* 95, 583–586.
- Hartsuiker, E., Vaessen, E., Carr, A. M., and Kohli, J. (2001). Fission yeast Rad50 stimulates sister chromatid recombination and links cohesion with repair. *EMBO J.* 20, 6660–6671.
- Hopfner, K. P., Karcher, A., Craig, L., Woo, T. T., Carney, J. P., and Tainer, J. A. (2001). Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. *Cell* 105, 473–485.
- Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J., and Jackson, S. P. (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.* 8, 37–45.
- Kai, M., Tanaka, H., and Wang, T. S. (2001). Fission yeast Rad17 associates with chromatin in response to aberrant genomic structures. *Mol. Cell. Biol.* 21, 3289–3301.
- Kanu, N., and Behrens, A. (2007). ATMIN defines an NBS1-independent pathway of ATM signalling. *EMBO J.* 26, 2933–2941.
- Kommajosyula, N., and Rhind, N. (2006). Cdc2 tyrosine phosphorylation is not required for the S-phase DNA damage checkpoint in fission yeast. *Cell Cycle* 5, 2495–2500.
- Krogh, B. O., Llorente, B., Lam, A., and Symington, L. S. (2005). Mutations in Mre11 phosphoesterase motif I that impair *Saccharomyces cerevisiae* Mre11-Rad50-Xrs2 complex stability in addition to nuclease activity. *Genetics* 171, 1561–1570.
- Lavin, M. F. (2004). The Mre11 complex and ATM: a two-way functional interaction in recognising and signaling DNA double strand breaks. *DNA Rep.* 3, 1515–1520.
- Lavin, M. F., and Shiloh, Y. (1997). The genetic defect in ataxia-telangiectasia. *Annu. Rev. Immunol.* 15, 177–202.
- Lee, J. H., Ghirlando, R., Bhaskara, V., Hoffmeyer, M. R., Gu, J., and Paull, T. T. (2003). Regulation of Mre11/Rad50 by Nbs 1, effects on nucleotide-

- dependent DNA binding and association with ataxia-telangiectasia-like disorder mutant complexes. *J. Biol. Chem.* 278, 45171–45181.
- Lee, J. H., and Paull, T. T. (2004). Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* 304, 93–96.
- Lewis, L. K., Storici, F., Van Komen, S., Calero, S., Sung, P., and Resnick, M. A. (2004). Role of the nuclease activity of *Saccharomyces cerevisiae* Mre11 in repair of DNA double-strand breaks in mitotic cells. *Genetics* 166, 1701–1713.
- Lim, D. S., Kim, S. T., Xu, B., Maser, R. S., Lin, J., Petrini, J. H., and Kastan, M. B. (2000). ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* 404, 613–617.
- Limbo, O., Chahwan, C., Yamada, Y., de Bruin, R. A., Wittenberg, C., and Russell, P. (2007). Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. *Mol. Cell* 28, 134–146.
- Lisby, M., Barlow, J. H., Burgess, R. C., and Rothstein, R. (2004). Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. *Cell* 118, 699–713.
- Lisby, M., and Rothstein, R. (2004). DNA repair: keeping it together. *Curr. Biol.* 14, R994–R996.
- Luo, G., Yao, M. S., Bender, C. F., Mills, M., Bladl, A. R., Bradley, A., and Petrini, J. H. (1999). Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proc. Natl. Acad. Sci. USA* 96, 7376–7381.
- Manolis, K. G., Nimmo, E. R., Hartsuiker, E., Carr, A. M., Jeggo, P. A., and Allshire, R. C. (2001). Novel functional requirements for non-homologous DNA end joining in *Schizosaccharomyces pombe*. *EMBO J.* 20, 210–221.
- Maser, R. S., Mirzoeva, O. K., Wells, J., Olivares, H., Williams, B. R., Zinkel, R. A., Farnham, P. J., and Petrini, J. H. (2001). Mre11 complex and DNA replication: linkage to E2F and sites of DNA synthesis. *Mol. Cell. Biol.* 21, 6006–6016.
- Moreau, S., Ferguson, J. R., and Symington, L. S. (1999). The nuclease activity of Mre11 is required for meiosis but not for mating type switching, end joining, or telomere maintenance. *Mol. Cell. Biol.* 19, 556–566.
- Morishita, T., Tsutsui, Y., Iwasaki, H., and Shinagawa, H. (2002). The *Schizosaccharomyces pombe* rad60 gene is essential for repairing double-strand DNA breaks spontaneously occurring during replication and induced by DNA-damaging agents. *Mol. Cell. Biol.* 22, 3537–3548.
- Murray, J. M., Lindsay, H. D., Munday, C. A., and Carr, A. M. (1997). Role of *Schizosaccharomyces pombe* RecQ homolog, recombination, and checkpoint genes in UV damage tolerance. *Mol. Cell. Biol.* 17, 6868–6875.
- Murray, J. M., Tavassoli, M., al-Harithy, R., Sheldrick, K. S., Lehmann, A. R., Carr, A. M., and Watts, F. Z. (1994). Structural and functional conservation of the human homolog of the *Schizosaccharomyces pombe* rad2 gene, which is required for chromosome segregation and recovery from DNA damage. *Mol. Cell. Biol.* 14, 4878–4888.
- Myers, J. S., and Cortez, D. (2006). Rapid activation of ATR by ionizing radiation requires ATM and Mre11. *J. Biol. Chem.* 281, 9346–9350.
- Myung, K., and Kolodner, R. D. (2002). Suppression of genome instability by redundant S-phase checkpoint pathways in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 99, 4500–4507.
- Nairz, K., and Klein, F. (1997). mre11S—a yeast mutation that blocks double-strand-break processing and permits nonhomologous synapsis in meiosis. *Genes Dev.* 11, 2272–2290.
- Nakamura, T. M., Cooper, J. P., and Cech, T. R. (1998). Two modes of survival of fission yeast without telomerase. *Science* 282, 493–496.
- Nakamura, T. M., Moser, B. A., and Russell, P. (2002). Telomere binding of checkpoint sensor and DNA repair proteins contributes to maintenance of functional fission yeast telomeres. *Genetics* 161, 1437–1452.
- Nelms, B. E., Maser, R. S., MacKay, J. F., Lagally, M. G., and Petrini, J. H. (1998). In situ visualization of DNA double-strand break repair in human fibroblasts. *Science* 280, 590–592.
- Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., and Okayama, H. (1990). High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* 18, 6485–6489.
- Painter, R. B., and Young, B. R. (1980). Radiosensitivity in ataxia-telangiectasia: a new explanation. *Proc. Natl. Acad. Sci. USA* 77, 7315–7317.
- Petrini, J. H. (2000). The Mre11 complex and ATM: collaborating to navigate S phase. *Curr. Opin. Cell Biol.* 12, 293–296.
- Rhind, N., and Russell, P. (2000). Checkpoints: it takes more than time to heal some wounds. *Curr. Biol.* 10, R908–R911.
- Roseaulin, L., Yamada, Y., Tsutsui, Y., Russell, P., Iwasaki, H., and Arcangioli, B. (2008). Mus81 is essential for sister chromatid recombination at broken replication forks. *EMBO J.* 27, 1378–1387.
- Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S. P. (2007). Human CtIP promotes DNA end resection. *Nature* 450, 509–514.
- Sato, M., Dhut, S., and Toda, T. (2005). New drug-resistant cassettes for gene disruption and epitope tagging in *Schizosaccharomyces pombe*. *Yeast* 22, 583–591.
- Stewart, E., Chapman, C. R., Al-Khodairy, F., Carr, A. M., and Enoch, T. (1997). rqh1+, a fission yeast gene related to the Bloom's and Werner's syndrome genes, is required for reversible S phase arrest. *EMBO J.* 16, 2682–2692.
- Stewart, G. S., Maser, R. S., Stankovic, T., Bressan, D. A., Kaplan, M. I., Jaspers, N. G., Raams, A., Byrd, P. J., Petrini, J. H., and Taylor, A. M. (1999). The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell* 99, 577–587.
- Symington, L. S. (1998). Homologous recombination is required for the viability of rad27 mutants. *Nucleic Acids Res.* 26, 5589–5595.
- Symington, L. S. (2002). Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol. Mol. Biol. Rev.* 66, 630–670, table of contents.
- Takeda, S., Nakamura, K., Taniguchi, Y., and Paull, T. T. (2007). Ctp1/CtIP and the MRN complex collaborate in the initial steps of homologous recombination. *Mol. Cell* 28, 351–352.
- Tavassoli, M., Shayeghi, M., Nasim, A., and Watts, F. Z. (1995). Cloning and characterisation of the *Schizosaccharomyces pombe* rad32 gene: a gene required for repair of double strand breaks and recombination. *Nucleic Acids Res.* 23, 383–388.
- Tomita, K., et al. (2003). Competition between the Rad50 complex and the Ku heterodimer reveals a role for Exo1 in processing double-strand breaks but not telomeres. *Mol. Cell. Biol.* 23, 5186–5197.
- Trenz, K., Smith, E., Smith, S., and Costanzo, V. (2006). ATM and ATR promote Mre11 dependent restart of collapsed replication forks and prevent accumulation of DNA breaks. *EMBO J.* 25, 1764–1774.
- Usui, T., Ohta, T., Oshiumi, H., Tomizawa, J., Ogawa, H., and Ogawa, T. (1998). Complex formation and functional versatility of Mre11 of budding yeast in recombination. *Cell* 95, 705–716.
- Wen, Q., Scorch, J., Phear, G., Rodgers, G., Rodgers, S., and Meuth, M. (2008). A mutant allele of MRE11 found in mismatch repair deficient tumor cells suppresses the cellular response to DNA replication fork stress in a dominant negative manner. *Mol. Biol. Cell* 19, 1693–1705.
- Williams, R. S., et al. (2008). Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell* 135, 97–109.
- Williams, R. S., Williams, J. S., and Tainer, J. A. (2007). Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. *Biochem. Cell Biol.* 85, 509–520.
- Willis, N., and Rhind, N. (2009). Mus81, Rhp51(Rad51) and Rqh1 form an epistatic pathway required for the S-phase DNA damage checkpoint. *Mol. Biol. Cell* 20, 819–833.
- Wilson, S., Tavassoli, M., and Watts, F. Z. (1998). *Schizosaccharomyces pombe* rad32 protein: a phosphoprotein with an essential phosphoesterase motif required for repair of DNA double strand breaks. *Nucleic Acids Res.* 26, 5261–5269.
- Wilson, S., Warr, N., Taylor, D. L., and Watts, F. Z. (1999). The role of *Schizosaccharomyces pombe* Rad32, the Mre11 homologue, and other DNA damage response proteins in non-homologous end joining and telomere length maintenance. *Nucleic Acids Res.* 27, 2655–2661.
- Xiao, Y., and Weaver, D. T. (1997). Conditional gene targeted deletion by Cre recombinase demonstrates the requirement for the double-strand break repair Mre11 protein in murine embryonic stem cells. *Nucleic Acids Res.* 25, 2985–2991.
- Yazdi, P. T., Wang, Y., Zhao, S., Patel, N., Lee, E. Y., and Qin, J. (2002). SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev.* 16, 571–582.
- Young, B. R., and Painter, R. B. (1989). Radioresistant DNA synthesis and human genetic diseases. *Hum. Genet.* 82, 113–117.
- Zhang, X., and Paull, T. T. (2005). The Mre11/Rad50/Xrs2 complex and non-homologous end-joining of incompatible ends in *S. cerevisiae*. *DNA Rep.* 4, 1281–1294.
- Zhao, S., et al. (2000). Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. *Nature* 405, 473–477.
- Zhu, J., Petersen, S., Tessarollo, L., and Nussenzweig, A. (2001). Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. *Curr. Biol.* 11, 105–109.