IDENTIFICATION OF NOVEL DNA DAMAGE RESPONSE GENES USING FUNCTIONAL GENOMICS

by

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ABSTRACT

The genetic information required for life is stored within molecules of DNA. This DNA is under constant attack as a result of normal cellular metabolic processes, as well as exposure to genotoxic agents. DNA damage left unrepaired can result in mutations that alter the genetic information encoded within DNA. Cells have consequently evolved complex pathways to combat damage to their DNA. Defects in the cellular response to DNA damage can result in genomic instability, a hallmark of cancer cells. Identifying all the components required for this response remains an important step in fully elucidating the molecular mechanisms involved. I used functional genomic approaches to identify genes required for the DNA damage response in *Saccharomyces cerevisiae*. I conducted a screen to identify genes required for resistance to a DNA damaging agent, methyl methanesulfonate, and identified several poorly characterized genes that are necessary for proper S phase progression in the presence of DNA damage. Among the genes identified, *ESC4*/*RTT107* has since been shown to be essential for the resumption of DNA replication after DNA damage. Using genome-wide genetic interaction screens to identify genes that are required for viability in the absence of *MUS81* and *MMS4*, two genes required for resistance to DNA damage, I helped identify *ELG1*, deletion of which causes DNA replication defects, genomic instability, and an inability to properly recover from DNA damage during S phase. I also used two-dimensional hierarchical clustering of synthetic genetic interaction data determined by large-scale genetic network analysis to identify *RMI1*, which encodes a new member of the highly conserved Sgs1-Top3 complex that is an important suppressor of genomic instability.

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LIST OF ABBREVIATIONS

ATM: ataxia-telangiectasia mutated
ATR: ataxia-telangiectasia related
BIR: break-induced replication
BrdU: bromodeoxyuridine
BS: Bloom syndrome
CPT: camptothecin
DNA: deoxyribonucleic acid
DSB: double strand break
dsRNA: double-stranded RNA
GCR: gross chromosomal rearrangement
GFP: green fluorescent protein
HJ: Holliday junction
HR: homologous recombination
HU: hydroxyurea
MMS: methyl methanesulfonate
ORF: open reading frame
PCNA: proliferating cell nuclear antigen
IR: ionizing radiation
rDNA: ribosomal DNA
RNA: ribonucleic acid
RNAi: RNA interference
RFB: replication fork barrier
RFC: replication factor C
RTS: Rothmund-Thomson syndrome
SCE: sister chromatid exchange
SGA: synthetic genetic array
SGAM: synthetic genetic array mapping
siRNA: small interfering RNA
TAP: tandem affinity purification
tRNA: transfer RNA
UV: ultraviolet radiation
WS: Werner syndrome
YFP: yellow fluorescent protein
YPD: yeast extract/peptone/dextrose

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1. GENERAL INTRODUCTION

The genetic information required for all life is encoded in biological macromolecules of deoxyribonucleic acid (DNA), which is constantly being damaged as a consequence of normal cellular metabolism and by exposure to genotoxic agents. DNA damage left unrepaired results in mutations that can alter the genetic information stored in the DNA. Cells have consequently evolved complex mechanisms to combat mutation of their genetic material. Defects in the cellular response to DNA damage can result in genomic instability, a hallmark of cancer cells. When faced with DNA damage, cells respond by invoking DNA repair and DNA damage checkpoint pathways, altering gene expression, or inducing programmed cell death (i.e. apoptosis). Our lab is interested in understanding how cells cope with DNA damage during S phase, a period in the cell cycle when DNA is particularly susceptible to mutagenic alterations. My thesis work has largely focused on the identification of proteins required for the processing of DNA replication forks stalled by the presence of DNA lesions or by deoxyribonucleotide depletion. Proteins involved in the stabilization of stalled replication forks, and the recovery of functional forks from those that have collapsed, are needed for restarting DNA replication when replication is impeded, and are therefore important in ensuring cell viability and genomic integrity.

1.1 DNA DAMAGE CHECKPOINTS

In eukaryotic cells, the integrity of the genome is protected by an elaborate set of surveillance pathways designed to detect damage to the DNA, and to arrest cell cycle progression while the damage is repaired. These pathways, termed "checkpoints", ensure that cells do not undergo DNA replication or mitosis in the presence of DNA lesions, thereby preventing the chromosome rearrangement, chromosome loss, and cell death that could result (Hartwell and Weinert, 1989).

Checkpoint pathways are also important to cope with stalled DNA replication forks, preventing their collapse and ensuring that they are properly restarted. Four checkpoints that monitor chromosome replication and integrity have been described in eukaryotic cells (reviewed in (Elledge, 1996; Foiani et al., 2000; Melo and Toczyski, 2002; Rhind and Russell, 1998; Rhind and Russell, 2000)). The three DNA damage checkpoints function during G1, S, and G2 phases of the cell cycle. The fourth, the DNA replication or S/M checkpoint, delays mitosis and suppresses initiation of DNA replication in response to replication arrest. The proteins that comprise checkpoint pathways are highly conserved from yeast to humans (Figure 1). Mutations in checkpoint genes are associated with cancer predisposition syndromes in humans (Bertoni et al., 1999; Hartwell and Kastan, 1994; Hoekstra, 1997; Thacker, 1994), emphasizing the relevance of these pathways to carcinogenesis. Checkpoint mutants also typically display genomic instability, a hallmark of cancer cells.

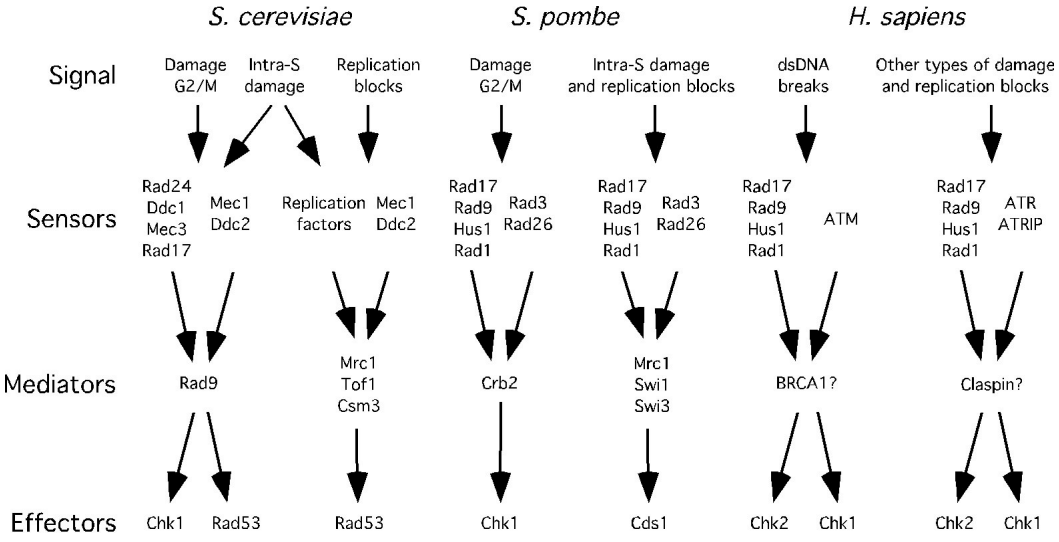

Figure 1. Checkpoint response pathways in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Homo sapiens*. See text for details. (Melo and Toczyski, 2002)

Checkpoint proteins can be divided into three groups, sensors, mediators, and effectors. Sensors are recruited to sites of DNA damage and are needed for the activation of effector kinases. Mediator proteins are important to facilitate this activation. Phosphorylation of target

proteins by the effector kinases results in a number of cellular responses important for cell viability and genomic integrity. The sensors comprise three groups of proteins: (i) the Mre11 complex and PI3-like protein kinases; (ii) the sliding clamp/clamp loader complexes; and (iii) replication proteins such as Pol2, Dpb11, Cdc7-Dbf4, Drc1, and Rfc5. The PI3-like kinases (ATM and ATR in humans, Tel1 and Mec1 in the budding yeast *Saccharomyces cerevisiae*) play a central role in all checkpoint responses. The Mre11 complex (MRN: Mre11/Rad50/Nbs1 in humans; MRX: Mre11/Rad50/Xrs2 in *S. cervisiae*) is an important sensor of DNA double strand breaks (DSBs) (Lisby et al., 2004). Activation of ATM/Tel1 (ATM in humans, Tel1 in *S. cerevisiae*) in response to DSBs requires the Mre11 complex (Carson et al., 2003; Lee and Paull, 2004; Lisby et al., 2004; Uziel et al., 2003). The ATR-like subfamily (ATR, Mec1) seems to be the primary sensor that responds to S phase damage by UV light, the DNA alkylating agent methyl methanesulfonate (MMS), and the replication inhibitors hydroxyurea (HU) and aphidicolin (Abraham, 2001; Foiani et al., 2000; Rhind and Russell, 1998). ATR/Mec1 forms a complex with the ATRIP/Ddc2 (ATRIP in humans, Ddc2 in *S. cerevisiae*) protein (Cortez et al., 2001; Edwards et al., 1999; Paciotti et al., 2000) and this complex binds to DNA strand breaks *in vivo* (Kondo et al., 2001; Melo et al., 2001; Zou and Elledge, 2003). The checkpoint proteins Ddc1, Mec3, and Rad17 form a complex (known in humans and the fission yeast *Schizosaccharomyces pombe* as the 9-1-1 complex) that is believed to be analogous to PCNA (Kondo et al., 1999; St Onge et al., 1999; Venclovas and Thelen, 2000; Volkmer and Karnitz, 1999). PCNA is a sliding clamp that encircles the DNA and tethers DNA polymerase to the template, and is loaded onto DNA by replication factor C (RFC) (Waga and Stillman, 1998). By analogy to PCNA, the 9-1-1 complex is loaded onto DNA by an RFC-like complex composed of Rad17 (Rad24 in *S. cerevisiae*) and 4 subunits of RFC (Green et al., 2000; Naiki et al., 2000). Like ATR-ATRIP, the 9-1-1 complex binds to sites of DNA damage, specifically

DNA breaks, *in vivo* (Kondo et al., 2001; Melo et al., 2001). Although ATR-ATRIP and the 9-1-1 complex are recruited to sites of DNA damage independently of each other (Melo et al., 2001), recruitment of both complexes requires the binding of replication protein A (RPA) to single-stranded DNA (Dart et al., 2004; Zou and Elledge, 2003; Zou et al., 2003). Thus it appears that all DNA damage must be modified to expose tracts of single-stranded DNA in order for the sensor proteins to recognize the damage and activate checkpoint pathways.

Two checkpoint effector kinases Chk1 and Chk2/Rad53 (Chk2 in humans, Rad53 in *S. cerevisiae*) function downstream of the sensors. Although some shuffling of their roles appears to have occurred in the course of evolution, there remains significant conservation of function. In *S. cerevisiae*, Rad53 is the key downstream component of both the replication stress and DNA damage signal transduction pathways (Allen et al., 1994; Sanchez et al., 1996; Sun et al., 1996; Weinert et al., 1994). In mammalian cells, Chk1 responds in an ATR-dependent manner to both DNA damage and HU-induced replication inhibition (Liu et al., 2000; Zhao and Piwnica-Worms, 2001). The Rad53 homologue Chk2 appears more specific for IR damage, acting downstream of ATM (Ahn et al., 2000; Blasina et al., 1999; Brown et al., 1999; Chaturvedi et al., 1999; Matsuoka et al., 1998; Matsuoka et al., 2000; Melchionna et al., 2000). Thus the roles of the effector kinases, particularly during S phase, are overlapping and complex. Activation of Rad53 requires Mec1-Ddc2 and one of two classes of mediator proteins. The prototypical mediator, Rad9, is required for Rad53 activation in response to DNA damage in the G1 and G2 phases of the cell cycle (de la Torre-Ruiz et al., 1998; Weinert and Hartwell, 1988), whereas Mrc1, Tof1, and Csm3 are critical during S phase where DNA damage causes stalling of DNA replication (Alcasabas et al., 2001; Foss, 2001; Katou et al., 2003; Nedelcheva et al., 2005; Osborn and Elledge, 2003; Tong et al., 2004; Xu et al., 2004).

Activation of checkpoint pathways causes cell cycle delay or arrest, presumably to allow repair of lesions to occur (Hartwell and Weinert, 1989; Weinert and Hartwell, 1988). In addition to cell cycle delay, checkpoints directly target DNA replication, recombination, and repair proteins, and cause increased transcription of a number of genes (Bashkirov et al., 2000; Brown and Kelly, 1999; Brush et al., 1996; D'Amours and Jackson, 2001; Gasch et al., 2001; Grenon et al., 2001; Kihara et al., 2000; Marini et al., 1997; Snaith et al., 2000; Usui et al., 2001; Weinreich and Stillman, 1999; Zhou and Elledge, 1993). Checkpoints also contribute to the stability of replication forks. When replication forks are impeded by DNA lesions or deoxyribonucleotide depletion, Mec1 and Rad53 prevent the collapse of stalled replication forks and allow the resumption of DNA synthesis after stalling (Cobb et al., 2003; Sogo et al., 2002; Tercero and Diffley, 2001; Tercero et al., 2003). Checkpoint defective mutants exhibit premature dissociation of DNA polymerases from stalled forks (Cobb et al., 2003; Lucca et al., 2004), and formation of abnormal DNA structures at replication forks, such as Holliday junctions (HJs) and DNA double strand breaks (DSBs) (Cha and Kleckner, 2002; Lopes et al., 2001; Sogo et al., 2002), which are detrimental to DNA replication, genomic integrity, and cell survival. In *mec1* and ATR mutants, these DSBs occur at specific chromosomal loci (Casper et al., 2002; Cha and Kleckner, 2002) that are thought to cause stalling of replication forks. The mediator proteins Mrc1, Tof1, and Csm3 interact directly with replication machinery at replication forks (Katou et al., 2003; Nedelcheva et al., 2005; Osborn and Elledge, 2003), and Mrc1 and Tof1 have been shown to be required for fork stability by facilitating activation of Rad53 when fork progression is inhibited (Katou et al., 2003; Osborn and Elledge, 2003). It is presently unclear how Mec1/ATR and Rad53/Chk2 act to stabilize stalled forks. Presumably phosphorylation of target proteins, such as RPA (Brush and Kelly, 2000; Brush et al., 1996), may be critical in this aspect. The identification of downstream targets of Mec1/ATR and

Rad53/Chk2 will be important in assessing the role of these checkpoint proteins at stalled replication forks.

1.2 RecQ DNA HELICASES

Over the last decade, strong evidence has accumulated which suggests that RecQ DNA helicases help process stalled replication forks to prevent genomic instability and cell death. *S. cerevisiae SGS1* is a member of the *recQ* DNA helicase family that unwinds DNA in a 3'-5' direction (Bennett et al., 1998; Gray et al., 1997; Harmon and Kowalczykowski, 2001; Karow et al., 1997; Puranam and Blackshear, 1994; Seki et al., 1994; Shen et al., 1998; Suzuki et al., 1997; Umezu and Nakayama, 1993; Umezu et al., 1990). RecQ helicases all have a central region of 350-400 residues that contains seven motifs found in many other DNA and RNA helicases. Five human homologues of *recQ* (*RECQL*, *BLM*, *WRN*, *RECQ4*, and *RECQ5*) have been identified to date. Loss of function mutations in *BLM*, *WRN*, and *RECQ4* give rise to Bloom syndrome (BS), Werner syndrome (WS), and Rothmund-Thomson syndrome (RTS), respectively (Ellis et al., 1995; Kitao et al., 1999; Yu et al., 1996). Although the spectrum of clinical features of each disease differs, they all result in a predisposition to cancer. Werner and Rothmund-Thomson syndromes are also characterized by premature aging. A detailed discussion of the clinical features of these diseases can be found elsewhere (German, 1995; Hickson, 2003; Shen and Loeb, 2000b; Vennos and James, 1995).

The major defects of cells with mutated RecQ helicases, including BS, WS, and RTS cells, are hyper-recombination and genomic instability. BS cells have elevated levels of sister-chromatid exchanges (SCEs) (Chaganti et al., 1974) and gross chromosomal rearrangements (GCRs) (German, 1993). Cells from WS patients display elevated levels of illegitimate recombination and large chromosomal deletions (Mohaghegh and Hickson, 2001; Shen and

Loeb, 2000a; Shen and Loeb, 2000b). RTS cells also have an increased frequency of chromosomal aberrations (Vennos and James, 1995). *S. cerevisiae sgs1* mutants show elevated levels of mitotic homologous recombination (HR), illegitimate recombination (Gangloff et al., 1994; Watt et al., 1996; Yamagata et al., 1998), SCEs (Onoda et al., 2000), and GCRs (Myung et al., 2001b; Myung and Kolodner, 2002). Cells lacking *SGS1* are also mildly sensitive to genotoxic agents such as methyl methanesulfonate (MMS), hydroxyurea (HU), and ultraviolet (UV) radiation (Gangloff et al., 1994; Watt et al., 1996; Yamagata et al., 1998).

Several observations suggest that RecQ helicases function during S phase to process abnormal replication intermediates resulting from stalled replication forks. In yeast and human cells, levels of RecQ helicases peak in S phase (Dutertre et al., 2000; Frei and Gasser, 2000). RecQ helicases co-localize with sites of DNA synthesis in yeast and *Xenopus laevis* (Chen et al., 2001a; Frei and Gasser, 2000). Furthermore, the human homologues, BLM and WRN, are required for normal S phase progression (Lonn et al., 1990; Poot et al., 1992). BS and WS cells accumulate abnormal replication intermediates or retarded replication forks, resulting in a prolonged S phase (Gianneli et al., 1977; Hanaoka et al., 1983; Hanaoka et al., 1985; Lonn et al., 1990; Poot et al., 1992). In contrast, S phase is completed faster than wild type in *sgs1Δ* cells as a result of faster moving DNA replication forks (Versini et al., 2003). However, completion of DNA replication is impeded at ribosomal DNA (rDNA), which contains a high density of replication fork barriers (RFBs), protein-DNA complexes which prevent replication forks from moving in the direction opposite to RNA polymerase I (Brewer and Fangman, 1988; Kaliraman and Brill, 2002; Versini et al., 2003). It is possible that human DNA contains more barriers to the progression of replication forks, which may account for the difference seen in yeast and humans. RecQ helicases may be needed to stabilize or restart stalled replication forks.

Indeed, Sgs1 is required to stabilize DNA polymerases α and ε at sites of stalled replication forks induced by HU treatment (Cobb et al., 2003).

Additional data support a role for RecQ helicases in processing stalled replication forks by suppressing unwanted and detrimental recombination events at sites of stalled forks. The *Escherichia coli* RecQ helicase, the founding member of the RecQ family, is part of the RecF recombination pathway, responsible for replication recovery following DNA damage (Horii and Clark, 1973; Kolodner et al., 1985). It has also been proposed that RecQ functions with the RecJ exonuclease in a process that leads to the formation of a triple stranded DNA and blocks formation of recombination intermediates until replication can restart (Courcelle and Hanawalt, 1999). In *S. cerevisiae*, *sgs1* mutants accumulate recombination-dependent cruciforms or X-structures, called Holliday junctions (HJs), at damaged replication forks (Liberi et al., 2005). As discussed above, HJs can form as a result of the regression of collapsed replication forks, as seen in *rad53* mutants that have been exposed to HU (Sogo et al., 2002). If left unprocessed, HJs can lead to arrest of DNA replication, genomic instability, and loss of cell viability. Interestingly, the defects of *S. pombe* strains lacking *rqh1*$^{+}$, the *recQ* homologue in fission yeast, can be partially suppressed by expression of RusA (Doe et al., 2000), a bacterial resolvase of HJs, suggesting that the presence of unresolved HJs is causing most, if not all, of the defects seen in *recQ* mutants. RecQ, Sgs1, BLM, and WRN are all able to unwind DNA structures that may be present at sites of stalled replication forks, including HJs (Bennett et al., 1999; Harmon and Kowalczykowski, 1998; Mohaghegh et al., 2001; Shen et al., 1998). These data are consistent with RecQ helicases functioning to stabilize stalled replication forks by reversing HJ formation.

The human RecQ homologue BLM has been found to be a part of the BRCA-associated genome surveillance complex, a complex that consists of proteins that have roles in the

recognition of aberrant DNA structures, in the repair of DNA damage, or in DNA damage checkpoint activation (Wang et al., 2000). Given the DNA structure-specific helicase activity of BLM, BLM may scan the genome for structural abnormalities (Oakley and Hickson, 2002), likely including abnormalities that arise from stalling of replication forks.

1.3 RecQ HELICASES AND TOPOISOMERASE III

RecQ helicases function in concert with additional proteins, in particular topoisomerase III (Top3). A subset of RecQ family members, including Sgs1, Rqh1, BLM, and RECQ5, physically interact with Top3 (Ahmad and Stewart, 2005; Bennett et al., 2000; Fricke et al., 2001; Johnson et al., 2000; Shimamoto et al., 2000; Wu et al., 2000). Moreover, the functionality of an Sgs1 N-terminal truncation mutant that can no longer interact with Top3 can be restored by replacing the truncated region with Top3, signifying the importance of the interaction between Sgs1 and Top3 (Bennett and Wang, 2001). Top3 possesses only weak DNA relaxation activity, suggesting that it is unlikely to participate in the maintenance of DNA supercoiling homeostasis (Kim and Wang, 1992). *E. coli* RecQ stimulates Top3 to catenate and decatenate covalently closed duplex DNA (Harmon et al., 1999). In addition, BLM stimulates the DNA strand passage activity of Top3 (Oakley and Hickson, 2002). Moreover, BLM and Top3 can work together to resolve a recombination intermediate containing a double Holliday junction (Wu and Hickson, 2003). Like Sgs1, Top3 is required to stabilize DNA polymerase ε at stalled replication forks, and both *sgs1Δ* and *top3Δ* mutants accumulate recombination-dependent X-structures, further suggesting that Top3 functions with Sgs1 to prevent replication fork collapse (Bjergbaek et al., 2004; Liberi et al., 2005). Two human topoisomerase III homologues, *TOP3α* and *TOP3β*, have been identified (Hanai et al., 1996; Ng et al., 1999). Deletion of murine *TOP3α* causes embryonic lethality (Li and Wang, 1998). Mice lacking

TOP3β develop to maturity, but have a reduced lifespan associated with multiple organ defects (Kwan and Wang, 2001). *S. cerevisiae* strains lacking *TOP3* exhibit a severe growth defect, sensitivity to DNA damaging agents, and hyper-recombination (Gangloff et al., 1994; Wallis et al., 1989). Most of the defects exhibited by *top3* mutants can be suppressed by deletion of *SGS1* (Chakraverty et al., 2001; Gangloff et al., 1994), a relationship that appears to be conserved in *S. pombe* where mutations in the *S. pombe rqh1*$^{+}$ can suppress the lethality of *top3Δ* mutants (Maftahi et al., 1999). This suggests that Top3 is required to resolve a toxic DNA structure that is generated by Sgs1.

1.4 RecQ-Top3 AND DNA DAMAGE CHECKPOINT FUNCTIONS

There is increasing evidence that RecQ helicases play a role in the checkpoint response during S phase. *S. cerevisiae* cells lacking *SGS1* are sensitive to the replication inhibitor HU, which arrests cells in S phase (Frei and Gasser, 2000). A fraction of these HU-treated cells extend microtubule spindles to mitotic length, a failure to completely arrest cells in S phase (Frei and Gasser, 2000). *sgs1Δ* mutants also fail to slow the progress of S phase in response to MMS-induced DNA damage (Frei and Gasser, 2000). Following exposure to HU or MMS, the checkpoint kinase Rad53 is activated by phosphorylation and functions to stabilize stalled replication forks, prevent the precocious firing of normally dormant replication origins, up-regulate DNA damage response genes, and delay cell cycle progression to allow time for repair of the damage (Paulovich and Hartwell, 1995; Pellicioli et al., 1999; Tercero and Diffley, 2001; Tercero et al., 2003). In the absence of the RFC-like checkpoint complex member Rad24, Sgs1 is needed for complete activation of Rad53 upon exposure to HU (Frei and Gasser, 2000). Consistent with a checkpoint role of Sgs1, Sgs1 co-localizes with Rad53 in S-phase-specific

foci, even in the absence of fork arrest (Frei and Gasser, 2000). These data support a role for Sgs1 in activating a Rad53-dependent checkpoint response upon exposure to genotoxic agents.

RecQ helicases may also be a downstream target of checkpoint pathways. BLM is phosphorylated by ATM in a cell-cycle-dependent manner, and in response to γ-irradiation, although the functional significance of this phosphorylation remains unclear (Ababou et al., 2000). Furthermore, BS cells are sensitive to HU and BLM is phosphorylated on two N-terminal residues by ATR (Davies et al., 2004). BS cells ectopically expressing BLM protein containing alanine substitutions of these two residues fail to recover from HU-induced replication blockage, and arrest at a G2/M checkpoint (Davies et al., 2004).

Top3 also has a role in activating the Rad53-dependent checkpoint response upon exposure to genotoxic agents. Like *sgs1Δ* mutants, *S. cerevisiae* strains lacking *TOP3* are sensitive to a variety of DNA damaging agents and are partially defective in slowing the rate of S phase progression following exposure to DNA damaging agents (Chakraverty et al., 2001). While *sgs1Δ* single mutants do not exhibit any detectable defects in Rad53 activation, *top3Δ* mutants fail to activate Rad53 fully after treatment with MMS (Chakraverty et al., 2001), indicating that the efficiency of sensing the existence of DNA damage or signaling to the Rad53 checkpoint kinase is impaired. Like many other defects associated with *top3* mutants, the defect in activating Rad53 in the presence of MMS can be suppressed by deletion of *SGS1* (Chakraverty et al., 2001). *top3Δ* mutants may have a compromised checkpoint due to impaired progression into and through S phase (Bjergbaek et al., 2004). A *rad24Δ top3Δ* double mutant, which does not exhibit these S phase defects or the slow growth exhibited by a *top3Δ* mutant, is fully competent in activating Rad53 upon exposure to HU (Bjergbaek et al., 2004).

In addition to having a role in checkpoint activation, several studies in *S. cerevisiae* and *S. pombe* have shown that *top3* mutants accumulate DNA damage that results in checkpoint

activation (Chakraverty et al., 2001; Win et al., 2004). *S. cerevisiae top3Δ* mutants exhibit a *RAD24*-dependent checkpoint delay in the G2 phase (Chakraverty et al., 2001). *S. pombe* cells lacking *top3*$^{+}$ arrest at G2/M in a Chk1-dependent manner (Win et al., 2004). These cells also show phosphorylated Chk1 checkpoint kinase (Win et al., 2004), a marker for checkpoint activation (Walworth et al., 1993). Thus, although *top3* mutants are defective in activating the S phase and S/M checkpoints, these mutants accumulate DNA damage that results in G2 checkpoint activation. *E. coli* RecQ has been shown to stimulate Top3 to catenate negatively supercoiled plasmids (Harmon et al., 1999), suggesting a role in decatenating linked chromosomes during the final stages of DNA replication. Such a role may explain the checkpoint-mediated delay in G2 as failure to complete replication might activate the G2 DNA damage checkpoint.

1.5 OTHER PROTEINS IMPORTANT FOR PROCESSING STALLED FORKS

Given the critical role of the RecQ-Top3 complex in preventing DNA replication fork collapse, several genetic screens have been performed in *S. cerevisiae* in an attempt to identify genes in parallel pathways to Sgs1-Top3-mediated fork stability (Mullen et al., 2001; Ooi et al., 2003; Tong et al., 2001). Characterization of a number of these genetic interactions has revealed additional, evolutionarily conserved proteins with putative roles in ensuring fork stability or fork restart after replication arrest. These include two heterodimeric endonucleases, Mus81-Mms4 and Slx1-Slx4, and two DNA helicases, Srs2 (also known as Hpr5) and Rrm3. Several of these genetic interactions are conserved in *S. pombe*, as deletion of the *S. pombe recQ* homologue *rqh1*$^{+}$ causes cell death or sickness in combination with deletions in *mus81*$^{+}$, *slx1*$^{+}$, *slx4*$^{+}$, and *srs2*$^{+}$ (Boddy et al., 2000; Coulon et al., 2004; Doe et al., 2002; Wang et al., 2001a).

Mus81-Mms4. *mus81* and *mms4* mutants are sensitive to genotoxic agents, such as MMS, HU, and camptothecin (CPT), which cause stalling of replication forks (Boddy et al., 2000; Interthal and Heyer, 2000; Mullen et al., 2001; Parsons et al., 2004). *Mus81*$^{-/-}$ mice are viable and fertile, but are hypersensitive to the DNA crosslinking agent mitomycin C, although not to γ-irradiation (McPherson et al., 2004). Both homozygous *Mus81*$^{-/-}$ and heterozygous *Mus81*$^{+/-}$ mice exhibit elevated levels of chromosomal aberrations and a predisposition to lymphomas and other cancers (McPherson et al., 2004). Extensive biochemical studies of Mus81-Mms4 have been performed with the *S. cerevisiae*, *S. pombe*, and human proteins. Mus81-Mms4 can resolve HJs (Boddy et al., 2001; Chen et al., 2001b; Gaillard et al., 2003), although it has also been shown to preferentially cleave 3'-flap or replication fork-like substrates (Bastin-Shanower et al., 2003; Constantinou et al., 2002; Doe et al., 2002; Kaliraman et al., 2001). HJs accumulate in a DNA polymerase α mutant, a mutant that likely causes elevated levels of fork stalling, that also lacks Mus81, providing evidence that HJs may be resolved by Mus81-Mms4 *in vivo* (Gaillard et al., 2003). Mus81-Mms4 may be required to resolve HJs formed from the collapsing of stalled forks, rather than to stabilize stalled forks. Consistent with this view, *S. pombe* Mus81 is not required to survive transient HU-induced stalled forks, but is required to survive fork collapse induced by CPT (Kai et al., 2005). HU inhibits ribonucleotide reductase function, causing replication fork stalling due to depletion of deoxyribonucleic acid pools (Reichard, 1988), while CPT is a topoisomerase I inhibitor which causes accumulation of single-stranded nicks that can cause replication fork collapse (Porter and Champoux, 1989a; Porter and Champoux, 1989b).

Slx1-Slx4. Slx1 and Slx4 form a second structure-specific endonuclease that cleaves branched DNA structures (Fricke and Brill, 2003), although phenotypic data suggests that it functions in a separate pathway than Mus81-Mms4. Though *slx1* and *slx4* are also sensitive to MMS and

CPT, (Deng et al., 2005; Fricke and Brill, 2003) they are less sensitive than *mus81* and *mms4* mutants, and are also not sensitive to chronic exposure to HU (Fricke and Brill, 2003; Mullen et al., 2001). In addition, although the *sgs1 mus81* and *sgs1 mms4* synthetic lethality can be suppressed by abolishing the homologous recombination (HR) pathway, the *sgs1 slx1* and *sgs1 slx4* synthetic lethality cannot (Bastin-Shanower et al., 2003; Fabre et al., 2002). This implies that Mus81-Mms4 acts on recombination-dependent structures while Slx1-Slx4 does not. The *sgs1 slx4* synthetic lethality results from an inability to replicate the rDNA repeats on chromosome XII properly (Kaliraman and Brill, 2002). Indeed, replication of the rDNA region, unlike the replication of the rest of the genome, is retarded in *sgs1Δ* mutants (Versini et al., 2003; Weitao et al., 2003). This effect is likely due to the presence of a replication fork barrier (RFB) in each rDNA repeat (Kaliraman and Brill, 2002; Versini et al., 2003). Thus, it appears that Slx1-Slx4 is required to process stalled forks at the RFB while Mus81-Mms4 is needed at damage-induced collapsed forks.

Srs2 and Rrm3. Srs2 and Rrm3 are antirecombinogenic DNA helicases (Aguilera and Klein, 1988; Fabre et al., 2002; Keil and McWilliams, 1993). Both *sgs1 srs2* and *sgs1 rrm3* synthetic growth defects can be suppressed by mutation of HR genes (McVey et al., 2001; Schmidt and Kolodner, 2004; Torres et al., 2004b). Srs2 suppresses recombination, potentially at sites of stalled forks, by removing Rad51 protein from single-stranded DNA, thus inhibiting the initial step in HR (Krejci et al., 2003; Veaute et al., 2003). Rrm3 is a DNA helicase whose absence causes replication forks to stall at over 1,000 discrete sites, including multiple sites in each of the 150 rDNA repeats, tRNA genes, centromeres, telomeres, and the silent mating-type loci (Ivessa et al., 2003; Ivessa et al., 2002; Ivessa et al., 2000). These sites are assembled into nonnucleosomal protein-DNA complexes and disruption of these complexes alleviates the need

for Rrm3 to prevent fork stalling (Ivessa et al., 2003; Torres et al., 2004a). Thus, Rrm3 likely acts to promote replication past protein-DNA complexes, although its exact mechanism of action has yet to be elucidated.

1.6 ROLE OF HOMOLOGOUS RECOMBINATION IN RESTARTING STALLED FORKS

The HR pathway, which is critical in repairing DSBs (Krogh and Symington, 2004), has also been implicated in restarting stalled replication forks. Indeed, the Sgs1-Top3 and Mus81-Mms4 complexes both act on structures that are generated by the HR pathway involving Rad51, Rad52, Rad54, Rad55, Rad57, and Rad59 (Bastin-Shanower et al., 2003; Kaliraman et al., 2001; Oakley et al., 2002; Shor et al., 2002). In *E. coli*, it has been estimated that the generation of DSBs at stalled replication forks occurs once every two to three rounds of replication (Michel et al., 1997), while in *S. cerevisiae*, 22% of S phase cells contain Rad52 foci, which form as a result of DSBs (Lisby et al., 2001). There are several ways in which a DSB could form at the site of a stalled fork (Figure 2). If the replication fork encounters a single strand nick on the template DNA, collapse of the fork and a DSB results. Regression of a stalled replication fork, whereby the newly-synthesized DNA strands anneal to each other, would form a HJ with a double-stranded DNA end (i.e. a DSB), that could be cleaved by endonucleases, also resulting in a DSB (Seigneur et al., 1998). In either scenario, the DSB would result in a strand invasion event from which replication can be restarted, a process termed break-induced replication, or BIR (Kraus et al., 2001; Symington, 2002). A HJ produced by a regressed fork could also allow limited synthesis using one of the nascent strands as template. Such a model would involve an uncoupling between leading and lagging strand DNA synthesis, which has been demonstrated in *E. coli* (Pages and Fuchs, 2003). The regressed fork could then reverse branch migrate to yield

a replication fork that has passed the stall-inducing lesion (Figure 2). This model may not involve a DSB but the HR proteins would likely be needed for the annealing and branch migration events.

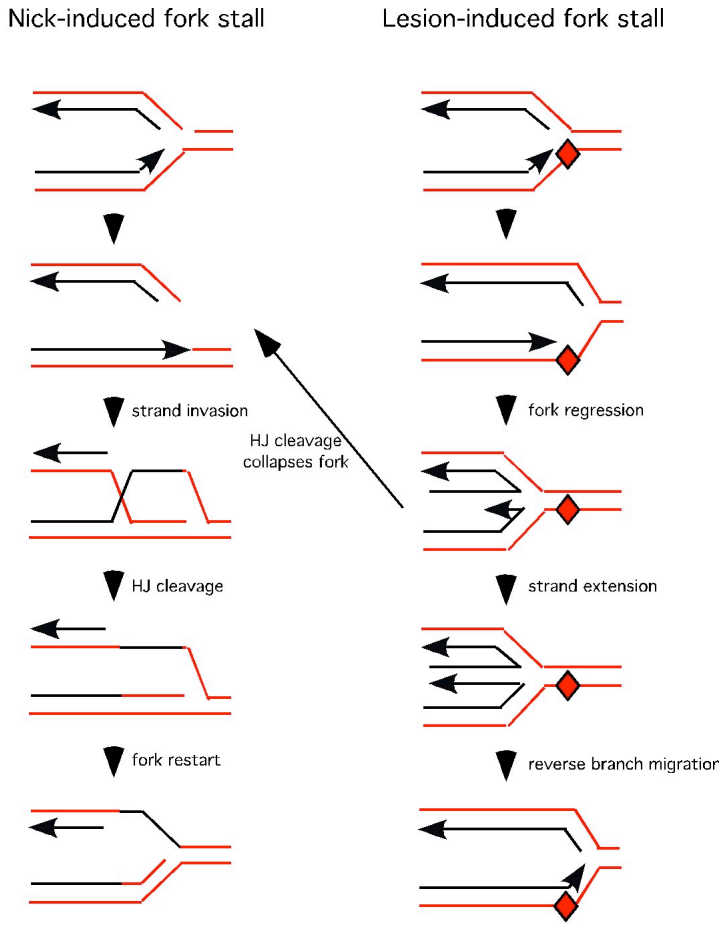

Figure 2. Role of HR in processing stalled replication forks. See text for details. (Krogh and Symington, 2004)

Despite the appeal of such models, the role of HR at stalled forks is still controversial. Although BIR has been shown to repair DSBs (Malkova et al., 1996), there is as yet no direct evidence for BIR having a role in restart of stalled replication forks. Also, even though there is evidence for regressed forks in *E. coli* (Courcelle et al., 2003; Grompone et al., 2004; Seigneur et al., 1998), they have only been observed in *S. cerevisiae* in HU-arrested cells lacking the checkpoint kinase Rad53 (Sogo et al., 2002). Furthermore, mutants that are defective in HR are not sensitive to transient exposure to HU (Meister et al., 2005), despite being sensitive to

prolonged exposure (Chang et al., 2002). HU-stalled forks likely only collapse after prolonged exposure to HU, a view supported by several observations. Induction of DSBs in *S. cerevisiae* leads to a relocalization of the normally diffuse, nuclear Rad52 into punctate nuclear foci (Lisby et al., 2001; Meister et al., 2003). In wild type cells, transient replication stress induced by exposure to HU does not induce Rad52 relocalization (Lisby et al., 2004) suggesting that collapsed forks and the resulting DSBs are not present. However, in S-phase checkpoint mutants that accumulate collapsed forks upon transient exposure to HU (Lopes et al., 2001; Sogo et al., 2002; Tercero and Diffley, 2001), Rad52 does form foci (Lisby et al., 2004) that colocalize with the fork-associated protein PCNA (Meister et al., 2005). Levels of spontaneous Rad52 foci are significantly reduced in wild type cells when exposed to HU, compared to untreated cells (Lisby et al., 2004), suggesting that Rad52 may be actively excluded from stalled forks in a checkpoint-dependent manner. The S-phase checkpoint may accomplish this by modulating the phosphorylation state of Srs2 (Liberi et al., 2000), which can suppress recombination at stalled forks (Fabre et al., 2002) by removing Rad51 from single-stranded DNA (Krejci et al., 2003; Veaute et al., 2003). Interestingly, Rad52 foci do form upon prolonged exposure to HU (Lisby et al., 2004), correlating with the sensitivity of HR mutants to chronic HU exposure (Chang et al., 2002). An interesting possibility is that during prolonged exposure to HU, replication fork collapse becomes increasingly likely, resulting in DSB formation, relocalization of Rad52, and a requirement for HR for cell survival. Altogether, these data suggest that HR may not be important for recovery of transient replication fork stalling, but may become essential for viability when forks collapse.

Recent work by Lambert *et al.* in *S. pombe* has shown that recombination proteins localize to sites of forks stalled at the fission yeast RFB *RTS1*, causing elevated levels of recombination (Lambert et al., 2005). Recombination is important in this context for cell

viability, but also induces site-specific GCRs (Lambert et al., 2005). The authors propose that recombination helps prevent cell death when forks stall, although at the expense of genomic stability. This apparent discrepancy with data obtained using HU-induced stalled forks likely illustrates a difference in response to different types of stalled forks. To generate an inducible fork stall, Lambert *et al*. exploited the polar RFB near the *S. pombe mat* locus needed for mating type switching (Dalgaard and Klar, 2001). Fork stalling at this RFB is a programmed event that has evolved to permit mating type switching, and as such, is likely processed differently than stalled forks induced by exposure to HU or DNA damaging agents. Unlike damage-induced stalled forks, forks stalled at this RFB may be preferentially collapsed to yield a DSB needed for mating type switching (Beach, 1983). This emphasizes the need to study a variety of stalled forks to fully understand the complex mechanisms underlying this fundamental process.

1.7 YEAST FUNCTIONAL GENOMICS

Extensive genetic studies, especially in the budding yeast *S. cerevisiae* and fission yeast *S. pombe*, have uncovered many components required for proper and efficient execution of DNA damage response pathways. Many *S. cerevisiae* and *S. pombe* DNA damage response genes have human homologues and mutations in a number of these genes have been implicated in human cancers. For example, ataxia telangiectasia and Li Fraumeni syndrome have been linked to mutations in *ATM* and *CHK2*, whose homologues in *S. cerevisiae* are *TEL1* and *RAD53*, respectively (Bell et al., 1999; Morrow et al., 1995; Savitsky et al., 1995). Therefore the identification of DNA damage response genes in *S. cerevisiae* is likely to uncover novel genes mutated in human cancers. Recent advances in genomic, proteomic, and bioinformatic techniques have significantly increased the utility of model organisms, especially *S. cerevisiae*. In particular, the construction of a complete collection of *S. cerevisiae* gene deletion mutants

(Giaever et al., 2002; Winzeler et al., 1999), along with libraries of conditional alleles of essential genes (Kanemaki et al., 2003; Mnaimneh et al., 2004), has allowed for systematic genetic analyses to determine gene function.

Of the ~6000 known or predicted genes in *S. cerevisiae*, about 75% are nonessential (Giaever et al., 2002; Winzeler et al., 1999). This emphasizes the ability of yeast cells to tolerate individual deletions of most genes, likely reflecting redundant pathways that have evolved to buffer the phenotypic consequences of genetic variation (Hartman et al., 2001). This high degree of genetic redundancy makes it difficult to determine the function of many genes, but studying synthetic genetic interactions can circumvent this problem. A synthetic genetic interaction occurs when a mutation in a gene suppresses, enhances, or modifies the phenotype of a second mutation. In particular, if two mutations cause cell sickness or cell death, the synthetic genetic interaction is termed synthetic sick or synthetic lethal, respectively. The creation of the *S. cerevisiae* gene deletion mutants have enabled genome-wide, high-throughput synthetic genetic interaction screens by using an approach termed synthetic genetic array (SGA) analysis (Tong et al., 2001; Tong et al., 2004). Large-scale SGA analysis has allowed prediction of gene function because genetic interactions often occur between functionally related genes, and similar genetic interaction profiles tend to identify components of the same pathway (Tong et al., 2004). To organize large-scale SGA data, two-dimensional hierarchical clustering is used. The algorithm groups genes according to the similarity of their genetic interactions (Tong et al., 2004). These and other functional genomic tools have greatly increased the speed at which functional information can be obtained on uncharacterized genes, including genes with currently unknown functions in the DNA damage response. In this thesis, I use these yeast functional genomic tools to identify novel DNA damage response genes.

1.8 RATIONALE FOR THESIS PROJECT

Despite the fact that *S. cerevisiae* has been well studied over the span of several decades, approximately 2,000 of its predicted ~6,000 open reading frames (ORFs) have not yet been characterized experimentally (Hughes et al., 2004). Assigning functions to these uncharacterized genes will undoubtedly uncover additional genes with important roles in DNA damage response. Furthermore, many genes that have been characterized in other pathways may have yet to be defined roles in responding to DNA damage. The goal of my project was to use functional genomics to identify novel genes that function in the response to DNA damage, particularly genes that are required for processing stalled replication forks. I have accomplished this by utilizing an ordered array of *S. cerevisiae* mutants (a) to screen for genes required for resistance to MMS, a known DNA replication fork stalling agent, and (b) to identify genetic interactions with genes with known or putative roles in processing stalled replication forks, as genetic interactions often occur between functionally related genes, and similar genetic interaction profiles tend to identify components of the same pathway (Tong et al., 2004). Some of these novel genes were subsequently characterized further through more hypothesis-driven experiments.

2. MATERIALS AND METHODS

2.1 YEAST STRAINS AND MEDIA

Standard yeast media and growth conditions were used (Moreno et al., 1991; Sherman, 1991). *S. cerevisiae* strains used in this study are derivatives of BY4741 (Brachmann et al., 1998) and are listed in Table 1. Nonessential haploid deletion strains were made by the *Saccharomyces* Gene Deletion Project (Winzeler et al., 1999), are isogenetic to BY4741, and can be obtained from Open Biosystems (Huntsville, AL) or EUROSCARF (Frankfurt, Germany). To construct MCY16, *mec2-1* (also known as *rad53-11*) (Weinert et al., 1994) was amplified by PCR and co-integrated with *URA3* into Y3068 (Tong et al., 2001).

Table 1. Yeast strains used in this thesis.

Strain Names	Genotype	Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Brachmann et al., 1998)
MCY16	*MATα mec2-1::URA3 can1Δ::MFA1pr-HIS3 his3Δ1 leu2Δ0 ura3Δ0*	(Chang et al., 2002)
Y3597	*MATα mus81Δ::natR can1Δ::MFA1pr-HIS3 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(Bellaoui et al., 2003)
Y3561	*MATα mms4Δ::natR can1Δ::MFA1pr-HIS3 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(Bellaoui et al., 2003)
Y4521	*MATα elg1Δ::natR can1Δ::MFA1pr-HIS3 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(Bellaoui et al., 2003)
MCY236	*MAT***a** *mec2-1::URA3 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(Bellaoui et al., 2003)
KSC006	*MAT***a** *ade1 his2 trp1 ura3 leu2*	(Naiki et al., 2001)
KSC1372	*MAT***a** *RFC1-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Naiki et al., 2001)
KSC1373	*MAT***a** *RFC2-FLAG::TRP1 ade1 his2 trp1 ura3 leu2*	(Naiki et al., 2001)
KSC1374	*MAT***a** *RFC3-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Naiki et al., 2001)
KSC1375	*MAT***a** *RFC4-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Naiki et al., 2001)
KSC1376	*MAT***a** *RFC5-FLAG::TRP1 ade1 his2 trp1 ura3 leu2*	(Naiki et al., 2001)
KSC1377	*MAT***a** *RAD24-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Naiki et al., 2001)
YPH1483	*MAT***a** *ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ63 leu2Δ CTF18-13MYC::TRP1*	(Mayer et al., 2001)
MBY44	*MAT***a** *ELG1-13MYC::KanMX6 RFC1-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)
MBY103	*MAT***a** *ELG1-13MYC::KanMX6 RFC2-FLAG::TRP1 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)

MBY104	*MAT***a** *ELG1-13MYC::KanMX6 RFC3-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)
MBY105	*MAT***a** *ELG1-13MYC::KanMX6 RFC4-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)
MBY106	*MAT***a** *ELG1-13MYC::KanMX6 RFC5-FLAG::TRP1 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)
MBY107	*MAT***a** *ELG1-3HA::KanMX6 RFC5-FLAG::TRP1 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)
MBY108	*MAT***a** *ELG1-13MYC::KanMX6 RAD24-FLAG::URA3 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)
MBY110	*MAT***a** *ELG1-3HA::KanMX6 ade1 his2 trp1 ura3 leu2*	(Bellaoui et al., 2003)
MBY112	*MAT***a** *ELG1-3HA::KanMX6 CTF18-13MYC::TRP1 ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ63 leu2Δ*	(Bellaoui et al., 2003)
MBY46	*MAT***a** *rad24Δ::natR elg1Δ::kanR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Bellaoui et al., 2003)
MBY66	*MAT***a** *elg1Δ::natR ctf18Δ::kanR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Bellaoui et al., 2003)
MBY74	*MAT***a** *rad24Δ::natR ctf18Δ::kanR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Bellaoui et al., 2003)
MCY290	*MAT***a** *elg1Δ::natR rad24Δ::kanR ctf18Δ::kanR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Bellaoui et al., 2003)
MBY235	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 [p426GAL1]*	(Bellaoui et al., 2003)
MBY236	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 [p426GAL1-POL30]*	(Bellaoui et al., 2003)
MBY238	*MAT***a** *elg1Δ::kanR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 [p426GAL1]*	(Bellaoui et al., 2003)
MBY239	*MAT***a** *elg1Δ::kanR his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 [p426GAL1-POL30]*	(Bellaoui et al., 2003)
Y5646	*MATα rmi1Δ::natR lyp1Δ can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2*	(Bellaoui et al., 2003)
BY4742	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	(Brachmann et al., 1998)
MCY304	BY4742 with *MATa rmi1Δ::kanMX6*	(Chang et al., 2005)
MCY352	BY4742 with *SGS1-3HA-LEU2*	(Chang et al., 2005)
MCY312	BY4741 with *RMI1-TAP-HIS3*	(Chang et al., 2005)
MCY353	BY4741 with *MATα SGS1-3HA-LEU2 RMI1-TAP-HIS3*	(Chang et al., 2005)
MCY348	BY4742 with *TOP3-V5-VSV-kanMX6*	I. Stagljar
MCY355	BY4741 with *MET15 TOP3-V5-VSV-kanMX6 RMI1-TAP-HIS3*	(Chang et al., 2005)
MCY356	BY4742 with *LYS2 TOP3-V5-VSV-kanMX6 RMI1-TAP-HIS3 sgs1Δ::kanMX6*	(Chang et al., 2005)
MCY365	BY4741 with *MATα SGS1-3HA-LEU2 TOP3-TAP-HIS3 rmi1Δ::kanMX6*	(Chang et al., 2005)
MCY367	BY4741 with *MATα SGS1-3HA-LEU2 RMI1-TAP-HIS3 top3Δ::kanMX6*	(Chang et al., 2005)
MCY372	BY4741 with *sgs1::kanMX6* (pRS415)	(Chang et al., 2005)
MCY373	BY4741 with *sgs1::kanMX6* (pSM100-HA)	(Chang et al., 2005)
MCY374	BY4741 with *sgs1::kanMX6* (pSM100-hd-HA)	(Chang et al., 2005)

MCY375	MCY356 (pSM100-HA)	(Chang et al., 2005)
MCY376	MCY356 (pSM100-hd-HA)	(Chang et al., 2005)
MCY377	BY4742 with *LYS2 TOP3-V5-VSV-kanMX6 rmi1Δ::natMX6 sgs1Δ::kanMX6* (pSM100-HA)	(Chang et al., 2005)
MCY378	BY4742 with *LYS2 TOP3-V5-VSV-kanMX6 rmi1Δ::natMX6 sgs1Δ::kanMX6* (pSM100-hd-HA)	(Chang et al., 2005)
MCY379	BY4742 with *LYS2 top3Δ::natMX6 RMI1-TAP-HIS3 sgs1Δ::kanMX6* (pSM100-HA)	(Chang et al., 2005)
MCY380	BY4742 with *LYS2 top3Δ::natMX6 RMI1-TAP-HIS3 sgs1Δ::kanMX6* (pSM100-hd-HA)	(Chang et al., 2005)
MCY357	BY4741 (pWJ1344)	(Chang et al., 2005)
MCY358	MCY304 (pWJ1344)	(Chang et al., 2005)
MCY328	BY4741 with *top3Δ::kanMX6*	(Chang et al., 2005)
MCY359	MCY328 (pWJ1344)	(Chang et al., 2005)
MCY360	BY4741 with *sgs1Δ::kanMX6* (pWJ1344)	(Chang et al., 2005)
RDY9	*MATa mfa1::MFA1pr-HIS3 can1Δ::natR leu2ΔEcoRI::URA3-HOcs::leu2ΔBstII leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 lyp1Δ*	(Chang et al., 2005)
RDY10	*MATa sgs1Δ::kanMX6 mfa1::MFA1pr-HIS3 can1Δ::natR leu2ΔEcoRI::URA3-HOcs::leu2ΔBstII leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 lyp1Δ*	(Chang et al., 2005)
RDY14	*MATa rmi1Δ::kanMX6 mfa1::MFA1pr-HIS3 can1Δ::natR leu2ΔEcoRI::URA3-HOcs::leu2ΔBstII leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 lyp1Δ*	(Chang et al., 2005)
RDY15	*MATa top3Δ::kanMX6 mfa1::MFA1pr-HIS3 can1Δ::natR leu2ΔEcoRI::URA3-HOcs::leu2ΔBstII leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 lyp1Δ*	(Chang et al., 2005)
CZY106	*MATa mfa1::MFA1pr-HIS3 hxt13Δ::URA3 his3Δ1 ura3Δ0 lyp1Δ leu2Δ0 met15Δ0*	(Chang et al., 2005)
CZY211	*MATa sgs1Δ::kanMX6 mfa1::MFA1pr-HIS3 hxt13Δ::URA3 his3Δ1 ura3Δ0 lyp1Δ leu2Δ0 met15Δ0*	(Chang et al., 2005)
CZY232	*MATa sgs1Δ::natR mfa1::MFA1pr-HIS3 hxt13Δ::URA3 his3Δ1 ura3Δ0 lyp1Δ leu2Δ0 met15Δ0*	(Chang et al., 2005)
CZY212	*MATa top3Δ::kanMX6 mfa1::MFA1pr-HIS3 hxt13Δ::URA3 his3Δ1 ura3Δ0 lyp1Δ leu2Δ0 met15Δ0*	(Chang et al., 2005)
CZY213	*MATa rmi1Δ::kanMX6 mfa1::MFA1pr-HIS3 hxt13Δ::URA3 his3Δ1 ura3Δ0 lyp1Δ leu2Δ0 met15Δ0*	(Chang et al., 2005)
MCY340	BY4741 with *can1Δ::MFA1-HIS3 rmi1Δ::natMX6*	(Chang et al., 2005)
MCY323	BY4741 with *lyp1Δ rmi1Δ::natMX6 sgs1Δ::kanMX6*	(Chang et al., 2005)
MCY335	BY4741 with *sgs1Δ::kanMX6 top3Δ::natMX6*	(Chang et al., 2005)
MCY345	BY4741 with *sgs1Δ::kanMX6 top3Δ::kanMX6 rmi1Δ::natMX6*	(Chang et al., 2005)
MCY297	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0*	(Chang et al., 2005)
MCY370	*MATa/MATα rmi1Δ::kanMX6/rmi1Δ::natMX6 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/met15Δ0 LYS2/lys2Δ0 LYP1/lyp1Δ0*	(Chang et al., 2005)
GBY635	*SGS1-3HA-LEU2 TOP3-V5-VSV-kanMX6 RMI1-TAP-HIS3 leu2Δ0 his3Δ1 ura3Δ0*	(Chang et al., 2005)

2.2 HIGH-THROUGHPUT MMS SCREEN

An ordered array of 4644 *MATa* viable haploid yeast gene deletion mutants, in duplicate at a density of 768 colonies per plate, was replica pinned onto YPD and YPD+0.035% MMS. MMS (Aldrich) plates contained 0.035% (v/v) MMS in YPD and were used within 24 hours of preparation. The screen was performed 3 times using an automated pinning system, as described (Tong et al., 2001). Plates were incubated at 30°C for 2 days before scoring.

2.3 MMS, HU, AND UV SENSITIVITY MEASUREMENTS

Cells were grown in YPD overnight at 30°C, diluted to a concentration of $1x10^7$ cells/mL, and four additional ten-fold serial dilutions were made. 8 μL of each serial dilution was spotted onto the indicated media and incubated at 30°C for 2 or 3 days. MMS (Aldrich) plates contained 0.004%, 0.01%, or 0.035% (v/v) MMS in YPD and were used within 24h of preparation. Hydroxyurea (HU) plates contained 10 mM, 50 mM HU, or 200 mM in YPD. For the UV radiation sensitivity assay, cells were serially diluted, spotted onto YPD plates, exposed to UV light at 100 J/m^2, and incubated at 30°C. To determine viability after transient MMS treatment, mid-log-phase cultures were incubated with 0.004% or 0.035% MMS, or 10 mM HU, in YPD liquid at 30°C. Samples were collected at the indicated time points, diluted, plated on YPD, and colonies were counted after incubation at 30°C for 3 days. The wild type control strain used in MMS, HU, and UV sensitivity assays was BY4741 (Brachmann et al., 1998).

2.4 CELL CYCLE SYNCHRONIZATION

Cells were arrested in G1 by culturing in the presence of 2 μg/mL alpha mating factor for 2 h at 30°C in YPD pH3.9. Cells were released into the cell cycle by harvesting, washing, and resuspending in YPD.

2.5 FLOW CYTOMETRY

Cells were harvested and fixed in 70% ethanol. Samples were then resuspended in 0.5 mL 0.1 mg/mL RNase A in 50 mM sodium citrate. After an overnight incubation at 37°C, 0.5 mL of 2 μM SYTOX Green in 50 mM sodium citrate was added. The samples were sonicated briefly before analysis using a Becton-Dickinson FACScalibur.

2.6 SYNTHETIC GENETIC ARRAY (SGA) ANALYSIS

SGA analysis was carried out as described (Tong et al., 2001). The *MAT*α SGA starting strains containing *mus81Δ::natR* (Y3597), *mms4Δ::natR* (Y3561) and *elg1Δ::natR* (Y4521) were used to identify viable gene deletions that show synthetic genetic interactions with deletions in *mus81Δ, mms4Δ,* and *elg1Δ*, respectively. Genetic interactions were confirmed by tetrad analysis on YPD (for *mms4Δ* and *mus81Δ*) or on synthetic medium supplemented with sodium glutamate as a nitrogen source (for *elg1Δ*). Confirmed interactions and extent of fitness defect are listed in Table 5.

2.7 EPITOPE TAGGING, IMMUNOPRECIPITATION, IMMUNOBLOTTING, AND GEL FILTRATION

The construction of strains carrying 3HA- or 13MYC-tagged Elg1 was performed as described (Longtine et al., 1998). Immunoprecipitation was performed essentially as described (Naiki et al., 2001). Purified rabbit IgG Agarose (Sigma) was used to immunoprecipitate TAP-tagged proteins, and immunoprecipitates were washed extensively with buffer containing 100 mM NaCl. Proteins were resolved on 12% (for Chapter 4) or 7.5% (for Chapter 5) polyacylamide-SDS gels, transferred to nitrocellulose membranes and subjected to immunoblot analysis with anti-HA (16B12, Covance), anti-myc (9E10, Santa Cruz), anti-FLAG (M2, Sigma), anti-VSV (P5D4; Roche), anti-tubulin (TAT-1) (Woods et al., 1989) or anti-TAP (PAP: Peroxidase-Anti-Peroxidase Soluble Complex; Sigma) antibodies. Immunoblots were developed using Supersignal ECL (Pierce). For detection of Rad53 and *in situ* autophosphorylation assays, cells were fixed and extracts were prepared essentially as described (Pellicioli et al., 1999). Proteins were separated on 8% or 4-12% polyacrylamide gels (Invitrogen), and immunoblots were probed with anti-Rad53 (yC-19, Santa Cruz). Gel filtration of extracts of GBY635 was carried out on a Superose 6 HR 5/20 column, essentially as described (Fricke et al., 2001).

2.8 PLASMID LOSS, FORWARD MUTATION RATE, AND Can^r MUTATION SPECTRA

Plasmid loss rate was measured using the plasmid YCp1 (Tye, 1999). Transformants were streaked on YPD, single colonies were inoculated into YPD, and grown to saturation. Probabilities of plasmid loss represent the averages of 10 independent experiments for wild-type and *elg1Δ*, and 7 independent experiments for *ctf19Δ*, and were calculated as described (Boe and Rasmussen, 1996). Mutation rates were determined by measuring the rate of forward

mutation to canavanine resistance as described previously (Huang et al., 2002). Fluctuation tests were performed with 10 parallel cultures and median value from each was used to calculate the spontaneous mutation rate by the method of the median (Lea and Coulson, 1949). Values represent the average of three experiments. To examine the spectrum of Can^r mutations, the complete open reading frame of the *CAN1* gene was amplified by PCR from independent Can^r colonies. Amplified DNA was analyzed by Hph1 restriction digestion and by sequencing.

2.9 SGA MAPPING (SGAM) ANALYSIS

SGAM analysis was carried out as described (Jorgensen et al., 2002; Tong et al., 2001; Tong et al., 2004) to map the location of the extragenic suppressor in the *rmi1Δ::natR* query strain (Y5646). This essentially involved performing an SGA analysis using an *rmi1Δ* mutant strain that contained the suppressor (*supX*) as the query. This analysis identified a group of linked but functionally unrelated genes on chromosome XIII (Figure 16B). In the SGA technique, double mutants are created by the normal shuffling of genes that occurs during meiosis, and selected using the markers that are disrupting the query (*natMX6*) and array (*kanMX6*) strain genes. If the *SUPX* genomic locus is unlinked to the array gene deletion, then 50% of the double mutants will contain the *supX* suppressor allele, germinate, and give rise to a colony. However, if the *SUPX* locus is tightly linked to the deleted array gene, double mutants that also contain the *supX* suppressor will occur at a low frequency, because recombination is infrequent between tightly linked genes. Since I hypothesized that *rmi1Δ* cells lacking *supX* would exhibit a growth defect, the chromosomal location of *supX* was therefore identified by observing a lack of colony growth for a set of double mutants whose array gene deletions were linked to the *SUPX* locus.

2.10 FLUORESCENT MICROSCOPY

Cells containing the plasmid pWJ1344, which expresses Rad52-YFP, were grown to logarithmic phase at 23°C in SC medium lacking leucine. Microscopy was performed essentially as described (Lisby et al., 2004; Lisby et al., 2003; Lisby et al., 2001).

2.11 RECOMBINATION AND GCR ASSAYS

Recombination assays were performed using a *LEU2* direct repeat, as described (Smith and Rothstein, 1999). Fluctuation tests of five colonies were repeated three times. GCR assays were performed as described (Myung et al., 2001a). Fluctuation tests of three colonies were repeated at least four times.

2.12 PROTEIN HOMOLOGY SEARCHES

A variety of publicly available and commercial databases were used to search for homologues to the *RMI1* gene and protein sequences, including NCBI (http://www.ncbi.nlm.nih.gov); SGD (Saccharomyces Genome Database, (Christie et al., 2004)); Ensembl (http://www.ensembl.org, (Hubbard et al., 2002)) genome assemblies; Celera (http://www.celera.com, (Kerlavage et al., 2002)) human and mouse genome assemblies; DOE Joint Genome Institute fugu genome assembly (http://www.jgi.doe.gov/fugu/index.html); tetraodon (Tetraodon nigroviridis) reads and genome assembly at GENOSCOPE (http://www.genoscope.cns.fr/externe/tetraodon/); and the sea squirt (*Ciona savignyi*) genome at the Center for Genome Research at Whitehead institute (http://www-genome.wi.mit.edu/annotation/ciona/background.html) and at the DOE Joint Genome Institute (http://www.jgi.doe.gov/programs/ciona.htm).

Programs used for homology searches were: BLAST (local generic and on the Paracel

Blaster system (Paracel, Inc.), and web implementations), Smith-Waterman algorithm for identifying remote homologues (implemented at Paracel GeneMatcher2), and BLAT (web implementation). GeneMatcher2 was also used for Hidden Markov Model searches. Alignments were produced using ClustalW (Chenna et al., 2003) and ClustalX, and shaded using BOXSHADE. The OB-fold nucleic acid binding domain family is Pfam accession number PF01336.

3. A GENOME-WIDE SCREEN FOR METHYL METHANESULFONATE SENSITIVE MUTANTS REVEALS GENES REQUIRED FOR S PHASE PROGRESSION IN THE PRESENCE OF DNA DAMAGE

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Data attribution: I performed the majority of the experimental work presented in this chapter. Dr. Mohammed Bellaoui helped me perform some of the confirmations of MMS sensitivity by spot dilution assays. Dr. Charles Boone provided me with use of his automated pinning robots.

3.1 SUMMARY

I performed a systematic screen of the set of ~5000 viable *Saccharomyces cerevisiae* haploid gene deletion mutants and have identified 103 genes whose deletion causes sensitivity to the DNA damaging agent methyl methanesulfonate (MMS). In total, 40 novel alkylation damage response genes were identified. Comparison with the set of genes known to be transcriptionally induced in response to MMS revealed surprisingly little overlap with those required for MMS resistance, indicating that transcriptional regulation plays little, if any, role in the response to MMS damage. Clustering of the MMS response genes on the basis of their cross-sensitivities to hydroxyurea, ultraviolet radiation, and ionizing radiation revealed a DNA damage core of genes required for responses to a broad range of DNA damaging agents. Of particular significance, I identified a subset of genes that show a specific MMS response, displaying defects in S phase progression only in the presence of MMS. These genes may promote replication fork stability or processivity during encounters between replication forks and DNA damage.

3.2 INTRODUCTION

As described in Chapter 1, the budding yeast *Saccharomyces cerevisiae* has been an invaluable tool for studying DNA damage response pathways. Many *S. cerevisiae* DNA damage response genes have human homologues and mutations in a number of these genes have been implicated in human diseases. Although several screens for *S. cerevisiae* DNA damage response genes have been conducted over the past 30-40 years, additional genes are still being identified. The set of viable *S. cerevisiae* deletion mutants (Winzeler et al., 1999) has allowed for genome-wide studies to identify genes required for resistance to various cellular insults (Bennett et al., 2001; Birrell et al., 2002; Birrell et al., 2001; Chan et al., 2000; Hanway et al., 2002). Here I report a

systematic analysis of the complete set of ~5000 viable gene deletion mutants to identify genes that are required for resistance to the DNA damaging agent methyl methanesulfonate (MMS).

MMS is a monofunctional DNA alkylating agent and a known carcinogen (Beranek, 1990; Lawley, 1989), and primarily methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine (Pegg, 1984). Although the N^7-methylguanine adduct may be non-toxic and non-mutagenic, N^3-methyladenine is a lethal lesion which inhibits DNA synthesis and needs to be actively repaired (Beranek, 1990; Boiteux et al., 1984). The three pathways responsible for the removal of most N^3-methyladenine lesions are bypass repair (or postreplication repair), recombination repair, and base excision repair (Xiao et al., 1996). All three pathways are required for wild-type resistance to MMS induced DNA damage (Xiao et al., 1996). In addition, checkpoint proteins are required to maintain cell viability in the presence of MMS (Tercero and Diffley, 2001; Weinert et al., 1994).

Several studies have found that cells are most sensitive to MMS during progression through S phase (Fung et al., 2002; Schwartz, 1989; Tercero and Diffley, 2001). Exposure to MMS causes a checkpoint-independent reduction in the rate of replication fork progression, likely due to a physical impediment of fork progression caused by alkylated DNA or some intermediate in lesion processing (Tercero and Diffley, 2001). *rad53* and *mec1* checkpoint mutants have high rates of replication fork termination suggesting that damage-induced fork catastrophe is the cause of MMS sensitivity in checkpoint mutants (Tercero and Diffley, 2001). Thus, in addition to identifying proteins involved in repair of MMS lesions and in regulating cell cycle progression, a screen for MMS sensitive mutants may reveal novel proteins required for DNA replication fork stability and processivity following alkylation damage.

3.3 RESULTS AND DISCUSSION

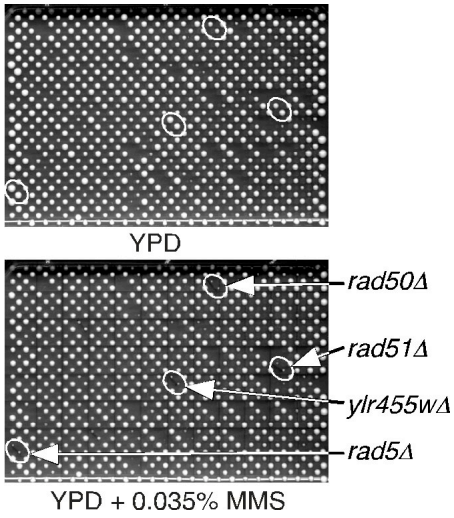

Figure 3. High-throughput MMS screen. The complete set of haploid yeast deletion mutants was arrayed in duplicate onto 16 plates and pinned onto YPD media or YPD + 0.035% MMS (array plate 11 of 16 is shown). Putative MMS sensitive mutants lead to the formation of smaller colonies when grown on MMS-containing media.

Screening for MMS sensitive deletion mutants

I performed a high-throughput MMS sensitivity screen by robotically pinning an ordered array of ~4700 haploid yeast deletion mutants onto YPD or YPD plus 0.035% MMS (Figure 3). From the 3 screens, 244 mutants were scored as MMS sensitive at least once, and 92 were scored at least twice. Strains scored as sensitive at least 2 out of 3 times were verified by spotting serial dilutions of the cells onto media containing 0.035% MMS (Figure 4). An additional 38 mutants (19 that were scored as sensitive in 1 out of 3 screens, and 19 that were not scored in any of the screens) were also chosen for verification because mutations in the deleted genes were previously reported to be MMS sensitive. Thus, a total of 130 deletion mutants were tested by spotting serial dilutions, of which 103 were confirmed to be MMS sensitive (Table 2). In total, 40 genes with no previously known role in MMS response were identified (Table 2, indicated in **bold**), including 15 uncharacterized genes. I also identified 48 genes that were not identified in genome-wide screens for sensitivity to UV or ionizing radiation

(Bennett et al., 2001; Birrell et al., 2001), illustrating the utility of performing screens with different DNA damaging agents. 55 of the MMS resistance genes have readily identifiable human homologues (Table 2, indicated with *), including 4 previously uncharacterized genes. Twelve of the MMS resistance genes have an established link to a human disease (Rebhan et al., 1997).

Table 2. MMS sensitive deletion strains.

Gene	Hits	MMS[S]	Cellular Role[1]
AAT2*	**3**	**+++**	**Amino-acid metabolism**
ANC1†*	0	+++	Pol II transcription
AOR1*	**1**	**+++**	**Unknown**
APN1	0	+++	DNA repair
ARO1	**2**	**+++**	**Amino-acid metabolism**
ARO7	**2**	**+++**	**Amino-acid metabolism**
*ASF1**	3	+++	DNA synthesis
*BDF1**	1	+++	Meiosis
BUD25	**3**	**+++**	**Cell polarity**
BUR2	0	+++	Pol II transcription
*CAC2**	1	+++	DNA repair
*CDC40**	0	+++	Meiosis
*CDC50**	2	+++	Cell cycle control
CHL1†*	0	++	Mitosis
CIK1	**2**	**+++**	**Meiosis**
CSE2	**3**	**+**	**Mitosis**
*CTF18**	0	+++	Cell cycle control
*CTF4/POB1**	2	+++	DNA synthesis
CTF8	1	+++	Chromatin/chromosome structure
DCC1	1	+++	Chromatin/chromosome structure
DDC1	1	+++	Cell cycle control
DEG1	**2**	**++**	**Protein synthesis**
*DOA1**	2	+++	Protein degradation
*DUN1**	1	+++	DNA repair
*ERG3**	3	++	Lipid, fatty-acid, sterol metabolism
ESC4	3	+++	Chromatin/chromosome structure
*GRR1**	3	+++	Amino-acid metabolism
HOF1†*	3	++	Cytokinesis
HPR1	0	+++	Recombination
HPR5/SRS2	2	+++	DNA repair
HTL1	0	+++	Unknown
ISC1*	**3**	**+**	**Lipid, fatty-acid, sterol metabolism**
KIM3/MMS1	2	+++	Cell stress
KRE22	1	+++	Unknown
LSM1*	**2**	**+++**	**RNA turnover**
LSM6*	**2**	**++**	**RNA splicing**
LYS7*	**2**	**+++**	**Amino-acid metabolism**
MAG1	2	+++	DNA repair

MEC3	1	+++	Cell cycle control
MED1	**2**	**++**	**Unknown**
MET18/MMS19	0	+++	Amino-acid metabolism
*MMS2**	3	+++	DNA repair
MMS22	3	+++	DNA repair
MMS4/SLX2	2	+++	DNA repair
MRE11†*	3	+++	DNA repair
MUS81	3	+++	DNA repair
NAT3*	**3**	**+++**	**Protein modification**
NCE4	**2**	**+++**	**Cell wall maintenance**
NPL6	**2**	**+**	**Nuclear-cytoplasmic transport**
NUP133	0	+	Nuclear-cytoplasmic transport
*NUP84**	1	+++	Nuclear-cytoplasmic transport
POL32	2	+++	DNA synthesis
RAD1†*	0	+	DNA repair
*RAD5/REV2**	2	+++	DNA repair
*RAD6**	0	+++	DNA repair
RAD9†*	3	++	Cell cycle control
RAD17	1	+++	Cell cycle control
*RAD18**	0	+	DNA repair
*RAD24**	2	+++	Cell cycle control
*RAD27**	3	+++	DNA synthesis
RAD50†*	3	+++	DNA repair
RAD51†*	3	+++	DNA repair
*RAD52**	3	+++	DNA repair
*RAD54**	3	+++	DNA repair
*RAD55**	3	+++	DNA repair
RAD57†*	3	+++	DNA repair
RAD59*	**1**	**+++**	**DNA repair**
REM50	3	+++	DNA repair
REV3*	**0**	**+++**	**DNA repair**
RPB9*	**2**	**++**	**Pol II transcription**
RRN10	**3**	**+**	**Pol I transcription**
RTT101*	**2**	**+++**	**Protein modification**
SAE2	2	+++	Meiosis
SEC66	**2**	**+++**	**Cell cycle control**
SGS1†*	3	+++	DNA repair
SIT4*	**2**	**+**	**Cell cycle control**
SLX4	**2**	**+++**	**DNA repair**
SOD1*†	**3**	**+++**	**Amino-acid metabolism**
*SPT4**	1	+	Recombination
SRB2	**2**	**+**	**Pol II transcription**
SRB5	**2**	**++**	**Pol II transcription**
SWI6*	**2**	**+++**	**Cell cycle control**
TOM37*	**2**	**++**	**Protein translocation**
*TOP3**	3	+++	DNA repair
*UBC13**	3	+++	DNA repair
UBP6*	**2**	**+++**	**Protein modification**
UME6	3	+	Meiosis
VID21	1	+++	Unknown
*VID31**	1	+++	Unknown
VMA21	**2**	**+++**	**Small molecule transport**

VPS36	**2**	**++**	**Amino-acid metabolism**
XRS2	3	+++	DNA repair
YBL006C	1	++	Unknown
YBR099C	**2**	**+++**	**Unknown**
YCK3*	**2**	**+++**	**Unknown**
YEL045C	**2**	**+++**	**Unknown**
YJL161W	**1**	**+**	**Unknown**
YLR218C*	**3**	**+**	**Unknown**
YLR235C	3	+++	Unknown
YLR376C	2	+++	Unknown
YMC2**†	**2**	**+++**	**Small molecule transport**
YMR031W-A	**2**	**+++**	**Unknown**
YOR275C*	**3**	**+++**	**Cell stress**

+, mildly sensitive; ++, moderately sensitive; +++, extremely sensitive. Novel MMS resistance genes are indicated in **bold**. *Genes with human homologues. †Genes involved in human disease. [1]Cellular role as indicated in YPD (http://www.proteome.com/).

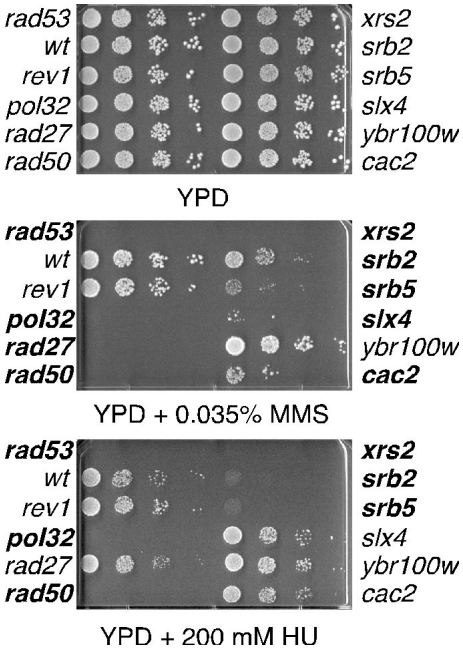

Figure 4. Confirmation of MMS sensitivity. Putative MMS sensitive strains were grown in YPD overnight at 30°C. Serial 10-fold dilutions were spotted onto YPD, YPD + 0.035% MMS, or YPD + 200 mM HU and incubated at 30°C for 3 days. Strains in **bold** were scored as sensitive. A *rad53* mutant was used as a positive control.

I estimate that 63 MMS resistance genes have been reported in the literature (Aboussekhra et al., 1989; Aguilera and Klein, 1990; Ajimura et al., 1993; Anderson, 1994; Bennett et al., 2001; Chakraverty et al., 2001; Chen et al., 1989; Chua and Roeder, 1995;

Costanzo et al., 2001; Fricke et al., 2001; Gellon et al., 2001; Hanway et al., 2002; Hodges et al., 1999; Hryciw et al., 2002; Huang et al., 2000; Leem et al., 1994; Longhese et al., 1997; Mullen et al., 2001; Naiki et al., 2001; Nasim and Brychcy, 1979; Nitiss et al., 1996; Paulovich et al., 1997; Petukhova et al., 1999; Piruat and Aguilera, 1996; Prakash and Prakash, 1977; Qian et al., 1998; Rattray et al., 2001; Sommers et al., 1995; Tyler et al., 1999; Winston et al., 1984; Wu and Wang, 1999; Xiao and Chow, 1998; Xiao et al., 2000), not including genes whose deletion mutants were reported as weakly sensitive to MMS (Bennett et al., 2001), or genes for which the haploid deletion mutant was not sensitive in my study. I successfully identified 78% of the known MMS resistance genes in at least 1 out of 3 screens. If I focus on those genes that were scored as sensitive in at least 2 out of 3 screens, I identified 72 mutants, 56% of known MMS resistance genes, and had a false-positive rate of 21.7%. I estimate that approximately 26 MMS resistance genes were not identified in my screen [(72/0.56) - 103 = 26]. Some of these 26 mutants may already be included in the mutants that were scored as sensitive in only 1 out of 3 screens.

In the course of my studies I noted that 10 deletion mutants that were reported to confer at least moderate MMS sensitivity in homozygous diploids (Bennett et al., 2001) were not sensitive when tested as haploids. To ensure that this was not due to a general difference in sensitivity between haploids and diploids I tested the MMS sensitivity of wild type diploids (Figure 5A). There was no difference in MMS sensitivity between the wild-type haploid and the corresponding diploid. Mohammed then tested the relevant homozygous diploid deletion mutants for MMS sensitivity (Figure 5B and data not shown). The *cnm67Δ/cnm67Δ*, *dhh1Δ/dhh1Δ*, *yif2Δ/yif2Δ* strains were found to be weakly sensitive to 0.035% MMS. The *bem1Δ/bem1Δ*, *nup12Δ/nup12Δ*, *hfi1/Δhfi1Δ*, *gos1Δ/gos1Δ*, *rvs161Δ/rvs161Δ*, *rvs167Δ/rvs167Δ* diploids were not sensitive to 0.035% MMS in Mohammed's hands. We were unable to isolate

a *clc1Δ/clc1Δ* strain. Thus only three deletions that confer MMS sensitivity in a diploid did not confer sensitivity in a haploid.

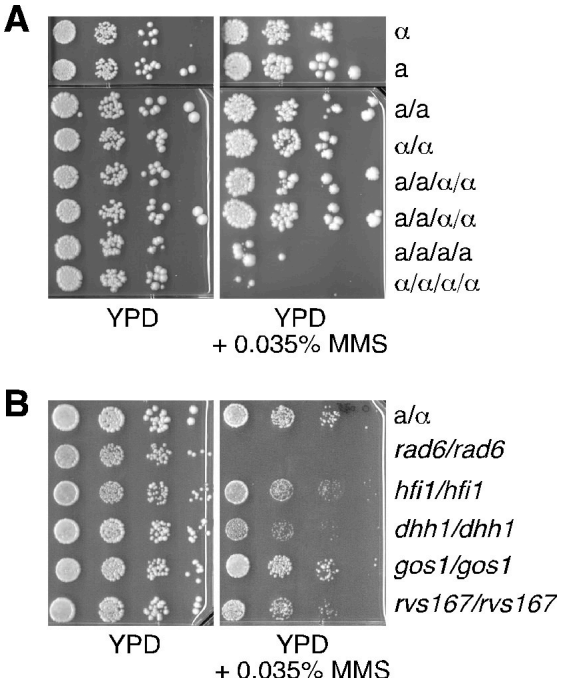

Figure 5. Effect of ploidy on MMS sensitivity. Strains were grown in YPD overnight at 30°C. Serial 10-fold dilutions were spotted onto YPD or YPD + 0.035% MMS plates and incubated at 30°C for 3 days.

Cross-sensitivity of MMS sensitive mutants to UV, IR, and HU

I also tested the MMS sensitive mutants for their sensitivity to HU by spotting serial dilutions of cultures onto YPD plates containing 200 mM HU (Figure 4). Two additional genome-wide screens have recently been reported, one for deletion mutants that confer sensitivity to UV radiation (Birrell et al., 2001) and one for deletion mutants that confer sensitivity to ionizing radiation (Bennett et al., 2001). I combined my data for MMS and HU sensitivity with the reported data for UV and IR sensitivity in order to cluster the MMS sensitive mutants based on their cross-sensitivity to UV, IR, and HU (Table 3). Since both these genome-wide studies missed known UV or IR sensitive mutants, I supplemented the data with literature reports (Ahne et al., 1997; Bai and Symington, 1996; Bennett et al., 2001; Birrell et al., 2002; Bressan et al.,

1999; Eckardt-Schupp et al., 1987; Fasullo et al., 1999; Hryciw et al., 2002; Interthal and Heyer, 2000; Kozhin et al., 1995; Lauder et al., 1996; Lee et al., 2001; Moore, 1978; Rattray and Symington, 1995; Wang and Elledge, 2002). I noted several interesting properties of these mutants. First, although several lines of evidence indicate that both HU and MMS exert their effects largely during S phase and cause stalling of replication forks, 13 of the 103 MMS sensitive mutants displayed no significant sensitivity to HU. This indicates that MMS and HU cause a different spectrum of DNA damage, and suggests that resistance to alkylation damage requires some activities that are distinct from those involved in HU resistance. Despite this clear difference, I also noted that a large cluster (37 genes out of 103) contained genes required for both MMS and HU resistance, but not for UV or IR resistance, indicating that MMS and HU are more alike in their action than they are to that of UV or IR. This cluster also contains most of the genes with unknown function that were identified in the MMS screen. These genes may have S phase specific roles in DNA damage response. Finally, I found just four genes whose deletion conferred MMS sensitivity but not sensitivity to HU, UV, or IR. These genes may function specifically to preserve viability following alkylation damage, and all have known roles in DNA metabolism. *MAG1* encodes a glycosylase that initiates base excision repair of N^3-methyladenine (Berdal et al., 1990; Chen et al., 1989; Chen et al., 1990). The *CAC2* gene product is a member of the chromatin assembly factor I (CAF-I) (Game and Kaufman, 1999; Kaufman et al., 1997). *REV3* encodes the catalytic subunit of DNA polymerase zeta and is required for DNA damage-induced mutagenesis (Lawrence and Maher, 2001; Nelson et al., 1996). *SLX4* was identified in a screen for mutants that are synthetically lethal with deletion of the DNA helicase gene *SGS1* (Mullen et al., 2001).

Table 3. Cross-sensitivity of MMS sensitive deletion mutants to HU, IR, and UV.

HU	IR	UV	n	Gene/ORF
S	S	S	**41**	***ASF1, BUR2, CDC40, CTF4, CTF8, DCC1, DUN1, GRR1, HOF1, HPR1, HTL1, KRE22, MEC3, MMS2, MMS4, MMS22, MRE11, MUS81, NPL6, NUP84, POL32, RAD5, RAD6, RAD17, RAD18, RAD24, RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, REM50, RPB9, SGS1, SRS2, VID31, XRS2, YBR099C, YLR235C***
		R	7	*ANC1, ERG3, NAT3, SOD1, TOM37, UME6, YBL006C*
	R	S	5	*DDC1, LSM1, MET18, MMS1, TOP3*
		R	37	*AAT2, AOR1, ARO1, ARO7, BDF1, BUD25, CDC50, CIK1, CSE2, CTF18, DEG1, DOA1, ESC4, ISC1, LSM6, LYS7, MED1, NCE4, RRN10, RTT101, SEC66, SIT4, SPT4, SRB2, SRB5, SWI6, UBP6, VMA21, VPS36, YCK3, YMC2, YEL045C, YJL161W, YLR218C, YLR376C, YMR031W-A, YOR275C*
R	S	S	8	*NUP133, CHL1, RAD1, RAD9, RAD27, SAE1, UBC13, VID21*
		R	1	*APN1*
	R	S	0	
		R	4	*CAC2, MAG1, REV3, SLX4*

As expected, I identified a large number of *RAD* genes in the MMS screen and all of these are sensitive to at least one other DNA damaging agent. Clustering of the cross-sensitivity data revealed a core subset of DNA damage response genes that are required for resistance to HU, MMS, UV, and IR (Table 3, **bold**). This core includes genes involved in recombination repair (*MRE11, RAD50*, *RAD51*, *RAD52*, *RAD54, RAD55*, *RAD57*, *RAD59, XRS2*) (Game, 2000), bypass repair (*RAD5, RAD6*, *RAD18, MMS2*) (Broomfield et al., 2001), DNA damage checkpoint activation (*RAD17*, *MEC3*) (Lowndes and Murguia, 2000), and processing of repair or recombination intermediates (*MMS4, MUS81*, *SGS1, SRS2*) (Boddy et al., 2001; Chen et al., 2001b; Kaliraman et al., 2001; Klein, 2001; Lee et al., 1999). Other genes present in this core

have less defined roles in the repair of DNA damage. *ASF1*, which is regulated by the checkpoint kinase Rad53, may function in chromatin assembly during DNA repair (Emili et al., 2001). *CTF4, CTF8* and *DCC1* encode proteins that are required for sister chromatid cohesion (Hanna et al., 2001; Mayer et al., 2001), defects in which might affect recombination repair (Hartsuiker et al., 2001). Deletion of *YBR099c* and *YLR235c* remove the 3' ends of the *MMS4* and *TOP3* open reading frames, and so the sensitivity of these mutants likely reflects loss of Mms4 or Top3 function.

Comparison of the MMS transcriptional profile with the set of genes required for MMS resistance

Exposure to DNA damaging agents often results in differential gene expression (Fornace et al., 1988; Herrlich et al., 1997; Kiser and Weinert, 1996; Zhan et al., 1993). Two groups have independently examined the genomic expression response to MMS by DNA microarray analysis (Gasch et al., 2001; Jelinsky and Samson, 1999). To determine the significance of the transcriptional response to MMS, I compared the MMS transcriptional profiles with the set of genes required for MMS resistance (Table 4). I found very little correlation between genes that are transcriptionally induced by MMS and genes that are required for MMS resistance. Of the 616 genes whose transcription was induced greater than 1.5-fold by 0.02% MMS (Gasch et al., 2001) there are only 8 whose deletion confers MMS sensitivity. Furthermore, none of these genes were included in the 50 most highly induced genes. Similar results were seen when my MMS sensitive data set was compared to those genes induced by 0.1% MMS (Jelinsky and Samson, 1999), with only 7 genes of the 320 genes transcriptionally induced greater than 4-fold conferring MMS sensitivity when deleted. Additionally, few MMS-induced genes are essential (Gasch et al., 2001; Jelinsky and Samson, 1999; Winzeler et al., 1999), so the poor overlap

between the data sets is not due to the absence of essential genes on the deletion strain array. I conclude that the transcriptional response plays little role in resistance to MMS induced cellular damage. Many of the genes induced by MMS may not be specifically required for a cellular response to MMS, but are coincidentally up-regulated because the cells have been stressed (Gasch et al., 2001). The important responses to MMS-induced cellular damage are likely effected immediately and require proteins that are already present in the cell. Consistent with my results, recent data has indicated that transcriptional response plays little, if any, role in sensitivity to IR, UV, cisplatin and hydrogen peroxide exposure (Birrell et al., 2002). Thus it appears that the cellular response to a wide range of DNA damaging agents is largely independent of transcriptional regulation.

Table 4. Transcriptional regulation of the 103 MMS response genes.

0.02% MMS, 60 minutes			0.1% MMS, 60 minutes		
Induced ≥ 1.5-fold[1]	rank	-fold induction[2]	Induced > 4-fold[3]	rank	-fold induction[4]
RAD51	62	2.85	***YJL161W***	62	6.9
DUN1[5]	114	2.36	*ARO1*	64	6.8
MAG1	205	2.0	***UBC13***	78	6.2
YJL161W	216	1.99	***UBP6***	107	5.6
UBP6	248	1.91	*TOM37*	112	5.6
UBC13	361	1.74	***MAG1***	117	5.5
SOD1	364	1.74	***DUN1***	244	4.3
AOR1	490	1.59			

[1]616 genes were induced ≥ 1.5-fold. [2]Maximum induction was 16.7-fold. [3]320 genes were induced ≥ 4-fold. [4]Maximum induction was 251-fold. [5]Genes induced at both MMS concentrations are indicated in **bold**.

Cell cycle progression analysis of selected MMS sensitive mutants

Several studies have suggested that it is passage through S phase in the presence of MMS damage that kills MMS sensitive mutants (Fung et al., 2002; Schwartz, 1989; Tercero and Diffley, 2001). Hence, I examined the S phase progression of a subset of the MMS sensitive mutants (Figure 6). I selected representative genes from pathways known to be involved in repair of MMS damage, as well as several genes with poorly defined roles in MMS resistance. Cells were arrested in G1 by alpha mating factor treatment and then released synchronously into

the cell cycle in either the presence or absence of MMS. Wild-type cells complete DNA replication within 60 minutes after release from the block in the absence of MMS. Consistent with previous reports, wild-type cells released in the presence of MMS show a slower progression through S phase (Paulovich and Hartwell, 1995; Paulovich et al., 1997) due to a slowing of replication fork progression and a checkpoint-dependent inhibition of late replication origin firing (Tercero and Diffley, 2001), completing S phase by 120 minutes (Figure 6, top panel).

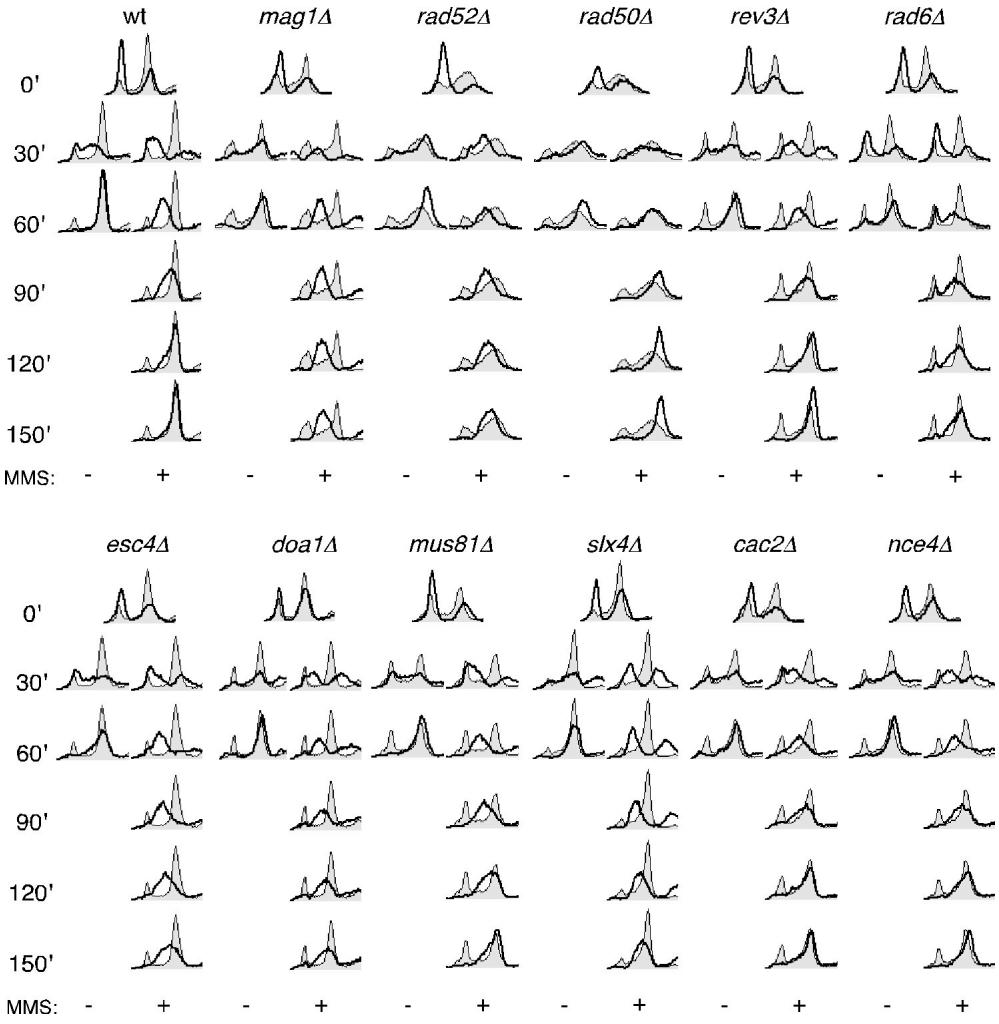

Figure 6. S phase progression analysis of selected MMS mutants. Cells were arrested in G1 and released either in the presence or absence of 0.035% MMS. Grey shaded histograms represent the cell cycle distribution of asynchronous culture prior to cell cycle synchronization. Overlaid histograms (black lines) represent the cell cycle distribution after release from G1 arrest ±0.035% MMS for the indicated times.

The *mag1Δ* mutant (defective in base excision repair) progresses normally in the absence of MMS, but arrests with an intermediate DNA content in the presence of MMS. This arrest was maintained for at least 20 h (data not shown), indicating that removal of N^3-methyladenine is essential for the completion of S phase, consistent with polymerases being unable to synthesize through or bypass this lesion (Boiteux et al., 1984). The *rad6Δ* and *rad52Δ* mutant strains (defective in bypass repair and homologous recombination repair, respectively) displayed significantly slower progression through S phase in the presence of MMS compared to the wild-type strain, suggesting that both bypass repair and recombination repair is required for replication through MMS-induced lesions. The replication defects in *rad6Δ* and *rad52Δ* were not as severe as in *mag1Δ*, perhaps indicating that Rad6 and Rad52 play a secondary role in processing MMS induced lesions, downstream of Mag1. The *rad50Δ* strain (defective in non-homologous end-joining and homologous recombination repair) progressed through S phase more rapidly than the wild-type strain, consistent with a role for *RAD50* in activating the intra-S phase checkpoint response to MMS. A similar result was obtained with *mre11Δ* (data not shown). *RAD50* and *MRE11* are required for Rad53 activation in response to double strand DNA breaks (D'Amours and Jackson, 2001; Grenon et al., 2001), and my data suggest a similar requirement following MMS damage. I found no significant role for the bypass polymerase gene *REV3* in S phase progression in the presence of MMS despite a role for *RAD6*. The *RAD6* epistasis group consists of two major pathways, one involved in post-replication repair and one in mutagenesis (Friedberg et al., 1995; Prakash et al., 1993). Rev3 is involved in mutagenesis while Rad6 plays a critical role in both pathways (Friedberg et al., 1995; Prakash et al., 1993), which may explain the differences seen between *rad6Δ* and *rev3Δ* in the S phase progression assay.

I next tested several mutants identified in the MMS screen with less defined roles in MMS response (Figure 6, bottom panel). Deletions in *CAC2* or *NCE4* do not affect the rate of bulk DNA synthesis, suggesting that their roles in resistance to MMS induced damage may be post-replicative. By contrast, both *doa1Δ* and *esc4Δ* are significantly defective in S phase progression in the presence of MMS, displaying cells with incompletely replicated DNA 120 minutes and 150 minutes after release from the G1 block. *DOA1* and *ESC4* might be required for replication fork stability or processivity when forks are stalled by DNA damage. Consistent with this idea, *DOA1* and *ESC4* are both members of the cluster of genes required for resistance to MMS and HU, drugs specifically affecting S phase progression, but not UV or IR (Table 3). *mus81Δ* and *slx4Δ* are also defective in S phase progression, but to a lesser extent. *MUS81* encodes a subunit of an endonuclease that is thought to act on stalled replication forks (Boddy et al., 2001; Chen et al., 2001b; Kaliraman et al., 2001). Interestingly, the sensitivity of *mec1* and *rad53* mutants to MMS is a result of replication forks terminating irreversibly at a high rate (Tercero and Diffley, 2001). Furthermore, Mus81 physically interacts with Rad53 (Boddy et al., 2000; Ho et al., 2002). *MUS81* may be acting in the same pathway as the checkpoint genes *MEC1* and *RAD53* in stabilizing or restarting stalled replication forks. Slx4, like Mus81, is required for viability in the absence of the DNA helicase Sgs1 (Mullen et al., 2001). However, *mus81Δ* mutants are sensitive to HU, UV, and IR while *slx4Δ* is not, suggesting that Slx4 may be required to process DNA structures that arise specifically in the presence of alkylated DNA. Thus, by utilizing a high-throughput genome wide screen for novel MMS resistance genes I have identified a new class of genes that are required for S phase progression in the presence of DNA damage.

4. Elg1 FORMS AN ALTERNATIVE RFC COMPLEX IMPORTANT FOR DNA REPLICATION AND GENOME INTEGRITY

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Data attribution: Dr. Mohammed Bellaoui performed the majority of the experiments in this chapter, with contributions from Jiongwen Ou, Dr. Grant Brown, and me. I initiated the *MUS81* and *MMS4* SGA analyses (Figure 7A), performed some of the checkpoint assays (Figure 11A), contributed to the confirmation of the *ELG1* SGA analysis (Figure 12; Table 5), and performed the plasmid loss assay (Figure 13B). Hong Xu in Dr. Charles Boone's lab helped with the SGA analyses.

4.1 SUMMARY

Genome-wide synthetic genetic interaction screens with mutants in the *mus81* and *mms4* replication fork processing genes identified a novel replication factor C (RFC) homologue, Elg1, which forms an alternative RFC complex with Rfc2-5. This complex is distinct from the DNA replication RFC, the DNA damage checkpoint RFC, and the sister chromatid cohesion RFC. As expected from its genetic interactions *elg1* mutants are sensitive to DNA damage. Elg1 is redundant with Rad24 in the DNA damage response and contributes to activation of the checkpoint kinase Rad53. We find that *elg1* mutants display DNA replication defects and genome instability, including increased recombination and mutation frequencies and minichromosome maintenance defects. Mutants in *elg1* show genetic interactions with pathways required for processing of stalled replication forks, and are defective in recovery from DNA damage during S phase. We propose that Elg1-RFC functions both in normal DNA replication and in the DNA damage response.

4.2 INTRODUCTION

DNA replication is typically highly processive. Replication fork stalling or arrest can result when replication forks encounter damage in the DNA, and at naturally occurring sequences such as replication fork barriers and replication slow zones (Cha and Kleckner, 2002; Rothstein et al., 2000). Several mechanisms by which replication forks can be restarted following arrest have been described in bacteria (reviewed in (Michel, 2000; Michel et al., 2001)). Stalled forks are susceptible to breakage and to replication fork reversal, both of which generate a double-strand DNA end. A replication fork can then be re-established by homologous recombination followed by Holliday junction resolution or by branch migration. By analogy, some of these same

processes are believed to occur in eukaryotes. In addition to these pathways, which are principally involved in restarting what are presumably collapsed replication forks, recent work has demonstrated that the S phase checkpoint pathway is responsible for stabilizing replication forks and preventing fork collapse and formation of DNA structures that are substrates for replication restart pathways (Lopes et al., 2001; Sogo et al., 2002; Tercero and Diffley, 2001).

Replication factor C (RFC) was first identified as a protein complex required for SV40 DNA replication *in vitro* (Tsurimoto and Stillman, 1989; Virshup and Kelly, 1989). RFC is a five subunit complex that recognizes the primer-terminus and catalyzes the loading of the sliding clamp PCNA (Hubscher, 1996; Mossi and Hubscher, 1998). PCNA acts as a processivity factor for DNA polymerase δ on the leading and lagging strands. At least two alternative forms of RFC have recently been identified in yeast and humans. In the first of these the large subunit of RFC, Rfc1, is replaced by the Rad24 protein (Green et al., 2000). Rad24-RFC loads a PCNA-like clamp consisting of Rad17, Ddc1, and Mec3 (Bermudez et al., 2003; Majka and Burgers, 2003), is required for DNA damage checkpoint responses in G1 and G2, and contributes to S phase checkpoint responses. In the second alternative RFC, Rfc1 is replaced by Ctf18 (Hanna et al., 2001; Mayer et al., 2001; Naiki et al., 2001). Mutants in *ctf18* have defects in sister chromatid cohesion (Hanna et al., 2001; Mayer et al., 2001), the process by which newly replicated chromatids remain physically associated until entry into anaphase. Ctf18-RFC also contains the Ctf8 and Dcc1 proteins, and mutations in *ctf8* or *dcc1* recapitulate the cohesion defects observed in *ctf18* mutants (Mayer et al., 2001). There is evidence that like Rad24, Ctf18 also contributes to the S phase checkpoint response. *ctf18 rad24* mutants have a mild S-M checkpoint defect that is not evident in the single mutants, and have a Rad53 activation defect in S phase (Naiki et al., 2001).

Here I describe a functional genomics approach to identify previously uncharacterized factors required for the DNA damage response, particularly those involved in replication fork progression. To this end, Mohammed and I conducted genome-wide synthetic lethality screens with deletion mutants in *mus81* and *mms4*. Mus81 and Mms4 are subunits of an endonuclease with a preference for branched DNA structures (Boddy et al., 2001; Chen et al., 2001b; Kaliraman et al., 2001). In addition to this substrate preference, several lines of evidence connect this enzyme to the processing of stalled DNA replication forks (Haber and Heyer, 2001; Kaliraman et al., 2001; Mullen et al., 2001).

Mohammed and I identified a previously uncharacterized *RFC1* homologue, *ELG1*. Mohammed finds that Elg1 forms an RFC-like complex with the Rfc2-5 proteins, but not with Rfc1 or its homologues Rad24 and Ctf18. *ELG1* is functionally redundant with *RAD24* in the DNA damage response, yet does not share its primary role in checkpoint activation. Of particular significance, *elg1* mutants display defects in DNA replication both in the presence and absence of DNA damage, suggesting that Elg1 functions directly in DNA replication. Cells lacking *elg1* require the intra-S phase checkpoint, homologous recombination proteins, and pathways involved in replication restart following replication fork stalling for wild-type growth, and are defective in recovery from DNA damage in S phase. We propose that Elg1-RFC functions in lagging strand DNA synthesis to prevent replication fork stalling and to facilitate re-start of stalled replication forks.

4.3 RESULTS

Genome-wide Synthetic Lethal Screens with *mus81Δ* and *mms4Δ* Identify *ELG1*

In order to identify novel genes that function to stabilize replication forks *in vivo* Mohammed and I conducted genome-wide synthetic genetic interaction screens (Tong et al., 2001) with

strains carrying deletions of *MUS81* or *MMS4*. Several lines of evidence suggest that Mus81 and Mms4 are involved in the processing of stalled replication forks (Haber and Heyer, 2001; Kaliraman et al., 2001; Mullen et al., 2001). In these screens the query mutant was crossed with the approximately 4600 strains that make up the complete set of *S. cerevisiae* viable haploid deletion mutants. Double mutants that show reduced fitness compared to single mutants, as evidenced by lack of growth or slow growth, were scored as positive. Each screen was performed three times and interactions that were scored at least two times were confirmed by tetrad analysis. The result of these screens is presented as a genetic interaction network in Figure 7A, with tetrad analysis of the *elg1Δ* interactions shown in Figure 7B. Each line on the network represents a synthetic lethal or synthetic sick (slow growth) interaction between the linked genes. Consistent with models in which Mus81 and Mms4 function exclusively as a heterodimer (Boddy et al., 2001; Kaliraman et al., 2001), the screen with *mus81Δ* identified the same set of seven genes as the screen with *mms4Δ*. We found strong genetic interactions between *mus81Δ* and *mms4Δ* and deletions of either *sgs1* or *top3*. These interactions have been previously described and are believed to reflect the redundant roles of the Sgs1/Top3 and Mus81/Mms4 pathways in the repair of stalled replication forks and/or the resolution of recombination intermediates (Mullen et al., 2001). *YLR235C* overlaps the *TOP3* gene and so was likely identified due to its effect on Top3 function. In addition to these known interactions we identified four novel interactions, with *elg1Δ*, *esc2Δ*, *nce4Δ*, and *vid22Δ*. We also found that *elg1Δ* is synthetic lethal with *mec2-1*, a checkpoint-defective allele of *RAD53* (Figure 7B), which indicates that *elg1Δ* mutant cells require a Rad53-dependent checkpoint for viability. Together, these data suggest that Elg1 is important for the integrity of DNA replication forks *in vivo*.

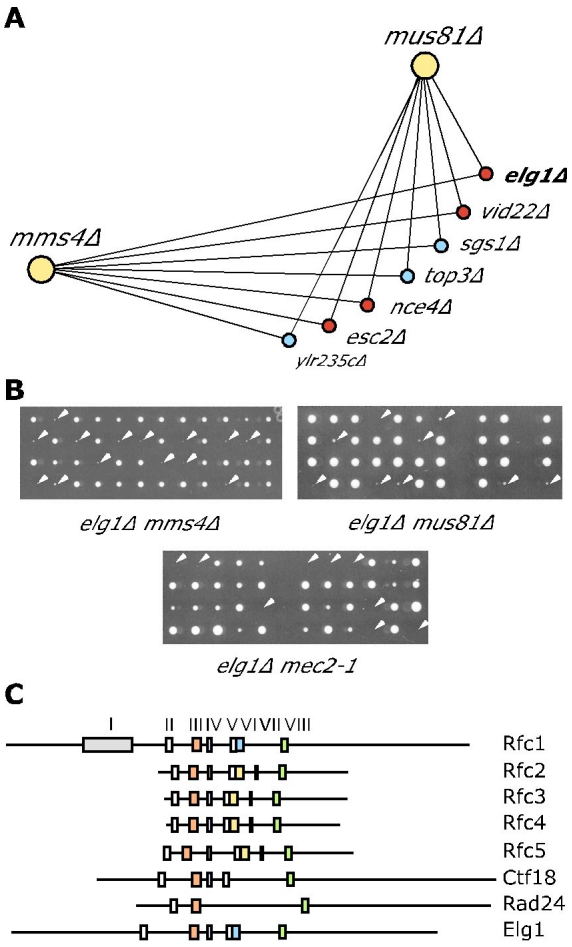

Figure 7. Genome-wide synthetic lethal screens with *mus81Δ* and *mms4Δ* identify the RFC homologue Elg1. (A) The results from synthetic genetic array analysis with *mus81Δ* and *mms4Δ* presented as a genetic interaction map. Lines connecting genes represent synthetic lethality or synthetic slow growth. Red circles indicate novel genetic interactions. (B) Tetrad confirmation of the *elg1Δ* crosses. Each column represents the four spores from a single ascus. Double mutant colonies, as detected by selection for the dominant selectable marker linked to each gene, are indicated by white arrowheads. (C) Schematic representation of the conserved sequence blocks in the *S. cerevisiae* RFC-family genes. Elg1 contains six of the seven RFC boxes found in Rfc1.

ELG1 is a Member of the Replication Factor C Family

ELG1 (Enhanced Level of Genome instability) was first identified in a screen for increased Ty transposon mobility, and *elg1* mutants have been reported to confer an increase in direct repeat recombination (Scholes et al., 2001). Using a *LEU2* direct repeat assay (Smith and Rothstein, 1999), Grant confirmed that deletion of *elg1* causes a 7-fold increase in recombination rate (data

not shown). Detailed examination of the hypothetical translation product of *ELG1* revealed extensive similarity to the Replication Factor C (RFC)-like protein family. Eight regions of sequence similarity have been defined in RFC proteins (Cullmann et al., 1995). As indicated in Figure 7C and in Figure 8, Elg1 contains all of the RFC boxes present in Rfc1 with the exception of the ligase homology region, RFC box I. The Rfc-specific boxes II, IV, VI, and VIII are present in Elg1, as is the ATP-binding motif contained in boxes III and V. RFC box VII is present only in the small Rfc subunits, Rfc2-5, and is absent from Elg1. The RFC box VI in Elg1 bears greater similarity to box VIa, found in the large Rfc subunits, than it does to box VIb, found in the small subunits. Like the Rfc1 homologues Rad24 and Ctf18, Elg1 is conserved throughout *Eucaryota*. Homologues of Elg1 are readily identifiable in *Sch. pombe* (NP_595265), *Drosophila melanogaster* (AAF49530), mouse (BAC39389.1), and human (CAC44537). Together, these data indicate that Elg1 is an Rfc1 homologue, and by analogy with the Rfc1 homologues Rad24 and Ctf18 suggests that Elg1 forms an alternative RFC complex with the small RFC subunits Rfc2-5.

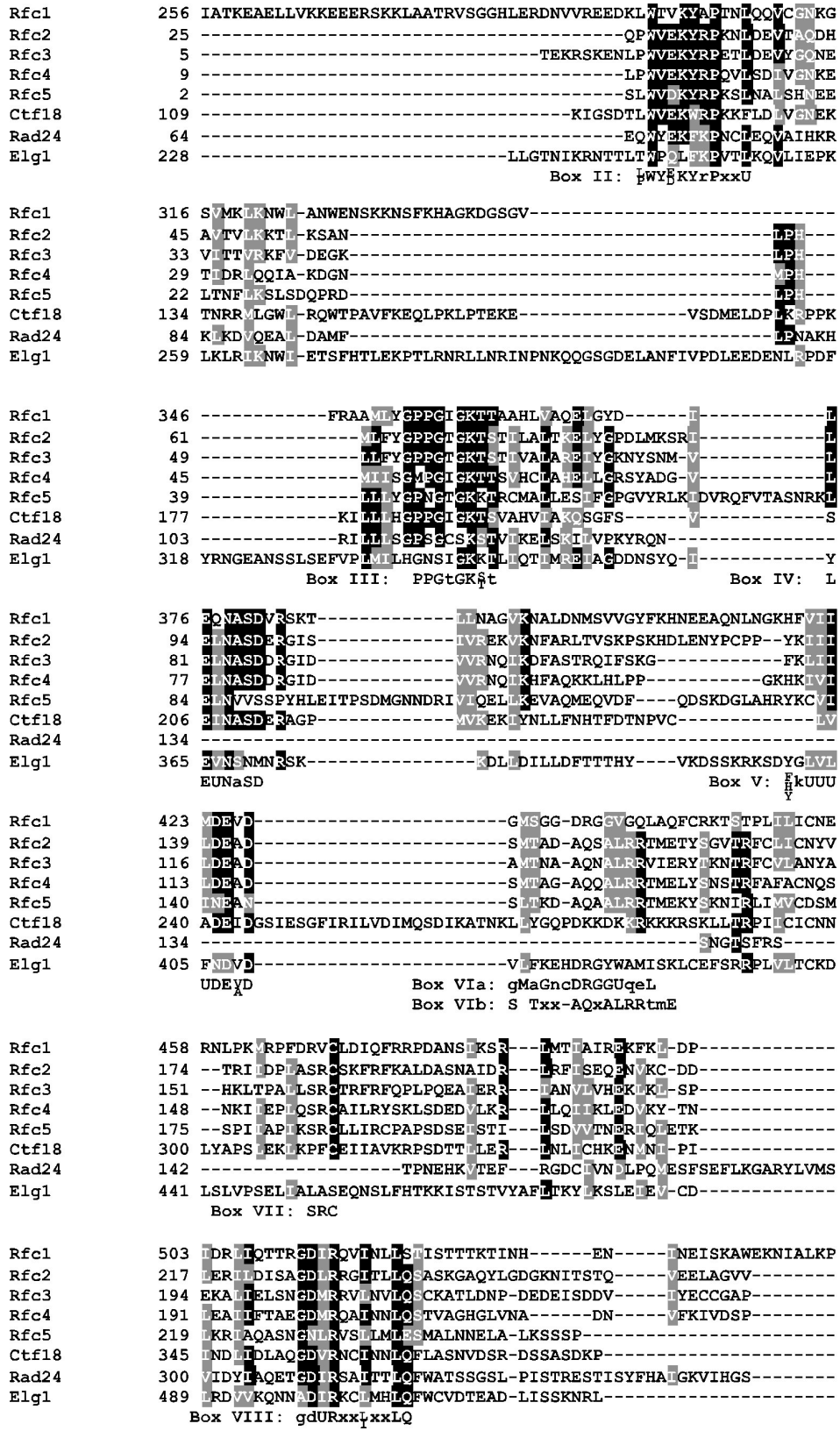

Figure 8. Alignment of *S. cerevisiae* RFC proteins. Conserved RFC boxes II-VIII are shown below the alignment. Sequences were aligned using DIALIGN (B. Morgenstern (1999). DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. Bioinformatics *15*, 211 - 21).

Elg1 Forms a Novel RFC Complex

High-throughput protein-protein interaction screens have detected Elg1 in complexes with Rfc2, Rfc4, and Rfc5 (Gavin et al., 2002; Ho et al., 2002). In order to test the possibility that Elg1 forms an RFC-like complex distinct from the canonical replication RFC Mohammed conducted co-immunoprecipitation experiments with Elg1 and the five subunits of RFC. Elg1 protein was not detectable in Rfc1 immunoprecipitates (Figure 9A, lane 4). In contrast, immunoprecipitates of Rfc2, Rfc3, Rfc4, or Rfc5 all contained Elg1 (Figure 9A, lanes 6, 8, 10, and 12). Elg1 was not immunoprecipitated in the absence of RFC, indicating that the immunoprecipitations were specific (Figure 9A, lane 2). Therefore, Elg1 forms a complex with Rfc2-5 but not with Rfc1. The simplest interpretation of these data is that Elg1 forms an alternative RFC complex in which it substitutes for Rfc1 in binding to Rfc2-5.

Although genetic data suggest that Elg1 is functionally distinct from Ctf18 and Rad24 as neither *ctf18Δ* nor *rad24Δ* shows as strong a genetic interaction with *mus81Δ*, *mms4Δ*, or *rad53* (data not shown), it remained formally possible that Elg1 is a member of the Rad24-RFC or the Ctf18-RFC complexes. To exclude these possibilities Mohammed first immunoprecipitated Rad24 and assayed for the presence of Elg1 (Figure 9B). Elg1 was detected in Rfc5 complexes, but not in Rad24 complexes. Mohammed next immunoprecipitated Elg1 and assayed for the presence of Ctf18 (Figure 9C). Ctf18 was not present in Elg1 complexes, although again Rfc5 was present. We conclude that Elg1 forms a novel RFC complex with Rfc2-5.

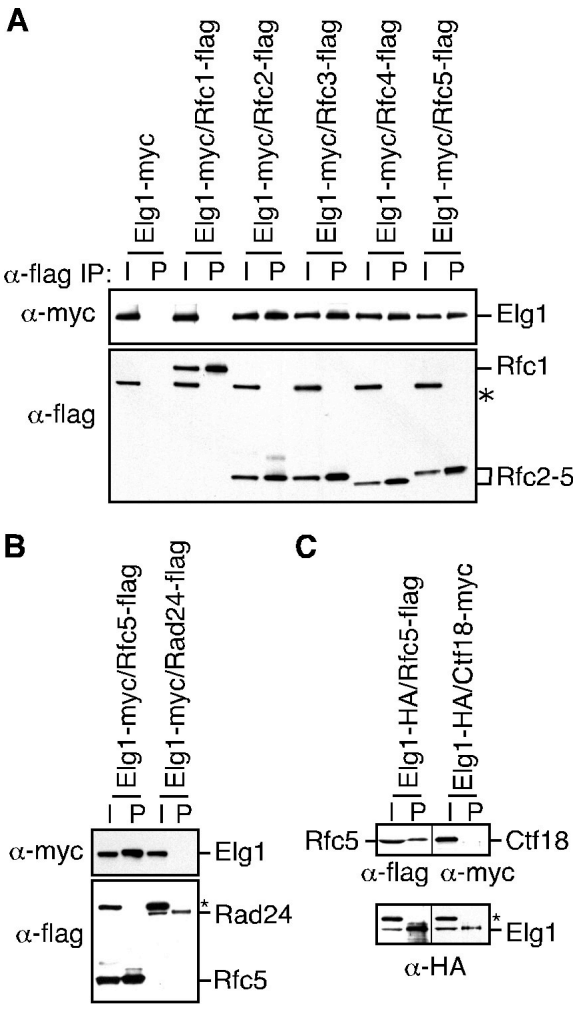

Figure 9. Elg1 forms complexes with Rfc2, 3, 4, and 5, but not with Rfc1, Rad24, or Ctf18. (A) Extracts from yeast strains expressing the indicated epitope-tagged RFC proteins were immunoprecipitated with antibody against the flag epitope. Ten percent of the input extract (I) and the immunoprecipitate (P) were fractionated on SDS-PAGE. Immunoblots were probed with anti-flag antibody to detect Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5, and with anti-myc antibody to detect Elg1. A non-specific cross-reacting polypeptide is indicated (*). (B) Extracts from strains expressing the indicated proteins were immunoprecipitated with anti-flag antibody to precipitate Rfc5 and Rad24. Immunoblots were probed with anti-flag antibody to detect Rfc5 and Rad24 and with anti-myc to detect Elg1. (C) Extracts were immunoprecipitated with anti-HA antibody to precipitate Elg1. Immunoblots were probed with anti-flag antibody to detect Rfc5, anti-myc to detect Ctf18, and anti-HA to detect Elg1.

Elg1 Function in the DNA Damage Response is Redundant with that of Rad24

Several lines of evidence have implicated RFC complexes in the checkpoint response to DNA damage, particularly during S phase. Mohammed tested the sensitivity of *elg1Δ, rad24Δ,* and

ctf18Δ mutants, as well as all pairwise double mutants, and the triple mutant, to the DNA alkylating agent MMS, the replication inhibitor HU, and UV radiation (Figure 10A). In addition, Mohammed measured mutant cell viability following exposure to 0.035% MMS in a 4 hour time course (Figure 10B). First, Mohammed found that the *elg1Δ* mutant was only mildly sensitive to MMS, and was resistant to HU and UV. This contrasts with *rad24Δ*, which was significantly sensitive to all three agents, and with *ctf18Δ*, which displayed sensitivity to MMS and to HU, but was only modestly UV sensitive. These different sensitivities distinguish the Rfc1 homologues, and indicate that Elg1 is not a central player in DNA damage repair.

Mohammed next examined the sensitivity of each possible double mutant combination and the triple mutant. Both double mutants with *rad24Δ* were significantly more sensitive to all agents than any of the single mutants, whereas the *rad24Δ ctf18Δ elg1Δ* triple mutant conferred the greatest sensitivity to MMS. These findings indicate that the Rfc1 homologues are in partially redundant pathways for DNA damage resistance.

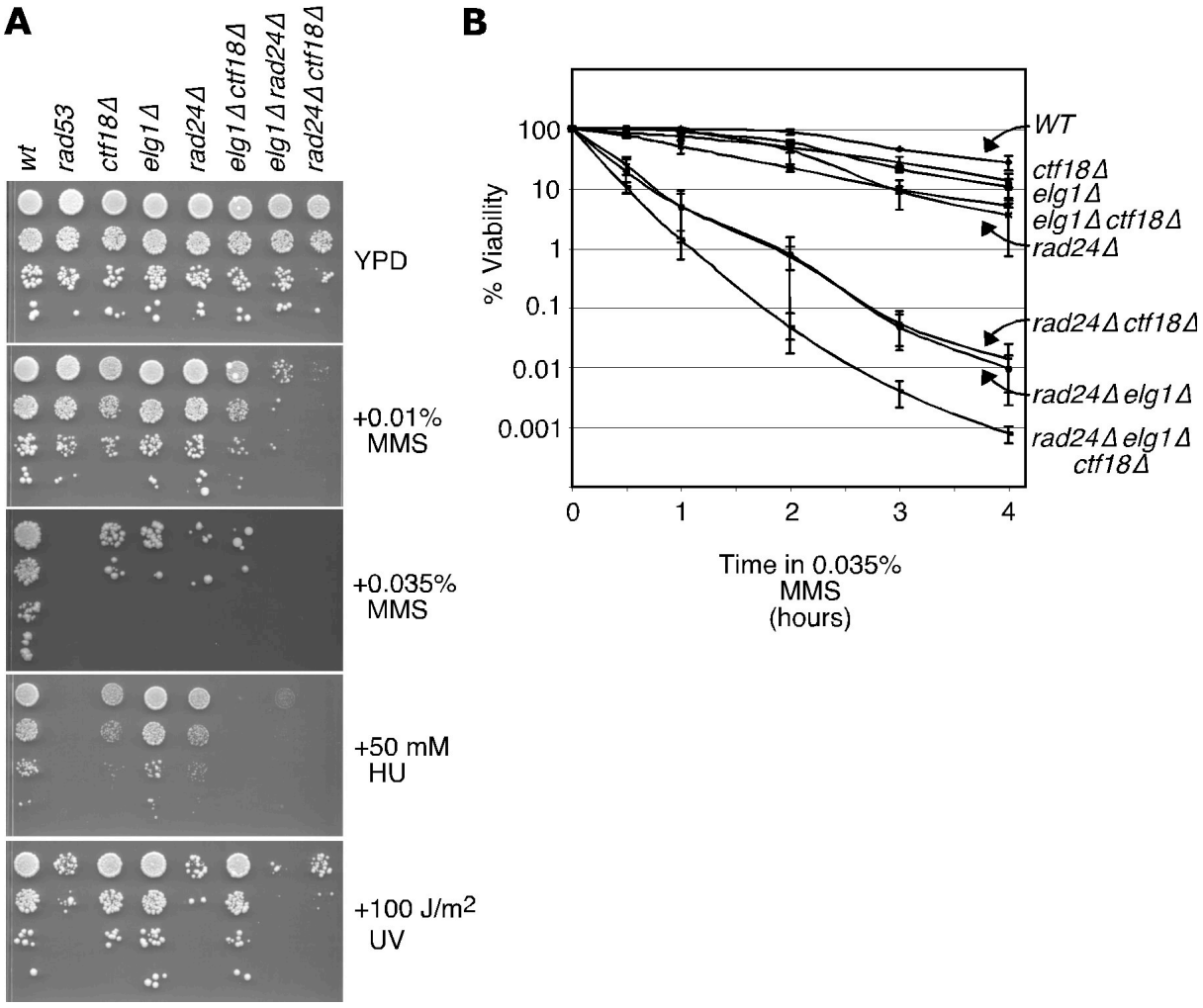

Figure 10. Elg1 is required for the DNA damage response. (A) Ten-fold serial dilutions of cultures of the indicated mutants were spotted on YPD, YPD containing 0.01% (v/v) MMS, 0.035% (v/v) MMS, 50 mM HU, or on YPD that was subsequently exposed to 100 J/m^2 UV. Plates were incubated at 30°C for 2-3 days. (B) Logarithmically-growing cultures of the indicated mutants were incubated in YPD containing 0.035% (v/v) MMS at 30°C. At the indicated times samples were withdrawn and plated on media lacking MMS to determine the number of viable cells. Percent of viable cells relative to the number of viable cells at t=0 is shown. Plots represent the average of three experiments and error bars span one standard deviation.

Elg1 Contributes to Rad53 Activation

Rad24 has a well-documented role in activation of the checkpoint kinase Rad53 (Naiki et al., 2000; Pellicioli et al., 1999; Shimomura et al., 1998). Activation of Rad53 can be readily assessed by immunoblot detection of a phosphorylation-dependent shift in Rad53 mobility (Pellicioli et al., 1999). I assayed Rad53 activation in HU-arrested cells to assess S-M

checkpoint function in *elg1Δ* cells (Figure 11). *ELG1* was not required for Rad53 activation following HU arrest, as I observed wild-type levels of Rad53 phosphorylation in the *elg1Δ* mutant (Figure 11A). The *ctf18Δ rad24Δ* mutant displayed a clear defect in Rad53 activation, as previously reported (Naiki et al., 2001) and consistent with the increased HU sensitivity of this double mutant relative to the *ctf18Δ* and *rad24Δ* single mutants (Figure 10). The *elg1Δ rad24Δ* and *elg1Δ ctf18Δ* double mutants displayed slight defects in Rad53 activation, as evidenced by the smaller fraction of Rad53 present in the most slowly-migrating form when compared with wild-type (Figure 11A). As with *ctf18Δ rad24Δ*, this might account for the increased HU sensitivity of the double mutants compared to the single mutants (Figure 10). In the *elg1Δ rad24Δ ctf18Δ* triple mutant phosphorylation of Rad53 was almost completely absent (Figure 11A). I conclude that *ELG1* contributes to the S-M checkpoint in the context of a *rad24* mutation. The near absence of Rad53 phosphorylation in the triple mutant indicates that all of the Rfc1 homologues can contribute to Rad53 activation in S phase.

Mohammed next treated cells with MMS to assess the role of Elg1 in the intra-S phase checkpoint (Figure 11B). Again, *elg1Δ* cells displayed no defect in Rad53 activation. However, when combined with *rad24Δ*, both *elg1Δ* and *ctf18Δ* have significant Rad53 activation defects, consistent with roles in intra-S checkpoint response. It is worth noting, however, that the *elg1Δ ctf18Δ* double mutant had little, if any, Rad53 activation defect, indicating that Rad24 plays the more important role in the intra-S checkpoint.

Since we had hypothesized a role for Elg1 in replication fork integrity, Mohammed assessed intra-S phase checkpoint function directly. Cells in asynchronous culture were treated with MMS, and their accumulation in S phase was monitored by flow cytometry (Figure 11C). Wild-type cells, with an intact intra-S phase checkpoint, accumulate in S phase by 2 hours, and remain blocked with an intermediate DNA content for the 4 hour duration of the experiment.

This S phase accumulation is due to checkpoint-independent slowing of DNA replication fork progression combined with a checkpoint-dependent inhibition of dormant and late origin firing (Tercero and Diffley, 2001). In the *rad53* mutant the checkpoint-dependent inhibition of origin firing is abrogated and cells appeared to move through S phase more rapidly, accumulating with a 2C DNA content by 3 hours. In the *rad24Δ* mutant, which is partially defective in the intra-S checkpoint (Paulovich et al., 1997), cells moved through S phase more rapidly than wild-type but not as rapidly as *rad53* mutant cells. The *elg1Δ* resembled wild-type, and deleting *elg1* did not enhance the intra-S checkpoint defect in *rad24Δ*. By contrast, the *ctf18Δ rad24Δ* mutant was more defective in the intra-S checkpoint, accumulating with 2C DNA content with kinetics similar to that observed with the *rad53* mutant.

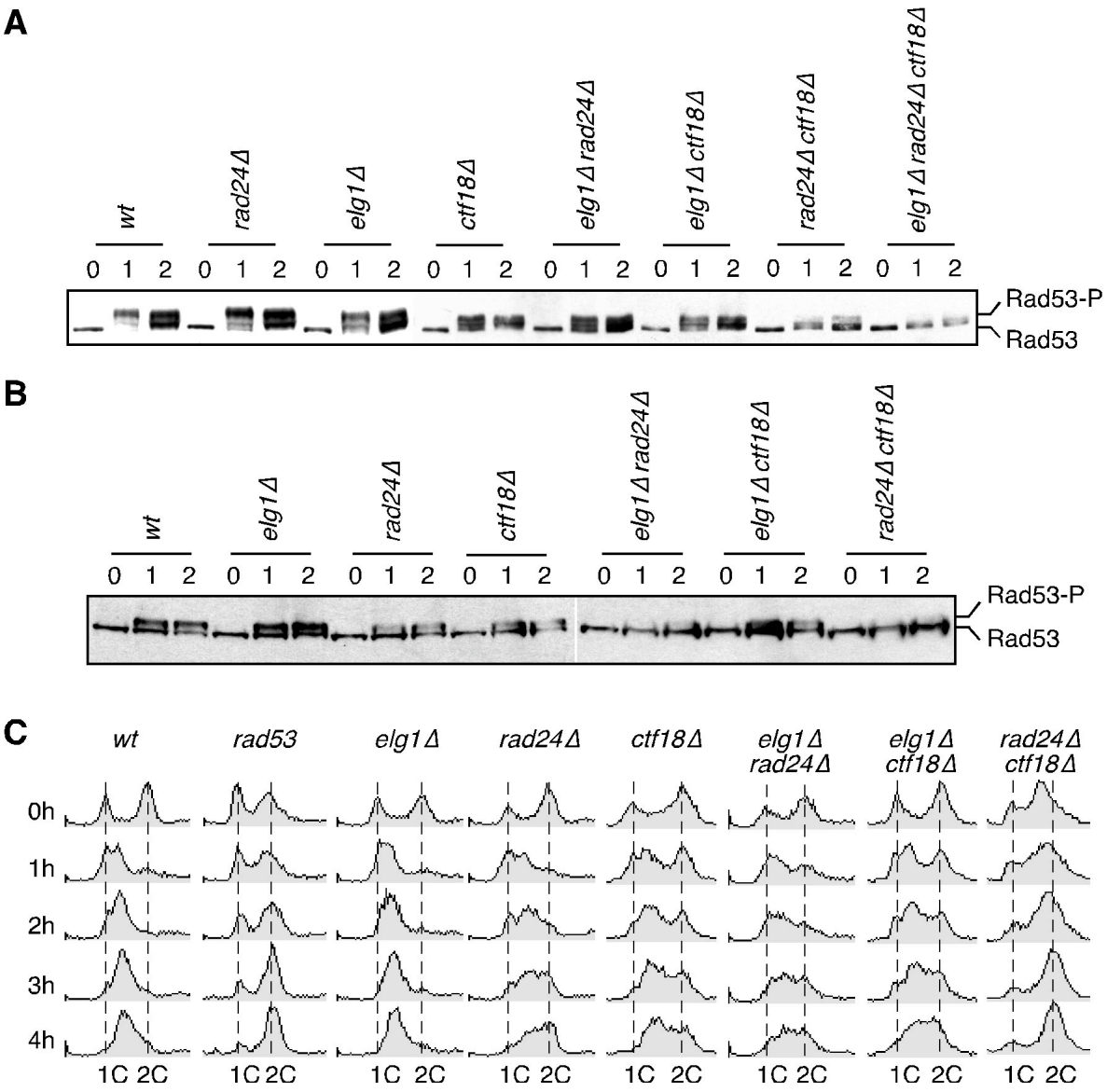

Figure 11. Rad53 activation defects in *elg1Δ*. (A) S-M checkpoint. Logarithmically growing cultures were arrested in G1 with α-factor and released into media containing 200 mM HU. At the indicated times (in hours) samples were fixed and extracts fractionated on SDS-PAGE. Following transfer the immunoblot was probed with anti-Rad53 antibody. Phosphorylation of Rad53 causes a shift in electrophoretic mobility (Rad53-P) and is a marker for checkpoint activation. (B) Intra-S phase checkpoint. Logarithmically growing cultures were treated with 0.035% (v/v) MMS. At the indicated times (in hours) samples were withdrawn and Rad53 activation was analysed by immunoblotting. (C) Cell cycle progression in the presence of MMS was assessed by flow cytometry. Logarithmically growing cultures were treated with 0.035% (v/v) MMS. At the indicated times samples were fixed and the DNA contents of cells in each sample were analysed by flow cytometry. The positions of cells with 1C and 2C DNA contents are indicated on the histograms.

Cells Lacking Elg1 Require Homologous Recombination and Replication Fork Re-start Pathways for Optimum Growth

To gain further insight into Elg1 function *in vivo* Mohammed and I performed a genome-wide synthetic lethal screen with *elg1Δ*. The synthetic genetic interactions confirmed by tetrad analysis are presented in Figure 12 and in Table 5. Several functional clusters of genes are readily apparent. First, *elg1Δ* interacts with members of the *RAD52* epistasis group (*rad51Δ, rad52Δ, rad54Δ, rad55Δ, rad57Δ*), which are required for homologous recombination. The genes *rad50Δ, mre11Δ,* and *xrs2Δ* were also identified. These interactions likely reflect the role of these genes in homologous recombination rather than in non-homologous end-joining, as *elg1Δ* is not synthetic lethal or sick with *dnl4Δ* or *yku80* (data not shown). Of particular significance, genes thought to be in redundant pathways for re-starting stalled replication forks (Kaliraman et al., 2001; Mullen et al., 2001) have synthetic genetic interactions with *elg1Δ*. *mus81Δ, mms4Δ, sgs1Δ, top3Δ*, *slx5Δ,* and *slx8Δ* all displayed a fitness defect in combination with *elg1Δ*. The last functional group of note comprises *rad24Δ, rad17Δ, mec3Δ, ddc1Δ, mrc1Δ,* and *tof1Δ*. These genes are all linked to defects in S phase checkpoints (Alcasabas et al., 2001; Foss, 2001; Osborn et al., 2002; Tanaka and Russell, 2001), and their identification in the screen is consistent with lack of Elg1 causing DNA lesions or arrested replication forks during S phase. Our comprehensive genetic analysis indicates that cells lacking Elg1 and homologous recombination, replication fork re-start, or S phase checkpoint pathways have a significant fitness defect, indicating that these pathways are required for optimal growth when *ELG1* is deleted. Finally, the functional classes of genes identified in the *elg1* synthetic genetic screen bear a striking similarity to those identified in screens with *rad27Δ* (Tong et al., 2001). Therefore, the genetic data suggest that Elg1 performs a function that is similar to that of Rad27,

which plays an important role in Okazaki fragment maturation (Merrill and Holm, 1998; Parenteau and Wellinger, 1999).

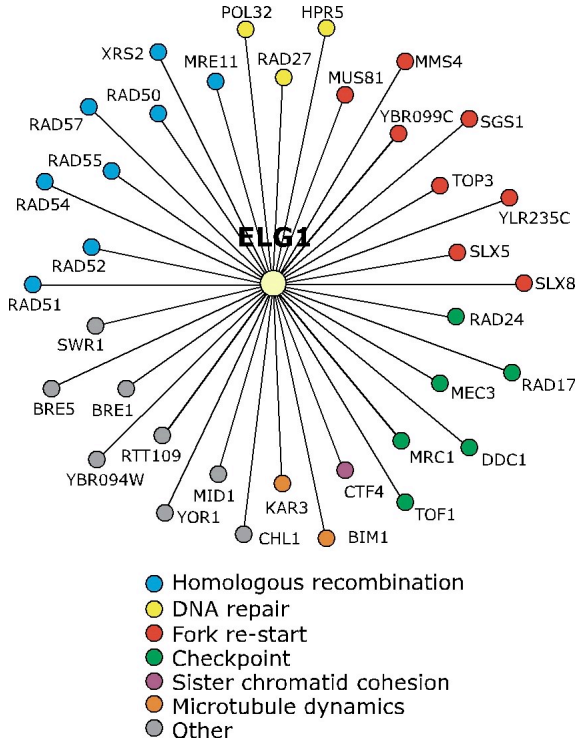

Figure 12. Genome-wide synthetic genetic screens with *elg1Δ* identify homologous recombination, fork re-start, and S phase checkpoint pathways. The results of synthetic genetic array analysis with *elg1Δ* presented as a genetic interaction map. Lines connecting genes represent synthetic lethality or synthetic slow growth. Colored circles designate the cellular role of the interacting genes, as determined by perusal of relevant literature.

Table 5. *elg1*Δ genetic interactions.

Gene	Growth defect[1]	Cellular role
RAD51	++	Homologous recombination
RAD52	++	Homologous recombination
RAD54	++	Homologous recombination
RAD55	++	Homologous recombination
RAD57	++	Homologous recombination
RAD50	+++	NHEJ, HR, checkpoint
XRS2	+++	NHEJ, HR, checkpoint
MRE11	lethal	NHEJ, HR, checkpoint
POL32	+++	DNA repair
RAD27	+	DNA repair, DNA replication
HPR5/SRS2	+	DNA repair, NHEJ, Checkpoint?, Fork re-start?
MUS81	+++	Fork re-start
MMS4	++	Fork re-start

YBR099C	+++	Fork re-start
SGS1	++	Fork re-start
TOP3	+	Fork re-start
YLR235C	+	Fork re-start
SLX5	+	Fork re-start
SLX8	+	Fork re-start
RAD24	+	Checkpoint
RAD17	+	Checkpoint
MEC3	+	Checkpoint
DDC1	+	Checkpoint
MRC1	++	Checkpoint
TOF1	+	Checkpoint
CTF4	+++	Sister chromatid cohesion
CHL1	++	Chromosome segregation
BIM1	+	Microtubule dynamics
KAR3	++	Microtubule dynamics
MID1	+	Small molecule transport
YOR1	+	Small molecule transport
YBR094W	+	Unknown
RTT109	+	Unknown
BRE1	+	Unknown
BRE5	+	Unknown
SWR1	+	Unknown

[1]Degree of synthetic fitness defect: '+++'=severe, '++'=moderate, '+'=mild.

Elg1 is Required for Replication Fidelity

Mutants defective in Okazaki fragment maturation, such as *rfc1-1*, *pol30-52*, and *rad27Δ* all have increased forward mutation rates, but differ in the spectrum of mutants produced (Xie et al., 1999; Xie et al., 2001). Whereas *rfc1-1* and *pol30-52* cause predominantly point mutations and small insertions or deletions, *rad27Δ* causes large rearrangements. Grant assessed the forward mutation rate to canavanine resistance of *elg1Δ*, and found that it was almost 8-fold higher than the wild-type strain (24.2×10^{-7} versus 3.24×10^{-7}), indicating that Elg1 is important for replication fidelity. To determine the spectrum of mutations caused by *elg1Δ* Grant examined the *CAN1* gene from 20 independent can^r mutants from both wild-type and *elg1Δ* for the presence of large insertion or deletion mutations. A difference in the size of a *CAN1*-derived fragment was observed in 2 out of 20 can^r wild-type strains, and in only 1 out of 20 can^r *elg1Δ* strains (data not shown). DNA sequencing revealed that the mutations in the *elg1Δ* strains were

predominantly base substitution mutations, with some small (< 5 bp) deletions and insertions. Therefore the *elg1Δ* mutation spectrum resembles that caused by defects in Rfc1 or PCNA.

elg1Δ Mutants are Defective in S Phase Progression

If *elg1Δ* cells are defective in fork re-start or have increased levels of fork stalling we would expect to detect defects in S phase progression. Mohammed arrested cells in G1 phase and released them synchronously into the cell cycle. Progression through S phase was measured by flow cytometry. As shown in Figure 13A, the wild-type culture completed S phase by 60 minutes after release, as evidenced by the accumulation of cells with a 2C DNA content. In the *elg1Δ* culture, however, a significant fraction of cells still had a 1C or intermediate (<2C) DNA content at 60 minutes. Even at 80 minutes post-release some *elg1Δ* cells had not completed S phase. Consistent with a role for Elg1 in S phase progression, I found that *elg1Δ* has a probability of plasmid loss that is 7.6 times higher than wild-type in a minichromosome maintenance assay (Figure 13B). This elevated plasmid loss was not suppressed significantly by the presence of additional origins of replication and 2D gel analysis did not reveal any initiation defects in *elg1Δ* (data not shown). Furthermore, Mohammed found that *elg1Δ* did not have any detectable defect in sister chromatid cohesion (data not shown), suggesting that plasmid loss in *elg1Δ* was not due to a segregation defect.

Since Elg1-RFC most likely functions as a clamp-loader, Mohammed tested whether overexpression of PCNA could suppress the *elg1Δ* phenotype. As shown in Figure 13C overexpression of PCNA rescued the MMS sensitivity of *elg1Δ*, as evidenced by improved growth on MMS plates when PCNA overexpression was induced by the presence of galactose. Taken together these results suggest that Elg1 plays a direct role in DNA replication, most likely during the elongation phase of DNA synthesis.

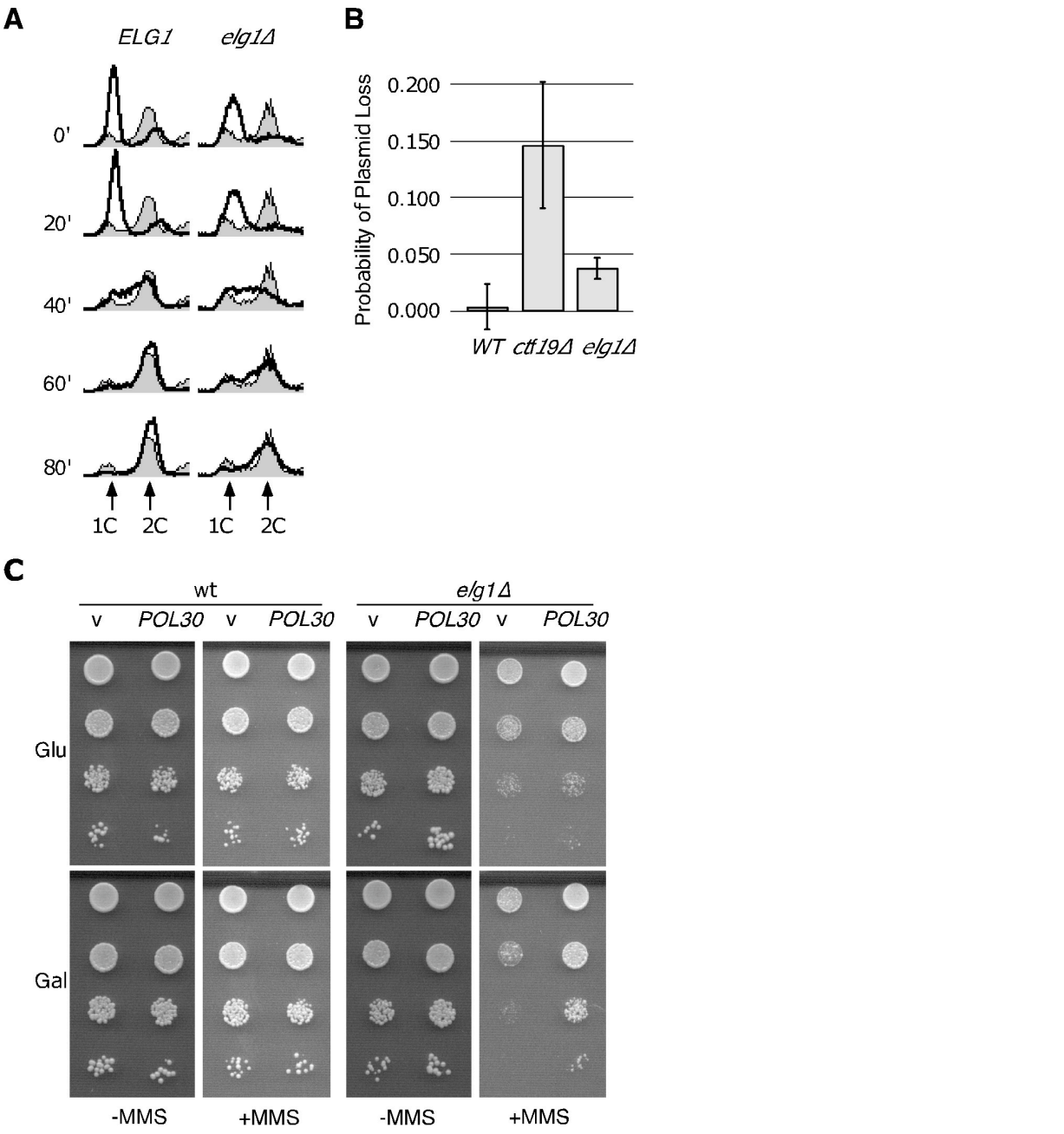

Figure 13. *elg1Δ* mutants display DNA replication defects. (A) Progression through S phase. Wild-type or *elg1Δ* cells were arrested in G1 (t=0) and released synchronously into the cell cycle. Samples were removed at the indicated times and analysed by flow cytometry. The shaded histograms represent the cell cycle distribution of the asynchronous cultures before the G1 arrest. Overlaid histograms represent the cell cycle distribution at the indicated times after release from the G1 arrest. The positions of cells with 1C and 2C DNA contents are indicated. (B) Plasmid loss in wild-type, *elg1Δ*, and *ctf19Δ*. The probability of plasmid loss per generation is plotted, and error bars span one standard deviation. (C) Suppression of *elg1Δ* MMS sensitivity by PCNA overexpression. Serial dilutions of wild type or *elg1Δ* cells carrying empty vector (v) or *GAL1-POL30* plasmid (*POL30*) were plated on synthetic media with 2% glucose (Glu; uninduced) or 2% galactose + 2% raffinose (Gal; induced), plus or minus 0.01% MMS.

Abnormal Recovery from Replication Fork Stalling in *elg1Δ*

Mohammed measured S phase progression in medium containing 0.035% MMS, a concentration of MMS known to cause extensive fork stalling in wild-type cells (Tercero and Diffley, 2001) (Figure 14A). In the presence of MMS S phase was prolonged in the wild-type cells, which take 120 minutes to accumulate with 2C DNA content. The *elg1Δ* mutant was clearly defective in S phase progression in the presence of MMS, with a significant fraction of cells containing less than 2C DNA at 160 minutes post-release. Thus the introduction of fork stalls causes further defects in DNA synthesis in *elg1* mutants, consistent with Elg1 functioning in preventing fork stalling or in re-starting stalled replication forks.

If Elg1 is required for replication fork integrity we expected to see prolonged activation of Rad53 following MMS treatment during S phase. Wild-type and *elg1Δ* cells were arrested in G1 and released into medium containing MMS for 1 hour to activate Rad53. The MMS was then washed out and cells were allowed to recover. Rad53 activation during recovery from MMS damage was assayed by immunoblot analysis (Figure 14B). In the wild-type strain Rad53 is dephosphorylated by 80 minutes following removal of MMS. By contrast, activated Rad53 persisted in the *elg1Δ* strain for at least 120 minutes, indicating that Elg1 is required for down-regulation of the intra-S checkpoint response. Since the *elg1Δ* strain is at least 10-fold less sensitive to MMS than mutants in MMS repair pathways (data not shown), these results suggest that Elg1 is required for efficient fork re-start rather than direct repair of DNA lesions.

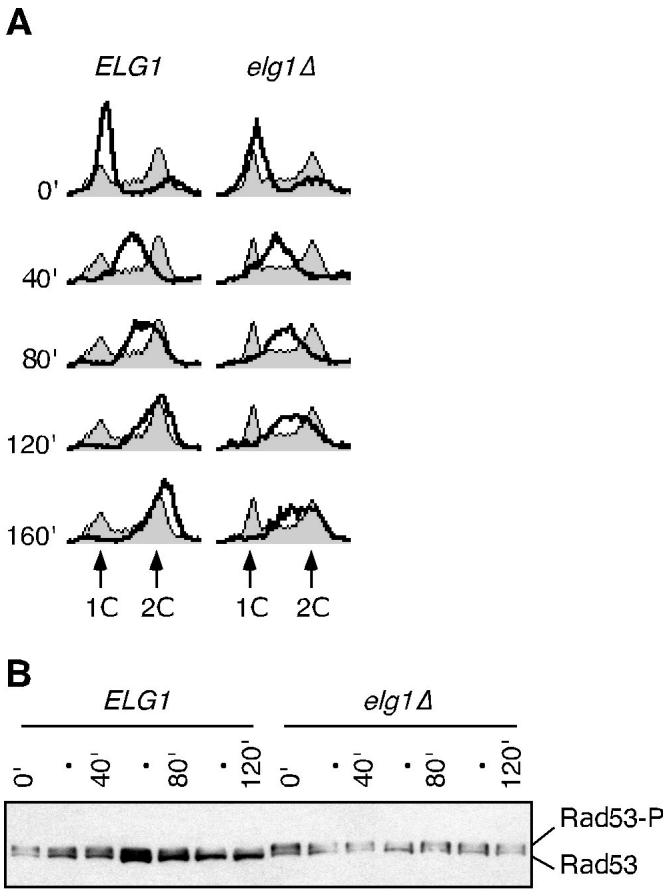

Figure 14. *elg1Δ* mutants are defective in recovery from MMS-induced replication fork stalling. (A) S phase progression in the presence of MMS. Wild-type or *elg1Δ* cells were arrested in G1 (t=0) and released synchronously into media containing 0.035% (v/v) MMS. Samples were removed at the indicated times and analysed by flow cytometry. The shaded histograms represent the cell cycle distribution of the asynchronous cultures before the G1 arrest. Overlaid histograms represent the cell cycle distribution at the indicated times after release from the G1 arrest. (B) Checkpoint activation of Rad53 during recovery from MMS damage. Cells were arrested in G1, released into MMS for 1 hour, and then transferred to medium lacking MMS (t=0). At the indicated times samples were withdrawn and Rad53 activation was analysed by immunoblotting.

4.4 DISCUSSION

A Novel RFC-like Complex

Using a functional genomics approach we have identified Elg1 as a novel DNA replication protein. Elg1 associates with Rfc2, Rfc3, Rfc4, and Rfc5, forming a fourth eukaryotic RFC-like complex that is functionally distinct from the canonical RFC, and from Rad24-RFC and Ctf18-

RFC. Whether Elg1-RFC functions as a pentameric complex, like RFC and Rad24-RFC (Green et al., 2000; Mossi and Hubscher, 1998), or requires accessory factors as found in Ctf18-RFC (Mayer et al., 2001), awaits purification of Elg1-RFC. Eukaryotic cells have at least two sliding clamps that are loaded by RFC-like enzymes, PCNA and the 9-1-1 complex. RFC loads PCNA during leading and lagging strand DNA synthesis (reviewed in (Mossi and Hubscher, 1998)), whereas Rad24-RFC loads the 9-1-1 clamp *in vitro* but cannot load PCNA (Bermudez et al., 2003; Majka and Burgers, 2003). Ctf18-RFC binds to PCNA *in vitro* and *in vivo* (Ohta et al., 2002), and so may function as a PCNA clamp loader under specialized circumstances. Our data suggest a role for Elg1-RFC in DNA replication, and we therefore propose that it functions to load or unload PCNA. In support of this Mohammed finds that overexpression of PCNA suppresses the MMS sensitivity of *elg1Δ*. Furthermore *elg1Δ* is synthetic sick when combined with *rad17Δ*, *mec3Δ*, and *ddc1Δ*, which encode the 9-1-1 clamp, but has no genetic interaction with *pol30-1*, a mutant in the gene encoding PCNA (data not shown), suggesting that Elg1 functions in the same pathway as PCNA, but in a pathway that is parallel to that in which the 9-1-1 clamp functions.

Elg1-RFC and Checkpoint Activation

A number of RFC-family proteins have been implicated in checkpoint activation. Rad24 is required for the G1 and G2 DNA damage checkpoints (Siede et al., 1994; Weinert et al., 1994), and is important (although not essential) for the intra-S damage checkpoint (Paulovich et al., 1997; Pellicioli et al., 1999). A role for Rad24 in the S-M checkpoint is revealed in the context of mutations in other RFC-family genes (Naiki et al., 2001; Shimomura et al., 1998). Similarly, deletion of *ctf18* has little effect on checkpoint activation unless *rad24* is also deleted (Naiki et al., 2001). Consistent with formation of Rad24-Rfc2-5 complexes (Green et al., 2000) mutants

in *rfc2, rfc4,* and *rfc5* with checkpoint defects have also been described (Kim and Brill, 2001; Noskov et al., 1998; Sugimoto et al., 1997; Sugimoto et al., 1996). Our data indicate that Elg1 does not play a primary role in checkpoint activation, as *elg1Δ* mutants show normal Rad53 activation in response to DNA damage in S phase and in response to replication fork arrest by HU. Double mutants in *rad24* and either *elg1* or *ctf18* were more defective in Rad53 activation in S phase, and the triple mutant lacked detectable Rad53 activation. Thus it appears that Ctf18, and to a lesser extent Elg1, can partially substitute for the primary role of Rad24 in Rad53 activation in S phase. Alternatively, Elg1 could contribute to Rad53 activation in an indirect manner, for example through its effects on DNA replication.

When assessed directly, *elg1Δ* mutants display an intact intra-S phase checkpoint response as these cells exhibit the slow progression through S phase in the presence of MMS that is seen in wild-type cells. Mohammed did note, however, that *elg1Δ rad24Δ* double mutants do not display an intra-S phase checkpoint defect that is greater than that seen with *rad24Δ* alone. This was unexpected, as *elg1Δ rad24Δ* was more defective in Rad53 activation than *rad24Δ*. By contrast, in *rad24Δ ctf18Δ* S phase progression in the presence of MMS was more rapid than wild-type or *rad24Δ*, and resembled the progression seen in the completely checkpoint-defective *mec2-1* strain. Thus deletion of *ctf18* in a *rad24Δ* strain exacerbates the *rad24Δ* checkpoint defect, whereas deletion of *elg1Δ* does not. The implications of these data are two-fold. First, the slow progression through S phase in *elg1Δ* is at least partially checkpoint-independent, which is consistent with the *elg1Δ* mutant having a defect in DNA replication fork integrity or re-start. Secondly, there is not a strict correlation between Rad53 activation and S phase progression in the presence of MMS in these mutants. This likely reflects that the slow progression through S phase that is the hallmark of the intra-S phase checkpoint (Paulovich and Hartwell, 1995; Paulovich et al., 1997) has both a checkpoint-dependent and a

checkpoint-independent component (Tercero and Diffley, 2001). Mohammed's data suggest that Elg1 functions in the checkpoint-independent component of the slow S phase progression, again consistent with a role for Elg1 in preventing replication fork stalling or in re-starting stalled forks.

Elg1-RFC and Genome Integrity

The phenotypes of *elg1Δ* indicate that Elg1 has an important role in maintaining genome integrity. Elg1 suppresses recombination, protects against mutation, and is important for recovery from DNA damage. Several aspects of the *elg1Δ* phenotype point to the presence of double-strand DNA breaks, including the importance of homologous recombination for growth of the *elg1Δ* mutant and the hyper-recombination phenotype. Other aspects of the phenotype, while consistent with the presence of double-strand breaks, point to a more fundamental role for Elg1 in DNA replication. These include the genetic requirement for the *SGS1/TOP3* and *MUS81/MMS4* replication fork re-start pathways (Doe et al., 2002; Fabre et al., 2002; Kaliraman et al., 2001) in the *elg1Δ* mutant cells, the slow S phase progression observed in both the presence and absence of DNA damage, the increase in plasmid loss, the increase in mutation rate, and the failure to recover efficiently from increased replication stalling induced by MMS. We propose that deletion of *ELG1* causes a decrease in replication fork processivity (an increase in fork stalling or a defect in re-start of stalled forks) perhaps due to defective Okazaki fragment maturation. Precedent for such a model comes from studies in both bacteria and yeast which have indicated that mutants in Okazaki fragment processing cause DSBs, stimulate recombination, confer DNA damage sensitivity, cause an increase in mutation frequency, and render homologous recombination essential for viability (Michel et al., 2001; Rothstein et al., 2000), phenotypes similar to those observed in *elg1Δ*. Furthermore, defects in Okazaki

fragment synthesis can cause replication fork stalling (Flores et al., 2001), and there is a strong correlation between replication stalling and genome instability (Aguilera et al., 2000; Michel, 2000; Michel et al., 2001; Rothstein et al., 2000).

Given the similarity of Elg1 to Rfc1 and its presence in RFC-like complexes, it is likely that Elg1-RFC functions as a clamp loader or unloader. Biochemical experiments *in vitro* indicate that a complex interplay between RFC, PCNA, and DNA polymerase δ governs appropriate polymerase switching and Okazaki fragment sealing during lagging strand DNA synthesis. Although it is clear that RFC is responsible for limiting the length of the nascent strand synthesized by pol α, and for loading of PCNA to initiate the switch to synthesis by pol δ (Ayyagari et al., 2003; Maga et al., 2001), it is not clear how PCNA is recycled on the lagging strand. Eukaryotic cells in S phase have a great excess of Okazaki fragments over molecules of PCNA (Mossi and Hubscher, 1998), and the stability of PCNA-DNA complexes suggests that an unloading activity is required to recycle PCNA (Yao et al., 1996). A role for Elg1 in unloading and recycling PCNA on the lagging strand fits well with the available data. Additionally, this model predicts that PCNA should be limiting in *elg1Δ* mutants, and indeed Mohammed found that PCNA overexpression suppresses the MMS sensitivity of *elg1Δ*. Since Elg1 is not essential its role in this process must be redundant, perhaps with RFC. Alternatively, Elg1 could be important for loading of PCNA during re-start of stalled replication forks, although in this model there must be a significant amount of fork stalling in an unperturbed S phase to account for the increased plasmid loss and the slow S phase progression observed in *elg1Δ*. It will be of great interest to determine if the human homologue of Elg1 plays a similar role in maintaining genome integrity.

5. *RMI1/NCE4*, A SUPPRESSOR OF GENOME INSTABILITY, ENCODES A MEMBER OF THE RecQ HELICASE/TopoIII COMPLEX

Published: Chang, M, Bellaoui, M, Zhang, C., Desai, R., Morozov, P., Delgado-Cruzata, L., Rothstein, R., Freyer, G.A., Boone, C., Brown, G.W. (2005). *RMI1/NCE4*, a Suppressor of Genome Instability, Encodes a Member of the RecQ Helicase/TopoIII Complex. EMBO J. 24: 2024-2033.

Data attribution: I performed the large majority of the experimental work presented in this chapter. Dr. Mohammed Bellaoui performed the viability assays in HU and MMS. Dr. Chaoying Zhang performed the gross chromosomal rearrangement assays. Ridhdhi Desai performed the recombination assays. Dr. Pavel Morozov at the Columbia Genome Center helped identify the human and mouse homologues of Rmi1. Lissette Delgado-Cruzata, a student in Dr. Greg Freyer's laboratory at Columbia University, performed the *S. pombe* experiments. Dr. Rodney Rothstein, also at Columbia University, provided me with use of his fluorescent microscope to do the Rad52-YFP localization experiments. Dr. Boone provided the SGA technology and expertise. Dr. Grant Brown performed the gel filtration experiment and *in situ* kinase assay.

5.1 SUMMARY

SGS1 encodes a DNA helicase whose homologues in human cells include the *BLM*, *WRN* and *RECQ4* genes, mutations in which lead to cancer-predisposition syndromes. Clustering of synthetic genetic interactions identified by large-scale genetic network analysis revealed that the genetic interaction profile of the gene *RMI1* (RecQ Mediated Genome Instability, also known as *NCE4* and *YPL024W*) was highly similar to those of *SGS1* and *TOP3*, suggesting a functional relationship between Rmi1 and the Sgs1/Top3 complex. I show that Rmi1 physically interacts with Sgs1 and Top3 and is a third member of this complex. Cells lacking *RMI1* activate the Rad53 checkpoint kinase, undergo a mitotic delay, and display increased relocalization of the recombination repair protein Rad52, indicating the presence of spontaneous DNA damage. Consistent with a role for *RMI1* in maintaining genome integrity, *rmi1*Δ cells exhibit increased recombination frequency and increased frequency of gross chromosomal rearrangements. In addition, *rmi1*Δ strains fail to fully activate Rad53 upon exposure to DNA damaging agents suggesting that Rmi1 is also an important part of the Rad53-dependent DNA damage response.

5.2 INTRODUCTION

As described in Chapter 1, *Saccharomyces cerevisiae SGS1* is a member of the *recQ* family of 3'-5' DNA helicases, which includes five human homologues (*RECQL*, *BLM*, *WRN*, *RECQ4*, and *RECQ5*) (Watt et al., 1996; Watt et al., 1995). Loss of function mutations in *BLM*, *WRN*, and *RECQ4* give rise to Bloom syndrome (BS), Werner syndrome (WS), and Rothmund-Thomson syndrome (RTS), respectively (Ellis et al., 1995; Kitao et al., 1999; Yu et al., 1996). Though the spectrum of clinical features of each disease differs, they all result in a predisposition to cancer. The major defects of cells with mutated *recQ* helicases are hyper-

recombination and genomic instability (Hickson, 2003). *S. cerevisiae sgs1* mutants show elevated levels of mitotic homologous recombination, illegitimate recombination (Gangloff et al., 1994; Watt et al., 1996; Yamagata et al., 1998), sister chromatid exchanges (Onoda et al., 2000), and gross chromosomal rearrangements (GCRs) (Myung et al., 2001b; Myung and Kolodner, 2002). Cells lacking *SGS1* are also moderately sensitive to genotoxic agents such as methyl methanesulfonate (MMS) and hydroxyurea (HU) (Chang et al., 2002; Gangloff et al., 1994; Watt et al., 1996; Yamagata et al., 1998).

A subset of RecQ family members physically interact with topoisomerase III (Top3) homologues (Gangloff et al., 1994; Goodwin et al., 1999; Johnson et al., 2000; Wu et al., 2000). *E. coli* RecQ stimulates Top3 to catenate and decatenate covalently closed duplex DNA (Harmon et al., 1999) and BLM is able to stimulate the DNA strand passage activity of Top3α (Oakley and Hickson, 2002). Furthermore, BLM and Top3α can work together to resolve a recombination intermediate containing a double Holliday junction (Wu and Hickson, 2003). *S. cerevisiae* strains lacking *TOP3* exhibit a severe growth defect, sensitivity to DNA damaging agents, and hyper-recombination (Chang et al., 2002; Gangloff et al., 1994; Wallis et al., 1989). Most of the defects exhibited by *top3* mutants can be suppressed by deletion of *SGS1* (Chakraverty et al., 2001; Gangloff et al., 1994), a relationship that is conserved in *Schizosaccharomyces pombe* where mutations in the *recQ* homologue $rqh1^{+}$ can suppress the lethality of *top3Δ* mutants (Maftahi et al., 1999). These data support models in which RecQ helicase action produces a toxic DNA structure that is resolved by Top3 (Gangloff et al., 1994; Ira et al., 2003; Wu and Hickson, 2003).

Two-dimensional hierarchical clustering of synthetic genetic interactions determined by large-scale genetic network analysis in *S. cerevisiae* has proven useful for identifying genes whose products function within the same pathway or complex (Tong et al., 2004). Such

clustering analysis revealed that the genetic interaction profile of the poorly characterized gene *RMI1* was highly similar to those of *SGS1* and *TOP3*. I show that Rmi1 associates with Sgs1 and Top3 and that strains lacking *RMI1* accumulate DNA damage in the absence of exogenous genotoxic agents. My results indicate that the actions of Sgs1, Top3, and Rmi1 are required in concert in order to maintain genome integrity.

5.3 RESULTS

Mutations in *SGS1* can suppress the growth defects of an *rmi1*Δ mutant

Two-dimensional hierarchical clustering of large-scale synthetic genetic array (SGA) data revealed that the set of genes that genetically interact with the uncharacterized gene *RMI1* was highly similar to that associated with *SGS1*, *TOP3*, and *YLR235C* (which overlaps the *TOP3* open reading frame such that a deletion of this ORF likely results in a *TOP3* hypomorph) (Tong et al. 2004; Figure 15). Synthetic genetic interactions are usually orthogonal to protein-protein interactions, but the products of genes with similar patterns of genetic interactions are often found in the same cellular pathway or protein complex (Tong et al., 2004), suggesting that *RMI1* might function in the *SGS1*/*TOP3* pathway.

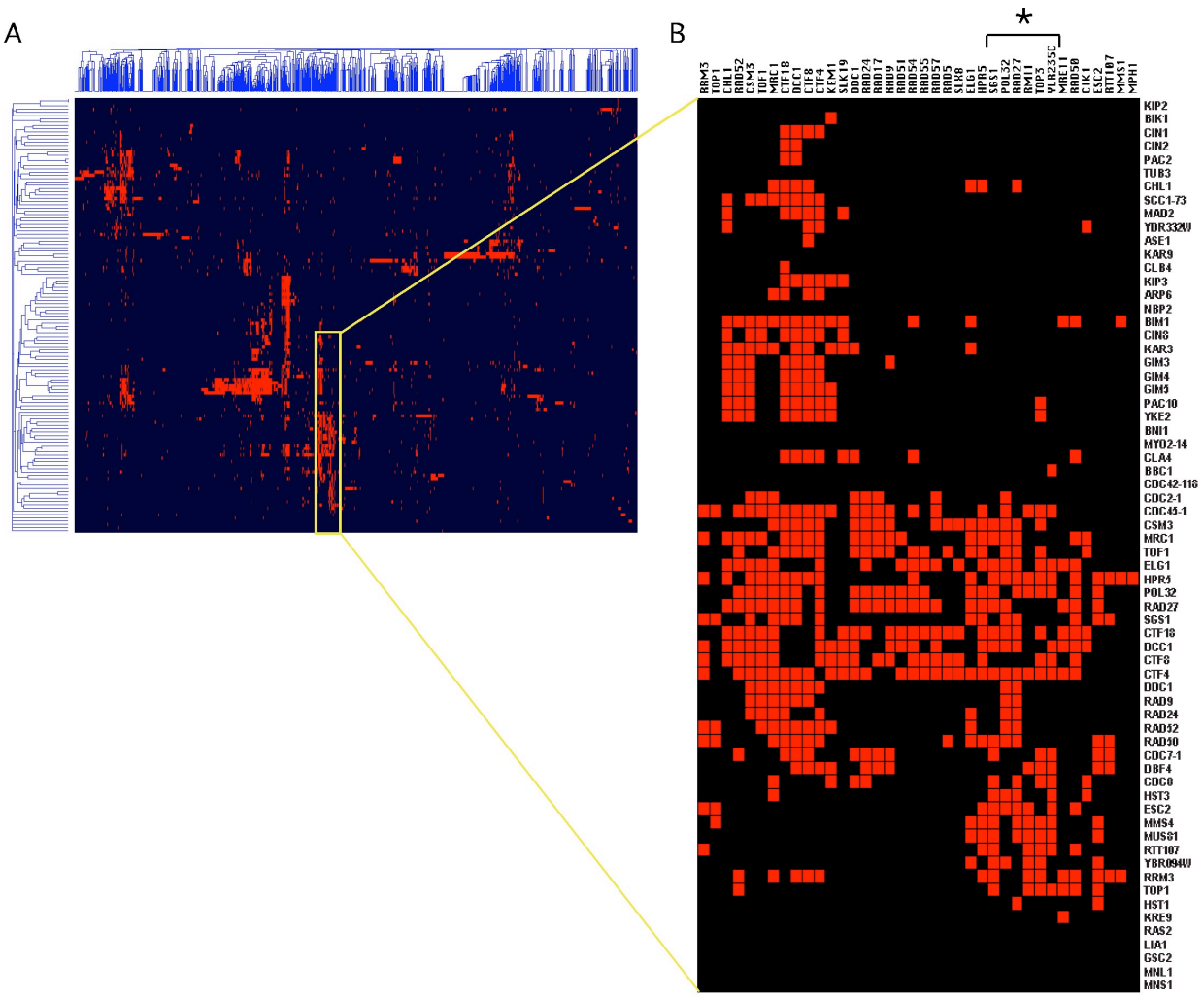

Figure 15. Two-dimensional hierarchical clustering of synthetic genetic interactions determined by SGA analysis (Tong et al., 2004). (*A*) Synthetic genetic interactions are represented as red lines. Rows, 132 query genes; columns, 1007 array genes. The cluster trees organize query (y-axis) and array genes (x-axis) that show similar patterns of genetic interactions. (*B*) The relevant section (yellow outline in *A*) is expanded to allow visualization of the *RMI1/SGS1/TOP3* array gene cluster. Synthetic genetic interactions are represented as red squares.

Crossing the *rmi1Δ* strain from the *Saccharomyces* gene deletion collection with a wild type strain revealed the presence of an extragenic suppressor in the *rmi1Δ* strain. Tetrads from this cross were dissected to analyze the products of individual meioses (Figure 16A). The resultant colonies were screened to identify those carrying the *rmi1Δ* mutation. I found that roughly half (10 of 24) of the *rmi1Δ* isolates exhibited a slow growth phenotype, whereas the other half (14 of 24) grew relatively normally, indicating that the original strain did indeed carry

a single extragenic suppressor mutation. To identify the suppressor (*supX*), I employed synthetic genetic array mapping (SGAM) methodology, in which an *rmi1Δ supX* query strain was crossed to an array of ~4600 viable gene deletion mutants (see Materials and Methods for details). This method maps a group of genes that are tightly linked to the suppressor (Jorgensen et al., 2002). Indeed, I identified a group of linked genes on chromosome XIII (Figure 16B), indicating that the suppressor was in this region. The *SGS1* gene was located in the middle of this linkage group, and the *sgs1Δ* strain was not identified in the SGAM experiment, suggesting that the suppressor might be a loss of function allele of *SGS1*. I crossed the *rmi1Δ* strain lacking the suppressor with an *sgs1Δ* strain and found that the double mutants had a normal growth phenotype (Figure 16C). The *SGS1* allele was also sequenced from the *rmi1Δ supX* strain and found that it carried a frame-shift mutation 691 nucleotides into the ORF and so encoded a truncated protein lacking the helicase catalytic domain of Sgs1. Therefore deletion of *RMI1* causes a slow growth phenotype that can be suppressed by deletion of *SGS1*. This is reminiscent of the *TOP3* gene, deletion of which causes slow growth that is suppressed by mutation of *SGS1* (Gangloff et al., 1994; Wallis et al., 1989).

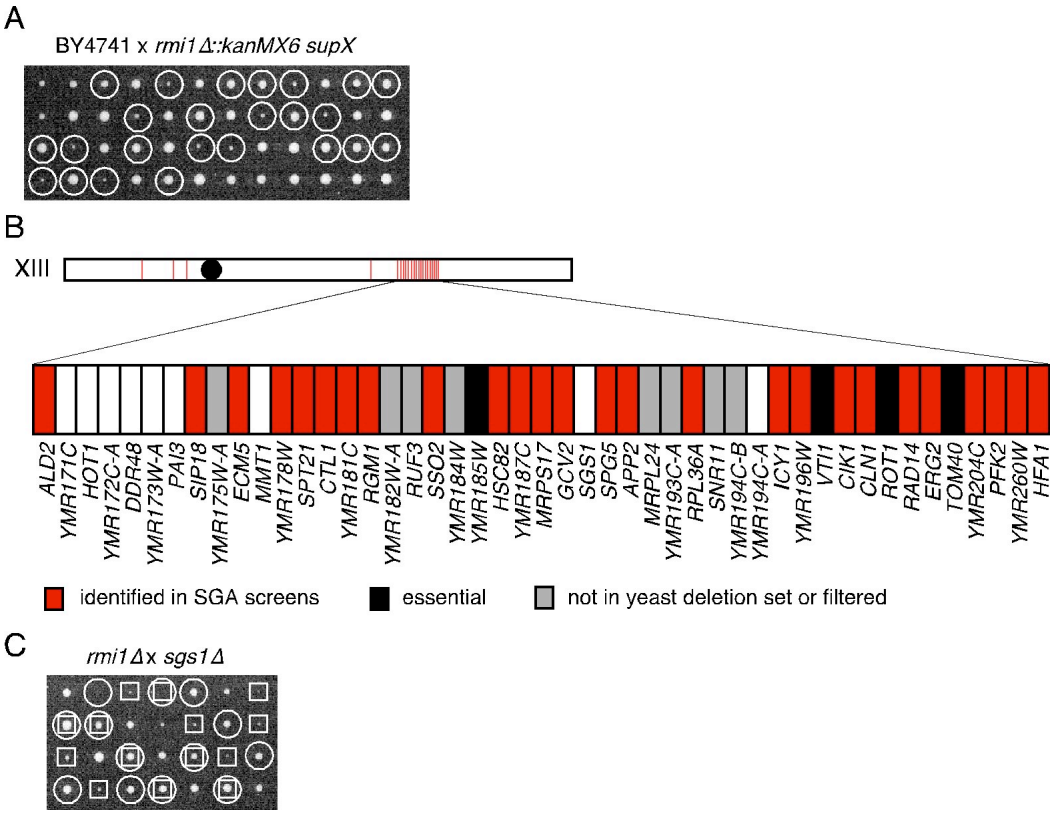

Figure 16. *rmi1Δ* mutants exhibit a growth defect that can be suppressed by mutation of *SGS1*. (*A*) The *rmi1Δ::kanMX6* strain was backcrossed to a WT strain (BY4741). The resulting diploids were sporulated and tetrads were dissected on YPD. Each column represents the four spores from a single tetrad. The genotypes of the resulting colonies are indicated with circles (○) for *rmi1Δ::kanMX6*. (*B*) SGAM analysis using an *rmi1Δ::natMX6* query strain (which contains *supX*) revealed a set of colinear synthetic genetic interactions on chromosome XIII (see Materials and Methods for details). A red bar indicates that deletion of the corresponding gene resulted in a genetic interaction. Black bars represent essential genes, which are not a part of the gene deletion collection. Grey bars indicate ORFs for which no deletion mutant was made as part of the *Saccharomyces* Gene Deletion Project (Winzeler et al., 1999) and genes that are often found in control screens using a WT query strain, and therefore are filtered from the results of SGA analyses. (*C*) An *rmi1Δ::natMX6* strain lacking *supX* was crossed to an *sgs1Δ::kanMX6* strain. The resulting diploids were sporulated for tetrad analysis as in *A*. The genotypes of the resulting colonies are indicated with boxes (□) for *rmi1Δ::natMX6* and circles (○) for *sgs1Δ::kanMX6*.

*rmi1*Δ synthetic genetic interactions

Since the *rmi1Δ* mutant readily accumulates mutations in *SGS1*, I was unable to conduct an *RMI1* SGA analysis. Instead I adopted a candidate approach, analyzing genes with connections to *SGS1* and *TOP3* function (Klein, 2001; Mullen et al., 2001; Tong et al., 2001; Tong et al.,

2004) (Table 6). I found that *rmi1Δ* is synthetic lethal when combined with mutations in genes thought to play roles in restarting stalled replication forks: *rrm3Δ, mus81Δ, mms4Δ, slx1Δ, slx4Δ, hex3Δ, slx8Δ,* and *hpr5Δ*. I also found that the slow growth phenotype of *rmi1Δ* was suppressed by *rad51Δ, rad52Δ,* and *rad54Δ*. This is consistent with models in which the presence of the homologous recombination pathway facilitates creation of DNA processing intermediates by Sgs1, which are toxic when Rmi1 is absent. Similar models have been proposed to account for the suppression of *top3Δ* phenotypes by mutations in recombination repair genes (Oakley et al., 2002; Shor et al., 2002). I also found that *rmi1Δ* did not display a detectable genetic interaction with *top3Δ*, consistent with *RMI1* and *TOP3* functioning in the same pathway. Finally, I found that homozygous *rmi1Δ/rmi1Δ* diploids are defective in undergoing meiosis to produce four spore asci (Figure 17), indicating that like Sgs1 and Top3 (Gangloff et al., 1999; Watt et al., 1995), Rmi1 is essential for proper meiotic cell division.

Table 6. *rmi1*Δ genetic interactions.

Gene	Interaction	Proposed Function
sgs1Δ	suppression	RecQ helicase
top3Δ	none	Type I topoisomerase
rad53-11	lethality	DNA damage checkpoint
mrc1Δ	sickness	S-phase DNA damage checkpoint
csm3Δ	sickness	S-phase DNA damage checkpoint
tof1Δ	sickness	S-phase DNA damage checkpoint
rad9Δ	none	G2 DNA damage checkpoint
rad24Δ	none	G2 DNA damage checkpoint
rrm3Δ	lethality	DNA helicase; fork re-start
mus81Δ	lethality	nuclease subunit; fork re-start
mms4Δ	lethality	nuclease subunit; fork re-start
slx1Δ	lethality	nuclease subunit; fork re-start
slx4Δ	lethality	nuclease subunit; fork re-start
hex3Δ	lethality	fork re-start
slx8Δ	lethality	fork re-start
hpr5Δ	lethality	DNA helicase; fork re-start
rad51Δ	suppression	homologous recombination
rad52Δ	suppression	homologous recombination
rad54Δ	suppression	homologous recombination

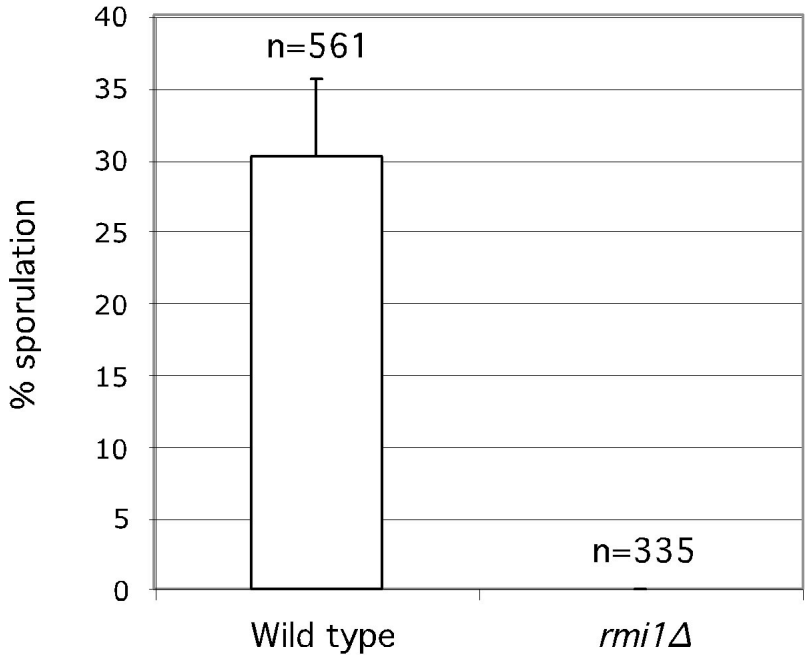

Figure 17. *rmi1Δ/rmi1Δ* diploids are sporulation defective. Sporulation efficiency, as determined by the percentage of cells that had formed four-spore asci, was scored following 5 days in sporulation medium at 30°C. The value presented for each strain is the average of three trials.

Rmi1 physically interacts with Top3 and Sgs1

Genetic analysis placed *RMI1* in the *SGS1/TOP3* pathway and indicated in several ways that *rmi1Δ* phenocopies *top3Δ*. To gain insight into the mechanism underlying these genetic observations, I tested whether Rmi1 physically associates with Sgs1 and Top3. Sgs1 and Top3 interact *in vivo* and *in vitro* (Bennett et al., 2000; Fricke et al., 2001; Gangloff et al., 1994) however the apparent molecular mass of Sgs1/Top3 complexes in yeast extracts suggests that the complexes are not heterodimeric and so may contain other proteins (Fricke et al., 2001). I used strains containing *SGS1*, *TOP3*, and *RMI1* epitope-tagged at their respective genomic loci to perform co-immunoprecipitations. Rmi1 was found in complex with both Sgs1 and Top3 (Figure 18A and 18B). This complex was not disrupted in the presence of DNase I, indicating that the interactions are not mediated by DNA (Figure 19). When Rmi1-TAP immunoprecipitations were quantified by densitometry Grant found that 39% of Rmi1 was

depleted from the extract compared with 42% of Sgs1 (data not shown), indicating that a significant fraction of Sgs1 is in complex with Rmi1. Grant next used gel filtration chromatography to fractionate extract from the tagged strain. He found that Sgs1-HA, Top3-VSV, and Rmi1-TAP co-elute in a high molecular weight complex (Figure 18C). Monomeric Rmi1 was not detected. Together these data suggest that Rmi1 is in a heteromeric complex with both Sgs1 and Top3 and functions as a subunit of the Sgs1/Top3 complex.

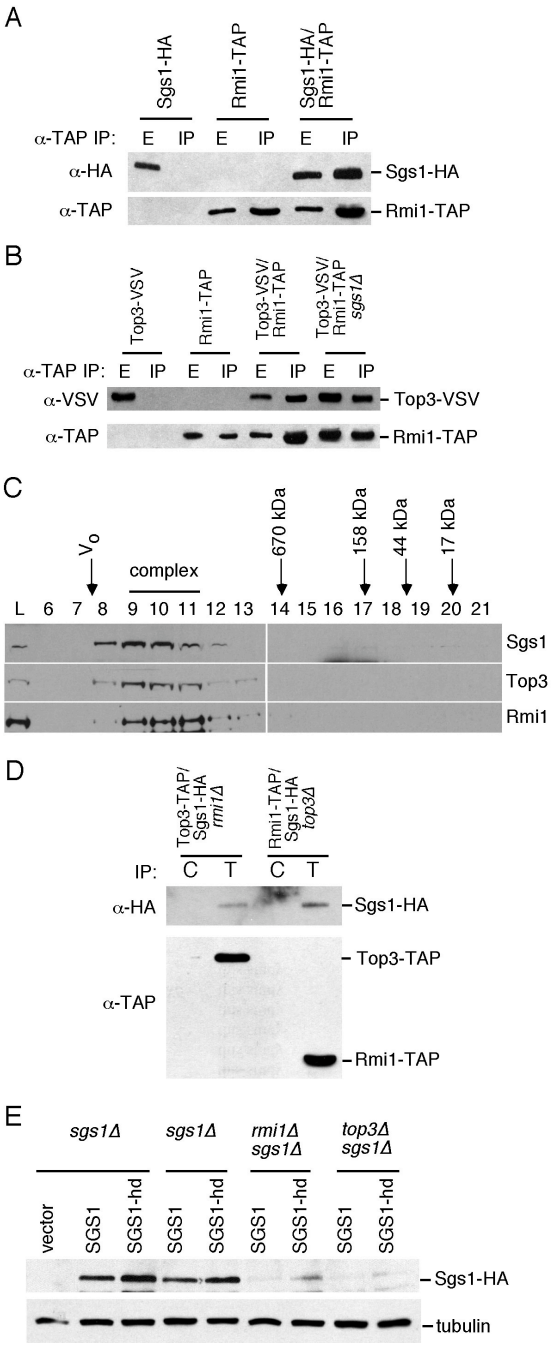

Figure 18. Rmi1 physically associates with the Sgs1/Top3 complex. (*A-B*) Extracts from yeast strains expressing the indicated epitope-tagged proteins were immunoprecipitated with IgG agarose. Ten percent of the input extract (E) and the immunoprecipitate (IP) were fractionated by SDS-PAGE. Immunoblots were probed with anti-HA antibody to detect Sgs1, with anti-VSV antibody to detect Top3, or with peroxidase-anti-peroxidase to detect Rmi1-TAP. (*C*) Extract from a yeast strain expressing Sgs1-HA, Top3-VSV, and Rmi1-TAP was fractionated on a Superose 6 gel filtration column. Fractions were precipitated with TCA and analysed by immunoblotting. The elution positions of molecular weight standards are indicated, as is the void volume of the column (V_o). (*D*) Extracts from yeast strains expressing Sgs1-HA and Top3-TAP or Sgs1-HA and Rmi1-TAP in an *rmi1Δ* or *top3Δ* background, respectively, were immunoprecipitated with IgG-agarose to precipitate the TAP tagged protein (lanes marked T) or with unconjugated agarose as a control (lanes marked C). The precipitates were immunoblotted and probed with anti-HA antibodies to detect Sgs1-HA (top panel) or with peroxidase-anti-peroxidase to detect the TAP tagged proteins. (*E*) *sgs1Δ*, *sgs1Δ rmi1Δ*, and *sgs1Δ top3Δ* strains were transformed with empty vector (vector) or low copy plasmids expressing HA-tagged Sgs1 (Sgs1) or helicase-dead Sgs1 (Sgs1-hd). TCA-fixed extracts were prepared and fractionated by SGS-PAGE. Immunoblots were probed with anti-HA antibody to detect Sgs1 or Sgs1-hd, and with anti-tubulin antibodies as a loading control.

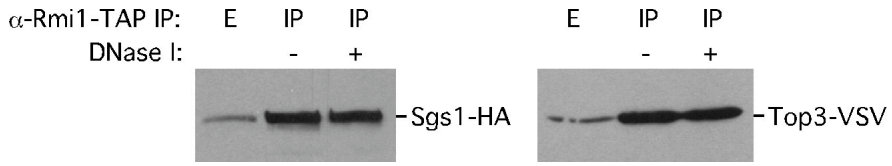

Figure 19. The Rmi1-Sgs1 and Rmi1-Top3 interactions are not mediated by DNA. Extract from a yeast strain expressing Sgs1-HA, Top3-VSV, and Rmi1-TAP was immunoprecipitated with IgG agarose following incubation for 15 minutes in the presence (+) or absence (-) of 10U of DNase I and 10 mM $MgCl_2$. Ten percent of the input extract (E) and the entire immunoprecipitate (IP) were fractionated by SDS-PAGE. Immunoblots were probed with anti-HA antibody to detect Sgs1 and with anti-VSV antibody to detect Top3.

Immunoprecipitates of Rmi1-TAP from extracts of an *sgs1Δ* strain contain Top3 (Figure 18B), indicating that the interaction of Rmi1 with Top3 does not require Sgs1. When attempting reciprocal experiments I found that deletion of either *TOP3* or *RMI1* caused a significant reduction in Sgs1 protein abundance (data not shown). Despite the reduced levels of Sgs1, I detected Sgs1 in both Top3 immunoprecipitates from *rmi1Δ* cells and in Rmi1 immunoprecipitates from *top3Δ* cells (Figure 18D). Both wild type and catalytically inactive helicase-dead mutant Sgs1 were poorly expressed in both *rmi1Δ* and *top3Δ* mutants (Figure 18E), indicating that the helicase activity of Sgs1 is not required for the observed reduction in Sgs1 levels.

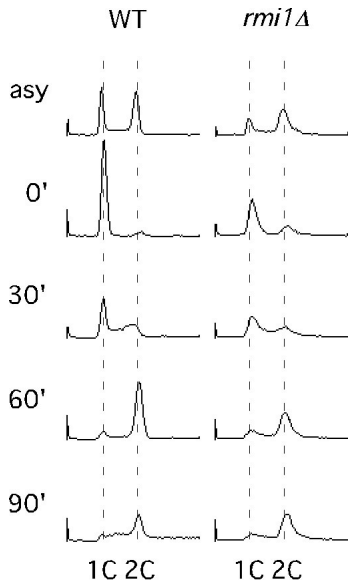

Figure 20. Bulk DNA synthesis is not affected in *rmi1Δ* cells. Wild-type or *rmi1Δ* cells were arrested in G1 (t = 0) and released synchronously into fresh YPD medium. Samples were removed at the indicated times and analyzed by flow cytometry. The positions of cells with 1C and 2C DNA contents are indicated.

Cells lacking *RMI1* display precocious checkpoint activation

RecQ helicases are thought to play a role in normal DNA replication. The human homologues BLM and WRN are required for S phase progression (Lonn et al., 1990; Poot et al., 1992). Completion of replication in the rDNA array is severely retarded in *sgs1Δ* mutants (Kaliraman and Brill, 2002; Versini et al., 2003), and *in vitro* replication in *Xenopus* egg extracts in the absence of Xblm results in DNA strand breaks (Li et al., 2004). I asked whether Rmi1 was also required for S phase progression. Using cells released synchronously from a G1 arrest, I could not detect a significant defect in bulk DNA synthesis, as assessed by flow cytometry (Figure 20). However, asynchronous *rmi1Δ* cultures exhibited an accumulation of budded cells with one nucleus suggesting a delay in the late S/G2 phase of the cell cycle (Figure 21A). These observations are similar to those made with *top3Δ* strains (Chakraverty et al., 2001; Gangloff et al., 1994) and may indicate a checkpoint dependent mitotic delay. Grant and I assayed for activation of the checkpoint kinase Rad53 in these cells, analysing both the phosphorylation

dependent mobility shift of Rad53 and Rad53 kinase activity (Figure 21B and 21C). I found that *rmi1Δ* mutants displayed a modest mobility shift of Rad53 when released from a G1 arrest in the absence of any DNA damaging agent (Figure 21B). This mobility shift is due to phosphorylation and correlates with activation of Rad53 kinase activity (Pellicioli et al., 1999). Grant measured Rad53 activation directly using an *in situ* kinase assay. Activation of Rad53 in *rmi1Δ* was clearly evident in this assay, even in the sample from the asynchronous culture and from the G1 arrested culture (Figure 21C). Activation of Rad53 was not evident in wild type cells in either assay. The precocious Rad53 checkpoint activation is likely the cause of the mitotic delay observed in *rmi1Δ*, suggesting that DNA damage is arising in cells lacking Rmi1 during an unperturbed cell cycle. Consistent with this interpretation, I found that *rmi1Δ* is synthetic lethal with *rad53-11* (Figure 21D), a checkpoint defective allele of *RAD53* (Weinert et al., 1994), indicating that an intact checkpoint response is essential for the viability of cells lacking Rmi1.

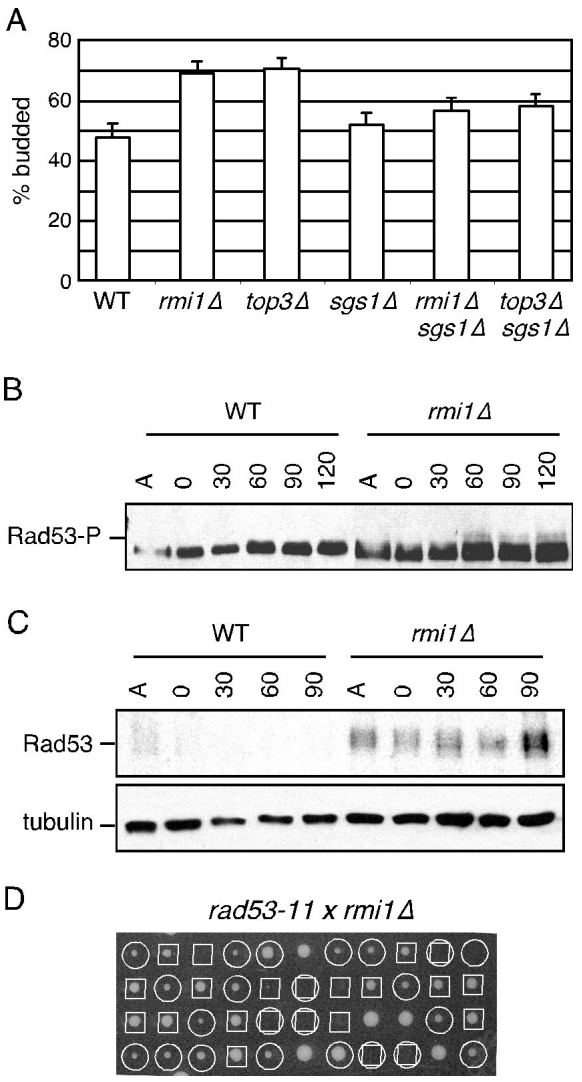

Figure 21. *rmi1Δ* mutants exhibit Rad53 checkpoint activation during an unperturbed cell cycle. (*A*) Asynchronous cultures of wild type, *rmi1Δ*, *top3Δ*, *sgs1Δ*, *rmi1Δ sgs1Δ*, and *top3Δ sgs1Δ* were examined microscopically to determine the % of cells with a bud. (*B*) Logarithmically growing cultures were arrested in G1 with alpha factor and released into fresh YPD media. At the indicated times, samples were fixed with TCA, extracts were fractionated on SDS-PAGE, and immunoblotted to detect Rad53. The position of the activated phosphorylated Rad53 is indicated. (*C*) Samples prepared as in *B* were fractionated on SDS-PAGE for *in situ* kinase assay of Rad53 (upper panel). A parallel blot was probed with anti-tubulin antibody as a loading control (lower panel). (*D*) An *rmi1Δ::kanMX6* strain was crossed to a *rad53-11::URA3* strain. The resulting diploids were sporulated and tetrads were dissected on YPD. The genotypes of the resulting colonies are indicated with boxes (□) for *rmi1Δ::kanMX6* and with circles (○) for *rad53-11::URA3*. Inferred double mutants are indicated with a box and circle.

I next investigated the requirement for other checkpoint proteins in *rmi1Δ* mutants. I found that deletion of the G1 and G2 DNA damage checkpoint genes *RAD24* or *RAD9* had no

detectable effect on the *rmi1Δ* mutant. However, deletion of the S phase checkpoint genes *MRC1*, *TOF1*, or *CSM3* in the *rmi1Δ* mutant caused a synthetic sick phenotype (Table 6). Therefore cells lacking *RMI1* require the S phase checkpoint response for optimal growth, suggesting that the DNA damage caused by deletion of *RMI1* results from DNA replication defects.

_rmi1_Δ mutants exhibit increased levels of Rad52 relocalization and genomic instability

RAD52 is essential for efficient homologous recombination. Rad52 relocalizes from a diffuse nuclear localization to distinct subnuclear foci in response to DNA damage, particularly double strand breaks (Lisby et al., 2004; Lisby et al., 2003; Lisby et al., 2001). Rad52 tagged with yellow fluorescent protein (Lisby et al., 2004; Lisby et al., 2003) was visualized by fluorescence microscopy in asynchronous mitotic haploid cells. As shown in Figure 22A, cells lacking *RMI1* display subnuclear Rad52 foci whereas wild type cells show infrequent and transient foci. Quantification of the data (Figure 22B) showed that *rmi1Δ*, *sgs1Δ*, and *top3Δ* all have elevated levels of spontaneous Rad52 focus formation, indicating the presence of DNA damage requiring homologous recombination for repair, likely double strand breaks. Elevated levels of Rad52 foci were observed both in S/G2/M (i.e. budded) cells and in G1 cells. Together with the data indicating that Rad53 is activated in *rmi1Δ* mutants, these results suggest that DNA replication in the absence of an intact Sgs1/Top3/Rmi1 pathway causes DNA lesions that result in genomic instability (Ajima et al., 2002; Gangloff et al., 1994; Myung et al., 2001b), similar to the effect observed in *Xenopus* egg extracts, in which replication in the absence of Xblm causes DNA strand breaks (Li et al., 2004).

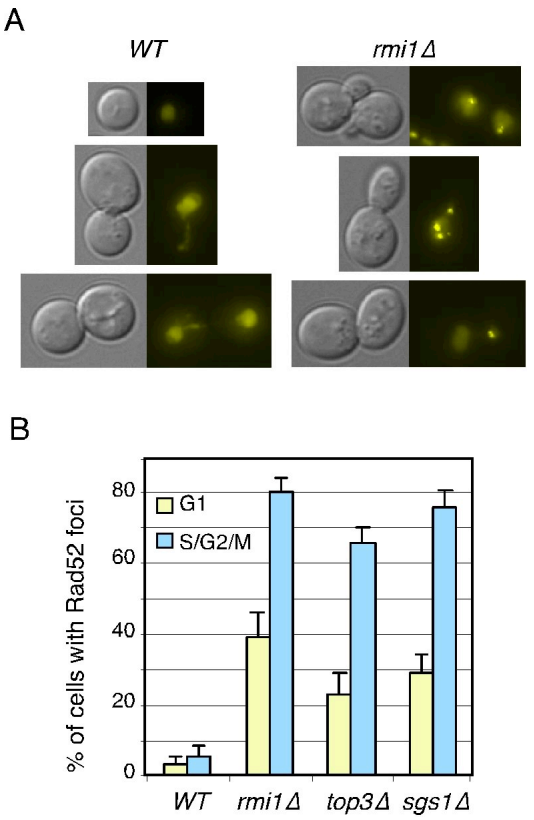

Figure 22. Spontaneous Rad52 focus formation in *rmi1Δ* cells. (*A*) Logarithmically growing cells expressing Rad52-YFP were visualized by fluorescence microscopy. For each pair of images, the left panel is a DIC image and the right panel is a fluorescence image showing Rad52-YFP. Representative cells are shown. (*B*) The percentage of cells with Rad52 foci was determined for the indicated strains. G1 cells with Rad52 foci are represented by the gold bars and S/G2/M cells with Rad52 foci are represented by the blue bars.

To assess the effect of the DNA damage that arises in *rmi1Δ* mutants, two assays for genomic instability were applied. Both *SGS1* and *TOP3* are suppressors of homologous recombination (Shor et al., 2002). Ridhdhi tested the effect of deletion of *RMI1* on homologous recombination using a *LEU2* direct repeat assay (Smith and Rothstein, 1999). Consistent with the observation that cells lacking *RMI1* have high levels of Rad52 foci, Ridhdhi found that *rmi1Δ* cells have an increased rate of recombination (Figure 23A), approximately 6-fold higher than wild type. Chaoying measured the rate of gross chromosomal rearrangements (GCRs) in *rmi1Δ*, using an assay that detects large interstitial deletions, translocations, chromosome fusions, and loss of a chromosome arm (Myung et al., 2001a). In this assay (Figure 23B), *sgs1Δ*

and *top3Δ* showed increased GCR rates of approximately 30-fold over wild type, similar to reported values (Myung et al., 2001b). In contrast, GCR rates in *rmi1Δ* cells were more than 150 times wild type levels. Thus, Rmi1 is a critical suppressor of gross chromosomal rearrangements.

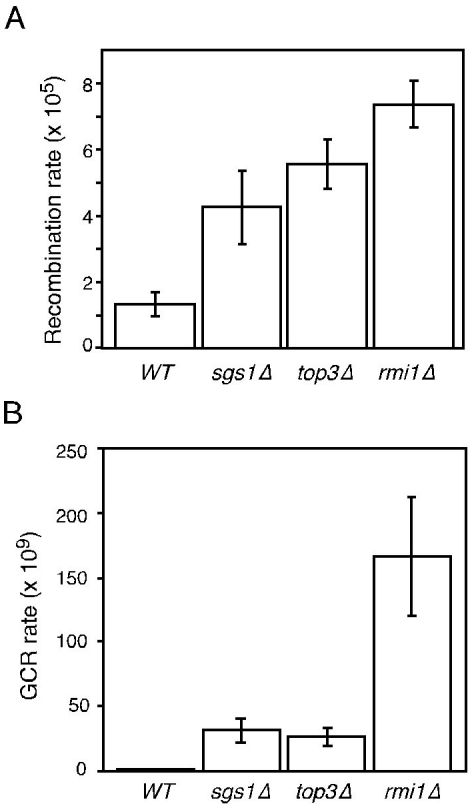

Figure 23. Deletion of *RMI1* causes genomic instability. (*A*) Recombination rate was measured using a direct repeat recombination assay. The average and standard deviation of three fluctuation tests are shown for each strain. (*B*) Gross chromosomal rearrangement rate was measured. The average and standard deviation of four fluctuation tests are shown for each strain.

RMI1 is required for the response to DNA damage

Both *SGS1* and *TOP3* are important for the response to DNA damage (Chakraverty et al., 2001; Davey et al., 1998; Frei and Gasser, 2000; Stewart et al., 1997), therefore I tested whether deletion of *RMI1* caused sensitivity to DNA damaging agents (Figure 24A, B). The *rmi1Δ* cells, like *top3Δ*, displayed slow growth on YPD. The presence of the alkylating agent MMS (at

0.004%) or the replication inhibitor HU (at 10 mM) reduced colony formation by *rmi1Δ* by at least an order of magnitude, indicating that the *rmi1Δ* mutant is sensitive to DNA damage and replication stress. Wild type cells were unaffected by the levels of MMS and HU used. Mohammed also tested whether *rmi1Δ* loses viability during transient exposure to the same concentrations of MMS or HU. The *rmi1Δ* mutant rapidly lost viability during exposure to MMS. During transient exposure to 10 mM HU the *rmi1Δ* mutant displayed little loss of viability, although the growth of *rmi1Δ* was significantly impaired (Figure 24B). These results are reminiscent of *top3Δ* which displays much greater sensitivity to transient MMS exposure than it does to transient HU exposure (Chakraverty et al., 2001; Oakley et al., 2002). The DNA damage sensitivity and loss of viability of *rmi1Δ* were suppressed by deletion of *SGS1* with the double mutant displaying growth similar to that of *sgs1Δ*.

Top3 is important for full activation of Rad53 in response to DNA damage (Chakraverty et al., 2001) while Sgs1 is necessary for Rad53 activation in the absence of Rad24 (Bjergbaek et al., 2004; Frei and Gasser, 2000). I tested whether *RMI1* was also important for Rad53 activation. Rad53 activation was measured after treatment with HU or MMS (Figure 24C). Wild type cells showed a robust checkpoint response, resulting in phosphorylation-dependent mobility shift of Rad53. By contrast, *rmi1Δ* mutants showed a defect in Rad53 activation in response to both HU and MMS, as evidenced by incomplete phosphorylation of Rad53. This defect can be suppressed by mutation of *SGS1* (data not shown), similar to the suppression of the Rad53 activation defect in a *top3Δ* mutant by deletion of *SGS1* (Chakraverty et al., 2001). Thus, in addition to causing DNA damage during S phase, deletion of *RMI1* impedes full checkpoint activation when cells are challenged with exogenous damaging agents, suggesting that like Sgs1 and Top3, Rmi1 is upstream of Rad53 in the S phase checkpoint response.

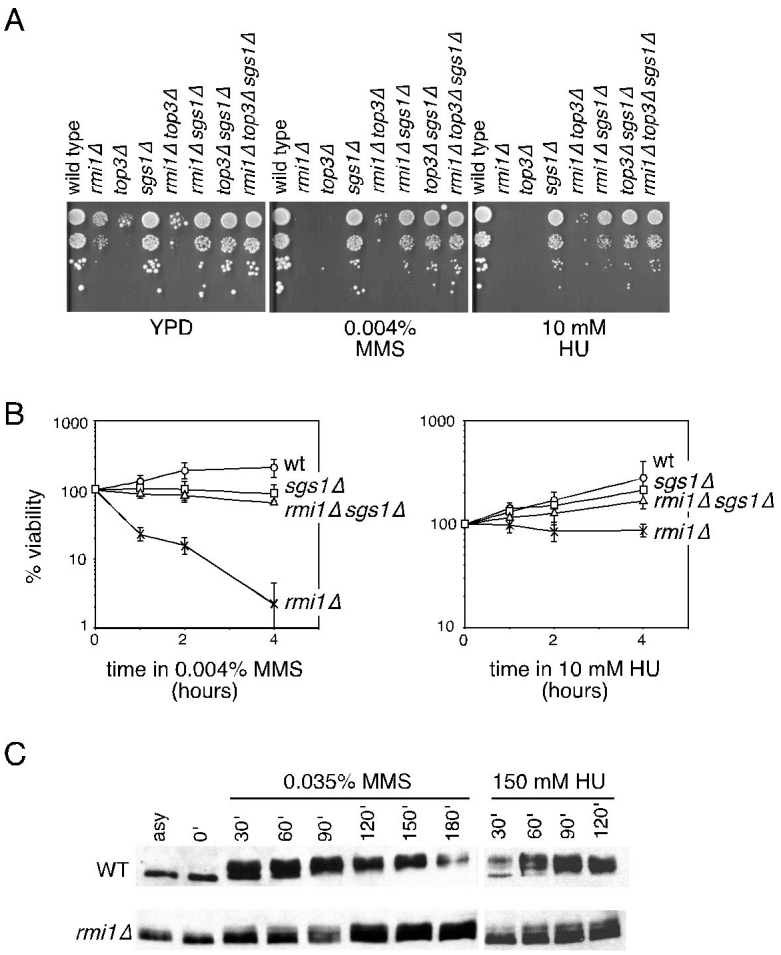

Figure 24. Rmi1 is required for the DNA damage response. (*A*) Ten-fold serial dilutions of cultures of the indicated mutants were spotted on YPD, YPD containing 0.004% (v/v) MMS, or YPD containing 10 mM HU. All plates were incubated at 30°C for 2-3 days. (*B*) Logarithmically growing cultures of the indicated mutants were incubated in YPD containing 0.004% (v/v) MMS or 10 mM HU at 30°C. At the indicated times, samples were withdrawn and plated on YPD to determine the number of viable cells. The percentage of viable cells relative to the number of viable cells at $t = 0$ is shown. Plots represent the average of three experiments, and error bars span 1 SD. (*C*) Logarithmically growing cultures were arrested in G1 with alpha factor and released into medium containing either 0.035% (v/v) MMS or 150 mM HU. At the indicated times, samples were fixed and extracts fractionated by SDS-PAGE. Following transfer, the immunoblot was probed with anti-Rad53 antibody.

Evolutionary conservation of Rmi1

Homologues of Sgs1 and Top3 are found throughout *Eucaryota*. Using local alignment searches, Grant identified homologues of Rmi1 in six other yeast species. Sequence alignments

of yeast Rmi1 homologues indicated that these proteins share three blocks of high sequence similarity (Figure 25A; Figure 26).

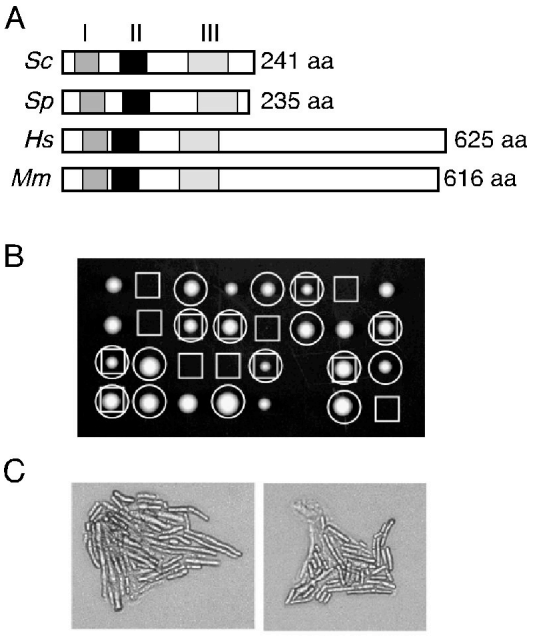

Figure 25. Rmi1 homologues. (*A*) Schematic diagrams of Rmi1 homologues from *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Homo sapiens* (Hs), and *Mus musculus* (Mm). Regions of high sequence identity are indicated by the three shaded boxes. (*B*) *S. pombe rmi1*$^{+}$ is a functional homologue of *RMI1*. *rmi1Δ::G418*^{R} *rqh1Δ::ura4*$^{+}$ was crossed to *rmi1*$^{+}$ *rqh1*$^{+}$ and tetrads were dissected on YE5S. The genotypes of the resulting colonies are indicated with boxes (□) for (inferred) *rmi1Δ::G418*^{R} and with circles (○) for *rqh1Δ::ura4*$^{+}$. (*C*) Micrographs of *rmi1Δ::G418*^{R} *rqh1Δ::ura4*$^{+}$ microcolonies from *C*.

In fission yeast, the *top3*$^{+}$ gene is essential for viability (Goodwin et al., 1999; Maftahi et al., 1999), a phenotype that is suppressed by deletion of the fission yeast RecQ homologue *rqh1*$^{+}$ (Goodwin et al., 1999; Maftahi et al., 1999). Lissette asked whether the fission yeast *rmi1*$^{+}$ gene is, like *top3*$^{+}$, essential for viability by replacing the *rmi1*$^{+}$ ORF with a G418 resistance gene in a haploid strain carrying a deletion of the *rqh1*$^{+}$ gene (*rqh1Δ::ura4*$^{+}$). This strain was viable, indicating that *rmi1*$^{+}$ is not essential in an *rqh1Δ* background. The *rmi1Δ::G418*^{R} *rqh1Δ::ura4*$^{+}$ strain was crossed to a wild type strain and meiotic progeny were examined following tetrad dissection (Figure 25B). All inferred *rmi1Δ* single mutants failed to form colonies, indicating that *rmi1*$^{+}$ is an essential gene. Examination of the resulting

microcolonies revealed that the *rmi1Δ* cells go through several divisions before arresting with an elongated morphology (Figure 25C), a phenotype similar to that found with *top3Δ* mutants (Maftahi et al., 1999). These results suggest that the fission yeast *rmi1*$^{+}$ is the functional homologue of budding yeast *RMI1*.

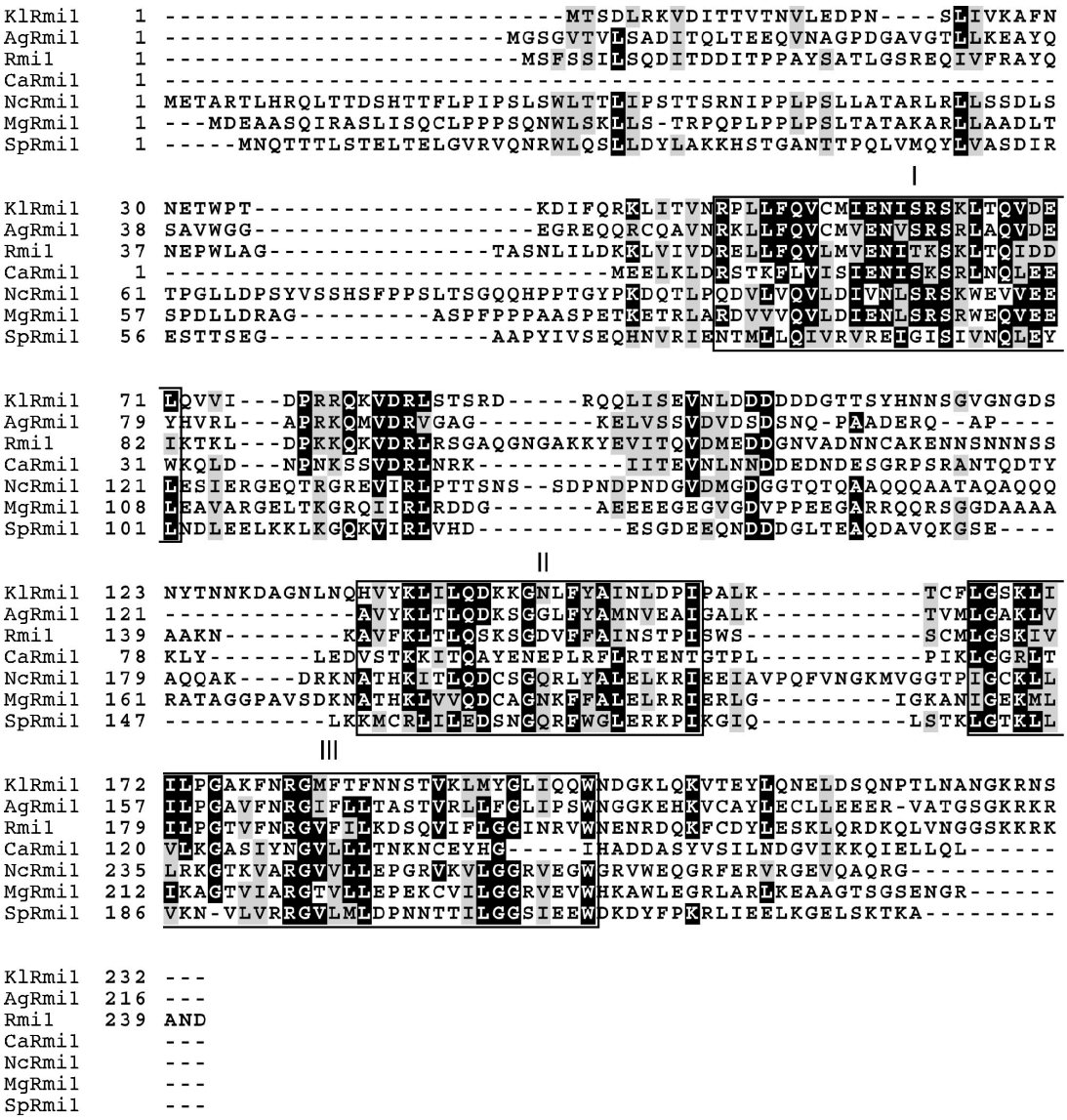

Figure 26. ClustalW alignment of yeast Rmi1 homologues. *Saccharomyces cerevisiae* Rmi1 (ScRmi1, Accession number NP_015301) is aligned with Rmi1 homologues from *Kluveromyces* lactis (KlRmi1, XP_453604), *Ashbya gossypii* (AgRmi1, AAS53829), *Candida albicans* (CaRmi1, EAK98148), *Neurospora crassa* (NcRmi1, EAA29355), *Magnaporthe grisea* (MgRmi1, EAA51673), and *Schizosaccharomyces pombe* (SpRmi1, CAA93226). Identical amino acids are shaded black and similar amino acids are shaded grey, at positions where the identity or similarity is shared by at least four of the homologues. Regions of extensive sequence similarity are designated I, II, and III.

Pavel extended Grant's homology search to metazoan species and found that homologues were not readily identified using local alignment searches such as BLAST. Pavel used the three regions of sequence similarity from the yeast analysis to build a Hidden Markov Model (HMM) for each region. The HMMs were then used to search the NCBI nonredundant protein database, resulting in the identification of homologous proteins in humans and mice. These putative Rmi1 homologues contain the three conserved regions that were evident in the yeast homologues, and also contain a C-terminal extension (Figure 25A; Figure 27). The human Rmi1 homologue is identical to the recently described BLAP75, a BLM-associated protein that is important for genome integrity in human cells (Yin et al., 2005).

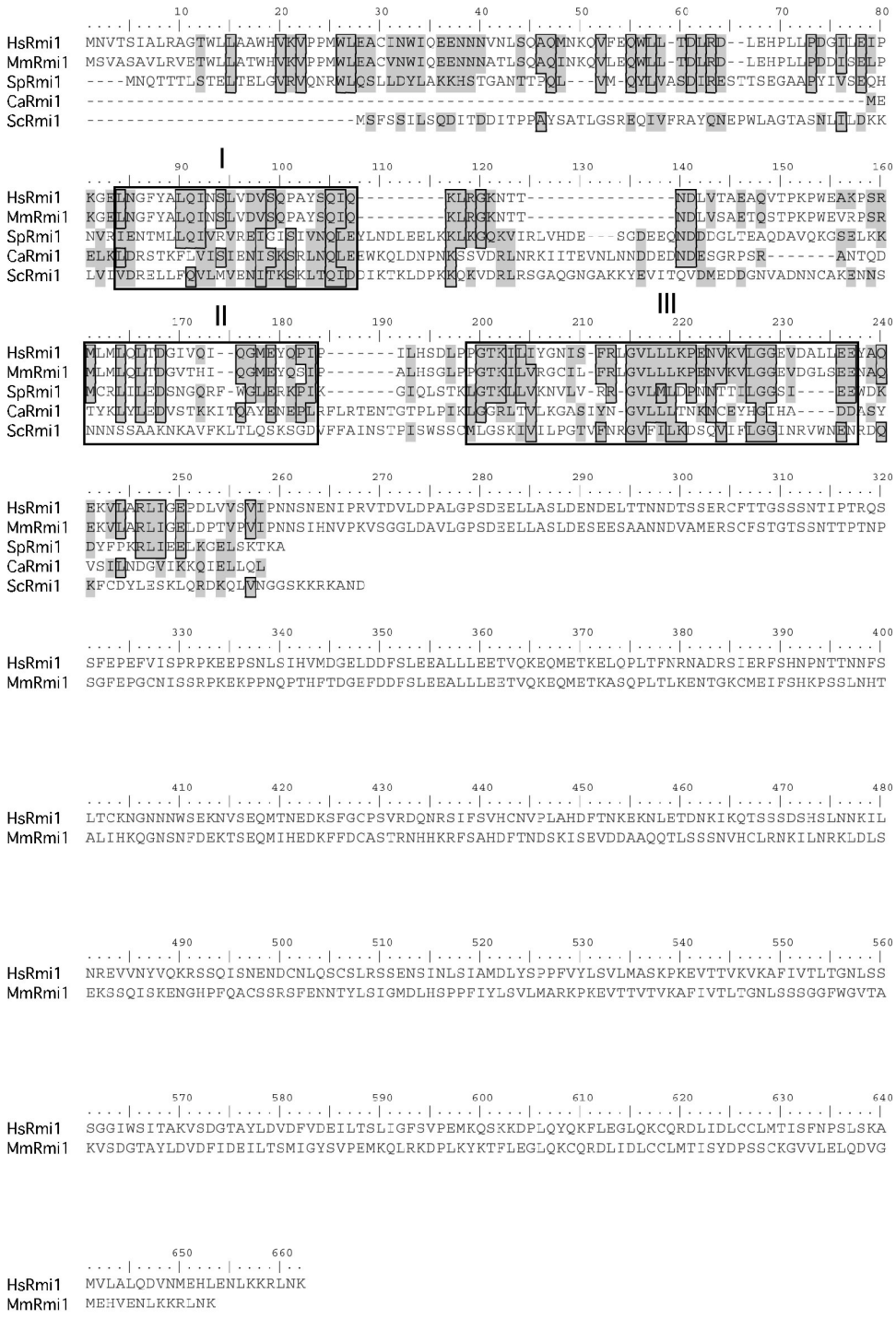

Figure 27. Alignment of Rmi1 homologues. *Saccharomyces cerevisiae* Rmi1 (ScRmi1, Accession number NP_015301) is aligned with Rmi1 homologues from *Candida albicans* (CaRmi1, EAK98148), *Schizosaccharomyces pombe* (SpRmi1, CAA93226), human (HsRmi1, NP_079221), and mouse (MmRmi1, NP_0833180. Identical amino acids are boxed in black and similar amino acids are shaded grey, at positions where the identity or similarity is shared by at least three of the homologues. Regions of extensive sequence similarity from the yeast analysis are designated I, II, and III.

5.4 DISCUSSION

Rmi1 is a novel member of the Sgs1/Top3 complex

I have found that Rmi1 physically associates with both Sgs1 and Top3. Fractionation of cell extracts by gel filtration chromatography and co-immunoprecipitation experiments indicated that Rmi1 is in a high molecular weight heteromeric complex that contains both Sgs1 and Top3. In the absence of Rmi1 the levels of Sgs1 decrease, an effect that is also observed in the absence of the Sgs1 binding partner Top3. This suggests that interactions with both Rmi1 and Top3 are important for Sgs1 stability. Finally, *rmi1Δ* shares many phenotypes with *top3Δ*, including slow growth and DNA damage sensitivities that are suppressed by deletion of *SGS1*, indicating that Rmi1 is required for Top3 function *in vivo* (or vice versa). The simplest interpretation of these data is that Rmi1 is a member of the functional Sgs1/Top3 complex. The exact stoichiometry and architecture of the native cellular Sgs1/Top3/Rmi1 complex remains elusive as Sgs1 is present in a very high molecular weight complex of some 1.3 MDa (Fricke et al., 2001), suggesting that other proteins may also be present. Thus the interaction of Rmi1 with Sgs1/Top3 may be direct or indirect.

The reduction of Sgs1 steady-state protein levels in an *rmi1Δ* or *top3Δ* background is especially intriguing given that deleting *SGS1* in these backgrounds improves cell viability. Therefore it appears that even a very low level of Sgs1 is detrimental to cells lacking Rmi1 or Top3. Although abolishing the helicase activity of Sgs1 improves viability of *rmi1Δ* (data not shown) and *top3Δ* mutants (Mullen et al., 2001), levels of helicase-dead Sgs1 were still greatly reduced in *rmi1Δ* and *top3Δ* compared to wild type (Figure 2E), indicating that the reduced Sgs1 levels are unlikely to be a response to Sgs1 activity. The mechanism by which Sgs1 levels are reduced is currently unknown, but the phenomenon appears to be evolutionarily conserved in that deletion of *top3*$^{+}$ in *S. pombe* results in a reduction in the level of a helicase-inactive

Rqh1 (Laursen et al., 2003). Although several models are consistent with my data, the simplest explanation is that absence of either Rmi1 or Top3 from the Sgs1/Top3/Rmi1 complex destabilizes Sgs1 but enough Sgs1 activity remains to cause reduced viability of *rmi1Δ* or *top3Δ* cells.

In vitro experiments using purified BLM and TOP3α have demonstrated that together, these proteins can resolve a recombination intermediate containing a double Holliday junction (Wu and Hickson, 2003). Deletion of *RMI1* results in a phenotype very similar to that displayed by *top3Δ* mutants, suggesting that Rmi1 may be important for the biochemical activity of Top3. However, deletion of either *TOP3* or *RMI1* causes a reduction in Sgs1 levels and so is likely to also compromise Sgs1 activity. Additionally, Rmi1 binds to both Top3 and Sgs1 further indicating that Rmi1 may influence the activity of both complex members. It will be of considerable interest to determine if and how the presence of Rmi1 affects the biochemical properties of RecQ/Top3 complexes.

Accumulation of DNA damage in cells lacking *RMI1*

The mitotic cell cycle delay, precocious Rad53 activation, and synthetic genetic interactions with genes required for DNA replication fork stability and the S phase checkpoint all point to the accumulation of DNA lesions. The genetic suppression data suggest that these lesions are generated from the processing of recombination intermediates by Sgs1. The exact nature of these lesions has yet to be determined but the elevated levels of GCRs, Rad52 foci, and recombination provide insight as to what these lesions may be. GCRs can take the form of non-reciprocal translocations, interstitial deletions, chromosome fusions, and loss of a chromosome arm followed by *de novo* telomere addition (Chen et al., 1998; Myung et al., 2001a). All of these rearrangements require the creation of DSBs. Thus we know that at least a significant

fraction of the lesions generated in an *rmi1Δ* mutant are, or result in, DSBs. Consistent with this hypothesis, Rad52 relocalizes into DSB repair foci in *rmi1Δ*, presumably reflecting the observed increase in recombination frequency. Recent work indicates that abnormal recombination structures accumulate in *sgs1Δ* and *top3Δ* mutants when alkylation damage is present (Liberi et al., 2005). Accumulation of these structures was not detected in the absence of DNA damage, however. I found increased levels of Rad52 recombination repair foci in cells lacking Rmi1, Sgs1, or Top3 in an otherwise unperturbed cell cycle, which argues that Sgs1/Top3/Rmi1 function is required to prevent DNA damage from occurring during normal cell cycle progression. Interestingly, Ridhdhi found that the direct repeat recombination rate is higher in an *rmi1Δ* mutant than in an *sgs1Δ* mutant, yet Rad52 foci form to the same extent in both. It has been shown that multiple DSBs can localize to one Rad52 focus, thus the formation of Rad52 foci may not be directly proportional to the extent of DNA damage (Lisby et al., 2003). As suggested by their slower growth rate and precocious checkpoint activation, it is likely that *rmi1Δ* mutants accumulate more damage than *sgs1Δ* mutants, resulting in the higher rate of recombination observed. Alternatively, the DNA lesions present in *rmi1Δ* cells may simply be more recombinogenic than those present in *sgs1Δ* cells.

Defects in Rad53 checkpoint activation

Similar to *top3Δ* mutants (Chakraverty et al., 2001), cells lacking *RMI1* are defective in fully activating Rad53 in response to DNA damage induced by HU or MMS, a defect that can be suppressed by the mutation of *SGS1*. The Sgs1/Top3/Rmi1 complex may be needed to process DNA lesions in order to generate DNA structures that can be recognized by the DNA damage checkpoint machinery, allowing for checkpoint activation. Sgs1 function in the absence of Rmi1 or Top3 could generate a toxic DNA intermediate that is not efficiently recognized by the

checkpoint machinery. Alternatively, Rmi1 could be required in a more direct way to facilitate Rad53 activation, perhaps by mediating localization of Rad53 to DNA lesions or stalled replication forks. Either model is consistent with the weak Rad53 activation seen in the Rad53 protein blots of extracts from *rmi1Δ* mutants treated with MMS or HU (Figure 24C). Although the failure of *rmi1Δ* mutants to support wild type checkpoint activation may seem at odds with my data demonstrating precocious checkpoint activation in *rmi1Δ* in the absence of DNA damaging agents, it is worth noting that Rad53 is in fact activated in *rmi1Δ* in response to MMS or HU, but to lower levels than in wild type cells. Thus the spontaneous damage present in *rmi1Δ* may cause more robust checkpoint activation if *rmi1Δ* mutants were not also compromised in checkpoint activation. In this regard, it is interesting that I see evidence of spontaneous DNA damage in G1 *rmi1Δ* cells (Figure 22B). In wild type cells, DNA damage accrued during G1 does not induce Rad52 foci formation until cells progress into S phase (Lisby et al., 2004). The presence of Rad52 foci in *rmi1Δ* G1 cells is likely due to progression through mitosis despite the presence of DNA lesions. Although a single DSB is typically sufficient to prevent passage through mitosis for several cell cycles (Lee et al., 1998), I would expect this checkpoint mediated mitotic delay to be abrogated in mutants such as *rmi1Δ* that display compromised checkpoint activation in response to DNA damage. Progression through mitosis in the presence of DNA lesions could be a principal cause of the poor viability of *rmi1Δ* mutants.

Recent data suggests that *top3Δ* mutants appear to have a compromised checkpoint due to impaired progression into and through S phase (Bjergbaek et al., 2004). A *rad24Δ top3Δ* double mutant, which does not exhibit these S phase defects or the slow growth exhibited by a *top3Δ* mutant, is fully competent in activating Rad53 upon exposure to HU (Bjergbaek et al., 2004). Flow cytometric analysis of *rmi1Δ* mutants failed to detect a significant delay in

progression into and through S phase, and I found that deletion of *RAD24* does not suppress the growth defect of an *rmi1Δ* mutant. Thus, the underlying mechanism by which the checkpoint is compromised in *rmi1Δ* may differ from that in *top3Δ*. Indeed, there are several aspects of the *rmi1Δ* phenotype that are different from that of *top3Δ*. *rmi1Δ* mutants grow slightly better than *top3Δ* mutants and there is a large difference in their GCR rates. These phenotypic differences are not surprising, given that loss of Top3 from the Rmi1/Sgs1/Top3 complex is likely to be biochemically distinct from loss of Rmi1.

RMI1 function in higher eukaryotes

Grant and Pavel have identified homologues of budding yeast Rmi1 in several yeast species, as well as mouse and human. The presence of three conserved regions in diverse species suggests that these regions may constitute functional domains. Human Rmi1 has similarity to nucleic acid binding OB-folds (Koonin et al., 2000) extending through conserved regions II and III, raising the possibility that Rmi1 might bind DNA directly. Of particular interest, the putative human Rmi1 homologue that Pavel identified by sequence similarity is identical to the recently described BLAP75 (Yin et al., 2005). Thus, the role of Rmi1 in RecQ/Top3 function appears to be conserved in all eukaryotes. Budding yeast Rmi1 is an important suppressor of DNA damage and genomic instability, and is also required for a robust checkpoint response to DNA damage and replication stress. It will be of great interest to determine if these functions are conserved in hRmi1/BLAP75, and if *hRMI1/BLAP75* polymorphisms are associated with human cancers.

6. GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 SUMMARY

DNA damage is a fairly common event and can lead to genomic instability if left unrepaired. My thesis has focused primarily on how cells cope with DNA damage during S phase, specifically replication fork stalling induced by DNA damage or replication stress. To fully understand how cells respond when faced with stalled forks, it is important that all the required components for this process are identified. To this end, I have taken functional genomic approaches to identify genes required for coping with DNA damage during S phase. In my screen for genes required for resistance to MMS-induced DNA damage, I identified several poorly characterized genes, including *ESC4* (also known as *RTT107*), that are necessary for proper S phase progression in the presence of DNA damage (Chang et al., 2002). Rtt107 contains four BRCT domains, which are found in many proteins involved in the DNA damage response or cell cycle regulation (Bork et al., 1997; Callebaut and Mornon, 1997). In agreement with my findings, Rtt107 has since been further characterized as a target of the checkpoint kinase Mec1 and is needed for the resumption of DNA replication after exposure to DNA damage (Rouse, 2004). *NCE4/RMI1* was also identified in my MMS screen (Chang et al., 2002), a gene which our group and others have found to be an important suppressor of genomic instability (Chang et al., 2005; Mullen et al., 2005). Using SGA to identify genes that are required for viability in the absence of *MUS81* and *MMS4*, I helped identify *ELG1*, deletion of which causes replication defects, genomic instability, and an inability to properly recover from DNA damage during S phase (Bellaoui et al., 2003). Lastly, I used two-dimensional hierarchical clustering of synthetic genetic interaction data determined by large-scale genetic network analysis in *S. cerevisiae* to identify *RMI1*, a novel component of the *SGS1/TOP3* pathway (Chang et al., 2005). Subsequent characterization of Rmi1 showed that it is a third

member of the Sgs1-Top3 complex (Chang et al., 2005). In the absence of *RMI1*, cells accumulate DNA damage and exhibit increased genomic instability (Chang et al., 2005). Thus, I have successfully utilized several functional genomic approaches to identify new genes important in the response to DNA damage and several of these genes are likely to have conserved roles in the DNA damage response. For example, the BRCT domains contained in Rtt107 are also found in many human DNA damage response proteins, and both Elg1 and Rmi1 have human homologues, which are candidate tumor suppressors. Taken together, my work demonstrates the utility of yeast functional genomics in identifying novel human genes that may function in the response to DNA damage and suppression of genomic instability.

6.2 FUNCTIONAL GENOMICS

My efforts have particularly emphasized the utility of the *S. cerevisiae* gene deletion collection. Indeed, the gene deletion collection has become the backbone of many functional genomic techniques. However, since haploid deletion mutants of essential genes are not viable, essential genes are conspicuously absent from studies using this collection. Thus, the approaches I have taken to identify novel DNA damage response genes can and should be applied to collections of conditional mutants of essential genes (Kanemaki et al., 2003; Mnaimneh et al., 2004). Screens for sensitivity to genotoxic agents can also be performed using the heterozygous deletion mutant collection (Deutschbauer et al., 2005; Giaever et al., 2004). Furthermore, it would be invaluable to have similar gene deletion collections in other organisms. However, generating such collections has not been feasible due to the high degree of difficulty in making gene deletions in most other organisms. The advent of RNA interference (RNAi) technology to inhibit gene expression, although not as effective as gene deletion, has circumvented this problem to some extent. Large-scale RNAi analysis has been employed in

Caenorhabditis elegans, *Drosophila melanogaster*, mouse cells, human cells, and *Arabidopsis* (Armknecht et al., 2005; Berns et al., 2004; Boutros et al., 2004; Helliwell and Waterhouse, 2003; Kamath et al., 2003; Paddison et al., 2004; Waterhouse and Helliwell, 2003). Therefore techniques such as SGA are, in principle, applicable to other model systems. Baugh *et al*. have done a small-scale, systematic synthetic lethal analysis in *C. elegans* by using existing mutants and RNAi to assemble a synthetic lethal matrix (Baugh et al., 2005). Living-cell microarrays have also been recently developed for the screening of RNAi-inducing double-stranded RNAs (dsRNAs) in *Drosophila* cells (Wheeler et al., 2004). In this technique, the dsRNA are printed onto a standard glass microarray slide and used to transfect cells added on top of the microarray. These microarrays can be used for cellular phenotyping and, by using multiple dsRNAs, genetic interaction screening (Wheeler et al., 2004). Although these techniques are still in their infancy, they hold tremendous promise in bringing the functional genomic approaches that have worked so well in yeast to metazoan systems.

6.3 REPLICATION DEFECTS IN *rmi1*Δ

Proteins required for the maintenance of genome integrity, including Sgs1 and Top3, often function during DNA replication. Thus, elucidating the molecular function of Rmi1 would be aided by examining the effect of the DNA damage generated in *rmi1Δ* mutants on DNA replication. This can be most directly observed by analyzing DNA replication intermediates by electron microscopy in an *rmi1Δ* mutant, and also in *sgs1Δ* and *top3Δ* mutants. This technique has been successfully used to show that *rad53* mutants exposed to HU exhibit extensive single-stranded gaps, hemi-replicated intermediates, and accumulation of Holliday junctions through fork reversal (Sogo et al., 2002). An alternative method to visualize replication intermediates is by using neutral-neutral two dimensional (2D) gel electrophoresis (Brewer and Fangman, 1987).

In this technique, DNA replication intermediates are run in 2D where the first dimension separates the DNA molecules proportional to their mass while the second dimension is run in conditions such that the mobility of non-linear molecules is drastically influenced by their shape. Considering *rmi1Δ* cells show evidence of DNA damage even in the absence of exposure to genotoxic agents, abnormal replication intermediates might be observed even without exposing *rmi1Δ* cells to HU or other genotoxic agents. However, using 2D gel electrophoresis, both *sgs1Δ* and *top3Δ* mutants show elevated levels of recombination-dependent X-structures only in the presence of MMS (Liberi et al., 2005). Although *rmi1Δ* mutants share many phenotypes with *sgs1Δ* and *top3Δ* mutants, *rmi1Δ* cells exhibit greatly elevated levels of GCR compared to *sgs1Δ* and *top3Δ* cells (Chang et al., 2005), indicating that loss of *RMI1* likely results in DNA damage that is different, and more damaging to genomic integrity, than loss of either *SGS1* or *TOP3*. This damage may be observable by electron microscopy or 2D gel electrophoretic analysis.

An alternative approach to assess the effect of the loss of *RMI1* on DNA replication is to use DNA combing, with which replication can be examined at the resolution of individual DNA molecules. DNA combing is a combination of dynamic molecular combing and fluorescent hybridization. In this technique, cells are released synchronously into S phase in the presence of bromodeoxyuridine (BrdU), a thymidine analogue that can be incorporated into DNA and visualized by a fluorochrome labeled antibody. Genomic DNA is stretched on a silanized cover slip, resulting in parallel, aligned DNA molecules on the surface of the cover slip. Sites of BrdU incorporation can be detected along individual DNA molecules with fluorescent antibodies. Since combed DNA molecules are stretched uniformly, DNA combing can be used to analyze many aspects of DNA replication including replication fork progression rate. Using this approach, Versini *et al*. showed that replication forks move faster in *sgs1Δ* mutants than in

wild type cells (Versini et al., 2003). If true for *top3Δ* and *rmi1Δ* mutants as well, this may imply that Sgs1-Top3-Rmi1 action at sites of stalled forks further delays replication fork progression. Indeed, replication pause sites have been found throughout the yeast genome (Cha and Kleckner, 2002; Deshpande and Newlon, 1996; Wang et al., 2001b). In a strain lacking *SGS1*, *TOP3*, or *RMI1*, replication forks may proceed rapidly through these pause sites at the expense of genomic integrity. If fork progression is not affected, or is slower, in *rmi1Δ* mutants compared to wild type, this would clearly indicate that the damage accumulated in these cells is different than that in *sgs1Δ* cells. The combination of the electron microscopy, 2D gel electrophoretic analysis, and DNA combing should help elucidate the nature of the DNA damage present in *rmi1Δ* cells, as compared to cells lacking *SGS1* or *TOP3*.

6.4 CHARACTERIZATION OF THE HUMAN Rmi1 HOMOLOGUE, *hRMI1/BLAP75*

My work on Rmi1 has shown that it functions with Sgs1 and Top3 to suppress genomic instability (Chang et al., 2005). Sequence homology searches identified a human homologue of Rmi1 which is identical to the recently described BLAP75 (Yin et al., 2005). hRmi1/BLAP75 was found to be an integral part of BLM complexes, and loss of hRmi1/BLAP75 resulted in reduced cell proliferation, destabilization of BLM complexes, and genomic instability as evidenced by elevated levels of SCEs (Yin et al., 2005). It will be of considerable interest to determine the effect of hRmi1/BLAP75 on BLM-associated activities.

In *E. coli*, RecQ can stimulate Top3 to catenate supercoiled DNA (Harmon et al., 1999). BLM is able to stimulate the ability of hTop3α to relax supercoiled DNA, but in these studies, no evidence of DNA catenation was detected (Wu et al., 2000). The bacterial and eukaryotic RecQ-Top3 complexes may have evolved to perform different functions, perhaps involving a different set of accessory proteins. Consistent with this, a bacterial homologue for Rmi1 has not

been identified (P. Morozov, personal communication). BLM can promote branch migration of HJs suggesting that the function of hTop3α may be to resolve HJs (Karow et al., 2000). However, hTop3α alone, or in combination with BLM, was unable to cleave a molecule containing one HJ, suggesting that the torsional constraints on the four arms of an HJ that would be present *in vivo*, but lacking in this *in vitro* substrate, are necessary for hTop3α action (Wu and Hickson, 2003). Thus, Wu and Hickson tested the activity of BLM-hTop3α on a substrate containing a double HJ, in which such torsional constraints are present, and found that together, BLM and hTop3α can resolve this substrate (Wu and Hickson, 2003). Double HJ structures exist *in vivo* and may be generated when both ends of a DSB invade a homologous sequence, as proposed in models of homologous recombination (Schwacha and Kleckner, 1995; Szostak et al., 1983). Since these experiments were performed *in vitro* using purified recombinant BLM and hTop3α, hRmi1/BLAP75 was not present in the reactions. Thus, it will be important to assess the role of hRmi1/BLAP75 on the activity of the BLM-hTop3α complex in these experiments. hRmi1/BLAP75 contains a putative nucleic acid binding OB-fold (Chang et al., 2005; Yin et al., 2005). Indeed, recent biochemical studies of yeast Rmi1 suggests that Rmi1 may be a structure-specific DNA binding protein with a preference for HJ structures (Mullen et al., 2005). Therefore hRmi1 may function to target BLM-Top3α to appropriate substrates, possibly by altering its substrate specificity. Rmi1 may also enhance or inhibit its activity, perhaps by modulating the interaction of BLM with hTop3α, or of the BLM-Top3α complex with other proteins. Our lab has purified hRmi1/BLAP75 to test these models.

The presence of a C-terminal extension in metazoan Rmi1, but lacking in yeast Rmi1, is intriguing as it may suggest a divergence or gain of function in metazoan Rmi1. The C-terminal extension could be the result of an ancestral gene fusing with *RMI1* in the course of evolution. Preliminary studies suggest that this may be the case, although all proteins identified which

exhibit homology to the C-terminal extension were either other Rmi1-like proteins, or were encoded by hypothetical, computationally-predicted genes of unknown function (P. Morozov, personal communication). Nevertheless, it will be of interest to perform mutational analysis on hRmi1/BLAP75 to determine the significance of the C-terminal extension.

Caretaker genes are tumor suppressor genes that, when mutated, result in genomic instability and predisposition to cancer (Kinzler and Vogelstein, 1997; Levitt and Hickson, 2002). This class of tumor suppressors includes a number of genes that are critical for genome integrity, including the RecQ helicase genes *BLM*, *WRN*, and *RTS*. *hRMI1/BLAP75* is an attractive candidate for a novel human caretaker gene as deletion of yeast *RMI1* (Chang et al., 2005; Mullen et al., 2005), as well as depletion of hRmi1/BLAP75 by siRNA (Yin et al., 2005) leads to significant genomic instability. *hRMI1/BLAP75* is located on chromosome 9q22.1, a region associated with frequent loss of heterozygosity in Chinese esophageal squamous cell carcinomas (Lichun et al., 2004). Thus, it will be of great interest to determine if loss-of-function mutations in *hRMI1/BLAP75* are associated with this or other human cancers. Furthermore, since yeast *rmi1Δ* mutants are readily suppressed by mutation of *SGS1* (Chang et al., 2005; Mullen et al., 2005), it is tempting to hypothesize that cases of Bloom, Werner, and Rothmund-Thomson syndromes may be the result of an initial mutation in *hRMI1/BLAP75*. It will be very interesting to determine if *hRMI1/BLAP75* is mutated in BS, WS, and RTS cells as it may provide insight into the molecular mechanisms behind the development of these diseases.

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