Brc1-mediated rescue of Smc5/6 deficiency; requirement for multiple nuclease and a novel Rad18 function

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Abstract

Smc5/6 is a Structural Maintenance of Chromosomes complex, related to the cohesin and condensin complexes. Recent studies implicate Smc5/6 as being essential for homologous recombination. Each gene is essential, but hypomorphic alleles are defective in the repair of a diverse array of lesions. A particular allele of smc6 (smc6-74) is suppressed by overexpression of Brc1, a six-BRCT domain protein that is required for DNA repair during S-phase. This suppression requires the post-replication repair protein Rhp18, and the structure-specific endonucleases Slx1/4 and Mus81/Eme1. However, we show here that the contribution of Rhp18 is via a novel pathway that is independent of PCNA ubiquitination and post-replication repair. Moreover, we identify Exo1 as an additional nuclease required for Brc1-mediated suppression of smc6-74, independent of mismatch repair. Further, the Apn2 endonuclease is required for the viability of smc6 mutants without extrinsic DNA damage, though this is not due to a defect in base excision repair. Several nucleotide excision repair genes are similarly shown to ensure viability of smc6 mutants. The requirement for excision factors for the viability of smc6 mutants is consistent with an inability to respond to spontaneous lesions by Smc5/6-dependent recombination.
Introduction

Eukaryotic cells contain three highly conserved multi-protein complexes that contain heterodimers of large ATPases known as the Structural Maintenance of Chromosomes (SMC) proteins (Harvey et al. 2002). These complexes are critical for chromosome integrity in interphase and for chromosome segregation at mitosis (Hirano 2006). The cohesin complex is essential for sister chromatid cohesion. In its absence, the lack of cohesion manifests as an inability to repair double stranded DNA breaks (DSBs) by homologous recombination (HR) in the G2 phase of the cell cycle (Birkenbihl and Subramani 1992; Nagao et al. 2004; Tatebayashi et al. 1998). In the absence of DSBs, the lack of cohesion manifests as chromosome segregation defects in mitosis (Hirano 2006). The condensin complexes, together with type II topoisomerases, are required for mitotic chromosome condensation. Without condensin, chromosomes are too disorganized to segregate into daughter cells at mitosis. However, genetic studies implicate condensin as also playing a role in DNA repair during G2 by an as yet undefined mechanism (Aono et al. 2002).

The function of these complexes in sister chromatid cohesion and chromosome condensation was readily inferred from yeast mutants and depletion experiments in Xenopus oocyte extracts. Unfortunately, this has not been the case for the third SMC complex, currently known as Smc5/6. This complex contains at least six stoichiometric subunits: Smc5 and 6, and four Non-Smc Elements, Nse1-4 (Hazbun et al. 2003; Sergeant et al. 2005). Genes for each of these subunits are essential, as is a sub-stoichiometric factor, Rad60 (Morishita et al. 2002). Two additional members of the
complex were recently identified, Nse5 and Nse6, which are not essential for viability though are required for DNA repair (PEBERNARD et al. 2006).

The Smc5/6 complex was initially defined by a hypomorphic allele of *smc6* in the fission yeast *Schizosaccharomyces pombe*. Initially known as *rad18-X*, and subsequently renamed *smc6-X*, this mutation resulted in DNA repair defects that by epistasis analysis were attributed to an HR defect (LEHMANN et al. 1995). An additional allele, *smc6-74*, was identified by virtue of DNA damage checkpoint maintenance defects (VERKADE et al. 1999). However, *smc6-74* cells are proficient in the initiation of a DNA damage checkpoint response as determined by activation of the Chk1 protein kinase (HARVEY et al. 2004), which is both necessary and sufficient to signal G2 arrest in response to DNA damage (DEN ELZEN and O’CONNELL 2004; LATIF et al. 2004). Like classical checkpoint mutants, irradiated *smc6-74* cells enter mitosis without completing the repair of DSBs, but unlike checkpoint mutants, an enforced delay to mitosis does not rescue their sensitivity to DNA damage. The recruitment of HR proteins to lesions is normal in *smc6-74* mutants, and a checkpoint response can be restored to these cells by deletion of HR genes (AMPATZIDOU et al. 2006; MIYABE et al. 2006; VERKADE et al. 1999). Together, these observations are consistent with the model that the *smc6-74* mutation defines a role for the Smc5/6 complex late in recombination, and the checkpoint machinery is unable to detect a defect in this to prevent mitotic entry with unresolved lesions still present in the chromosomes. Understanding this defect will be important both to define Smc5/6 function, and to understand how the checkpoint signal is terminated.

Brc1 is a six BRCT-domain protein that is required for the repair of a variety of lesions during S-phase, but not during G2 (SHEEDY et al. 2005). It was identified as an
allele-specific high-copy suppressor of smc6-74; modest overexpression of Brcl completely rescues the sensitivity of smc6-74 to DNA damage in both S- and G2-phase. *brcl* is not an essential gene, but it is essential for the viability of strains with compromised Smc5/6 function (Sheedy *et al.* 2005; Verkade *et al.* 1999). There is no evidence that Brcl physically associates with the Smc5/6 complex, and so this suppression is likely to be a functional bypass that enables cells to repair lesions that result from Smc5/6 dysfunction.

We have used this suppression of smc6-74 by Brcl as an assay to determine what factors are required for this response and as a means to define the nature of the repair and checkpoint maintenance defect in smc6-74 (Sheedy *et al.* 2005). Initially, we studied components of checkpoint control, HR, Nucleotide Excision Repair (NER) and Post-Replication Repair (PRR). In response to alkylation damage by high doses of Methyl Methanesulfonate (MMS), Brcl-mediated suppression of smc6-74 required PRR lesion bypass promoted by the Rad18 homolog Rhp18. This tolerance mechanism enables completion of replication and passage into G2 but not DNA repair. In response to lesions in G2 cells, Brcl-mediated suppression of smc6-74 was reliant upon the HR pathway, and two structure specific nucleases implicated in the recombination-dependent restart of stalled replication: Slx1/4 and Mus81/Eme1. Further, deletion of *slx1*, which by itself results in little phenotype, abolished the residual MMS resistance of smc6-74 cells. These data led to the model that DNA structures accumulate in smc6-74, which are not sensed by the checkpoint and hence cells enter mitosis without their repair. However, such lesions can, in a Brcl-dependent manner, be processed by the nucleases into alternative structures that both signal a checkpoint and can be repaired by HR even though Smc5/6
function is attenuated. Recently, Smc5/6 was shown to be essential for repair at collapsed replication forks (AMPATZIDOU et al. 2006). The combined roles for Smc5/6 in repair and checkpoint maintenance at these and other spontaneous lesions are a likely explanation as to why Smc5/6 genes are essential for viability, while HR genes are not.

These studies left open several unanswered questions. Under the assay conditions used, Rhp18 and lesion bypass were required for Brc1-mediated suppression of lesions in S-phase, which predicted that this response would act via mono-ubiquitination of Proliferating Cell Nuclear Antigen (PCNA) on lysine-164 (K164), though this had not been shown. Rhp18 is also required for the suppression of UV-C sensitivity during G2, where lesion bypass should be irrelevant. Further, Base Excision Repair (BER) and Mismatch Repair (MMR) needed to be considered as alternative mechanisms to reverse lesions in these assays. Finally, as Smc5/6 mutants are predicted to be defective in HR, they should have a heightened requirement for excision pathways to tolerate lesions, though we had only tested this with one gene involved in NER, the XP-G homolog rad13.

Here we address these issues. We show that the requirement for Rhp18 in this context defines a novel Rhp18 function that is independent of lesion bypass and PCNA ubiquitination. Moreover, we identify additional nucleases, Apn2 and Exo1, as being required for the repair of spontaneous and induced lesions in smc6-74 cells. Finally, we show that NER genes are indeed critical to the full viability of smc6-74, suggesting some redundancy for XP-G function in S. pombe. The requirement for several different nucleases suggests different types of lesions accumulate in smc6-74, and we speculate
that Brcl may facilitate the cleavage of these structures by the nucleases and subsequent processing into HR.

Materials and Methods

Fission yeast genetic methods

All strains used were derivatives of 972h− and 975h+. Standard procedures and media were used for propagation and genetic manipulation (MORENO et al. 1991). Methods for UV-C and MMS survival assays and transformation have been described previously (DEN ELZEN and O'CONNELL 2004; HARVEY et al. 2004; O'CONNELL et al. 1997; VERKADE et al. 1999; VERKADE et al. 2001). Table 2 shows a list of strains used in this study. Methods for Brcl-mediated suppression of smc6-74 were as described (SHEEDY et al. 2005). For these assays, expression from the attenuated (pRep41) promoter (referred to as pBrc1) under repressing conditions (5µM thiamine) was used, and compared to controls containing pRep41 only (referred to as vector). For suppression of MMS sensitivity, cultures were grown to 4 x 10^6 cells/ml, and 5µl of 10-fold serial dilutions were spotted into plates containing a range of MMS doses. For suppression of UV-C sensitivity, plates were inoculated in triplicate with 100, 1,000 or 10,000 cells, irradiated with 0 – 250J/m² of UV-C (254nm) using a Stratalinker (Stratagene), and colonies left to form for 4 days at 30°C. Data were normalized to unirradiated controls.

Construction of the RING domain mutant rhp18-1

Using a genomic clone of rhp18 (VERKADE et al. 2001), codons for Cysteine 44, Serine 45 and Histidine 46, at positions 3 and 4 of the cross-brace of the RING domain, were
mutated to Serine, Glycine and Alanine respectively using the method of Kunkel (Kunkel et al. 1987), and the oligonucleotide:

\[ 5'\text{-CGCGCCCCCTTTAATTACTTTTCGGAGCCACCTTTTGTTCGT} \]

(mutated residues are underlined). The mutated clone was then integrated back into the \textit{rhp18} locus by replacement of the \textit{ura4} marker in an \textit{rhp18::ura4} strain. Correct integration was confirmed by southern blotting, and strains were back-crossed prior to analysis.

\textit{Analysis of PCNA ubiquitination}

Ubiquitination of PCNA was detected by Western blotting using polyclonal anti-PCNA antisera as described (Frampton et al. 2006). Note that this antiserum detects a non-specific band that co-migrates with di-ubiquitinated PCNA. Extracts were prepared from untreated exponential cultures, or cells treated with 50J/m\(^2\) with a 30-minute recovery, or with 0.01% MMS for 3 hours.

\textit{Fluctuation rate analysis of mutation rate}

Rates of mutation in \textit{can1} were calculated using the method of the median as previously described (Fraser et al. 2003) from eleven independent cultures per treatment, with mutants selected on supplemented EMM2 medium containing 60\(\mu\)g/ml canavanine. UV-C induced mutagenesis was performed in cells irradiated with 100 J/m\(^2\). MMS-induced mutagenesis was performed on cells treated with 0.05% MMS for 60 minutes, with MMS then inactivated with 5% sodium thiosulfate; each condition was chosen to give \(\sim 50\%\) survival for \textit{rhp18} alleles (Sheedy et al. 2005).
Results

**Brc1 suppresses smc6-74 via a novel post-replication repair (PRR) pathway.**

The DNA damage sensitivity of the *S. pombe* smc6 hypomorphic allele *smc6-74* is efficiently suppressed by overexpression of Brc1. We have previously shown that this suppression was reliant on the Rad18 homolog, Rhp18, but was independent of Ubc13 (Sheedy et al. 2005). Based on current models of post-replication repair (Friedberg 2005; Prakash et al. 2004), these observations predicted that lesion bypass but not template switching was promoted by Brc1, and thus should be dependent on mono-ubiquitination of PCNA by Rhp18 and its E2 partner, Rhp6. *S. pombe* strains deleted for *rhp6* show very poor viability and are infertile (Reynolds et al. 1990). However, we have previously reported an allele of *rhp18*, *rhp18-40*, which encodes a truncated protein that lacks the Rhp6-binding domain (Verkade et al. 2001). We therefore used this allele to test the hypothesis that Brc1-mediated suppression of *smc6-74* was via Rhp18/Rhp6-catalyzed ubiquitination.

Whilst *rhp18Δ smc6-74* strains showed no rescue of MMS or UV-C sensitivity by Brc1 overexpression (Fig 1A, C; (Sheedy et al. 2005)), *rhp18-40 smc6-74* were indeed proficient for suppression at lower concentrations of MMS (0.0025%, Fig 1A). At higher concentrations, both strains failed to be rescued by Brc1. Considerable suppression of the UV-C sensitivity of *rhp18-40 smc6-74* was also seen upon overexpression of Brc1 (Fig 1D). These data suggested that the lack of suppression in *rhp18Δ smc6-74* strains was due to an Rhp6-independent function for Rhp18.
It was formally possible that, in these assays, Rhp18 was still acting as an E3 ubiquitin ligase, though utilizing another E2 enzyme. To test this hypothesis, we mutated conserved cysteines residues within the Rhp18 RING domain. This allele, \textit{rhp18-1}, showed a similar level of sensitivity to DNA damaging agents as \textit{rhp18}\textDelta and \textit{rhp18-40}. When combined with \textit{smc6-74}, \textit{rhp18-1} behaved essentially the same as \textit{rhp18-40} (Fig 1A, E). Each allele of \textit{rhp18} increased the MMS and UV-C sensitivity of \textit{smc6-74} and the MMS sensitivity of \textit{brc1}\textDelta (Fig 1 A-E, and data not shown). We conclude that Rhp18 has an E3-independent function, and that this function is required for Brc1 to suppress \textit{smc6-74}.

Lysine 164 (K164) on PCNA is the best-characterized site for Rhp18-catalyzed ubiquitination (LEHMANN 2006; MATUNIS 2002; WATTS 2006). We considered that in the \textit{rhp18-1} and -40 strains, another ubiquitin ligase might compensate for Rhp18. To test this, we assayed Brc1-mediated suppression in a background where K164 of PCNA had been mutated to arginine (\textit{pcn1-K164R}). Whilst this mutation significantly enhanced the MMS and UV-C sensitivity of \textit{smc6-74}, this was readily suppressed by Brc1 overexpression for UV-C and at lower (<0.005%), but not higher (≥0.005%), MMS concentrations (Fig 2A, B). Further, we confirmed that each allele of \textit{rhp18} was defective in PCNA ubiquitination (Fig 2C), and thus PCNA ubiquitination is not required for Brc1-mediated suppression of \textit{smc6-74}.

We had previously shown that translesion synthesis polymerases were required for Brc1 to suppress the MMS (but not UV-C) sensitivity of \textit{smc6-74}, but this was only observed at relatively high doses (0.005-0.01%) and also required the triple deletion of polymerases η, κ and ζ (SHEEDY et al. 2005). We therefore re-tested suppression at lower
levels (0.001-0.002%) of MMS, and indeed found that the triple polymerase deletion ($3TLS\Delta$) was unaffected for suppression of $smc6$-$74$, though the quadruple mutant had significantly enhanced MMS sensitivity (Fig 2A). Such enhancement of MMS sensitivity was also observed when the $3TLS\Delta$ mutants (Sheedy et al. 2005) or $pcn1-K164R$ (Supplementary Material) were combined with $brc1\Delta$.

Lesion bypass is error prone and leads to mutagenic DNA replication. We confirmed that both $rhp18$-$1$ and $rhp18$-$40$ are indeed defective in MMS and UV-C-induced mutagenesis that is a byproduct of lesion bypass. In fluctuation tests for canavanine resistance caused by mutations in $can1$, wildtype cells showed a 7.7-fold induction of mutation rate by MMS treatment ($6.7\times10^{-6}$) and a 18.4-fold increase by UV-C irradiation ($1.6\times10^{-5}$) compared to untreated cells ($8.7\times10^{-7}$). $rhp18$-$1$ and $rhp18$-$40$ showed $\sim$4-fold higher rates of spontaneous $can1$ mutations ($3.7\times10^{-6}$ and $3.4\times10^{-6}$ respectively). This is consistent with data for $S. cerevisiae$ rad18$\Delta$, which shows a 4.6-fold increase (Stelter and Ulrich 2003). MMS treatment increased this only 2.2-fold for $rhp18$-$1$ ($8.3\times10^{-6}$) and 1.5-fold for $rhp18$-$40$ ($5.2\times10^{-6}$). Similarly, UV-C irradiation increased $can1$ mutation rates only 1.9-fold for $rhp18$-$1$ ($7.2\times10^{-6}$) and 2.9-fold for $rhp18$-$40$ ($1.0\times10^{-5}$).

Combined, these data show that under high levels of alkylation damage, Rhp18-mediated lesion bypass via PCNA and the translesion synthesis polymerases are required for Brc1 to suppress lethality in $smc6$-$74$ cells, and for MMS resistance in $brc1\Delta$ and $smc6$-$74$ cells. This tolerance mechanism allows for completion of DNA replication and for subsequent repair. Our previous findings strongly implicate that HR is ultimately required for DNA repair in this context (Sheedy et al. 2005).
At lower doses of MMS, lesion bypass and PCNA ubiquitination are not required for Brc1 to suppress *smc6-74*. This pathway of repair is independent of the Rhp6-binding and RING domains of Rhp18 and thus defines a novel function for Rhp18 in *smc6-74* cells. We note that the *pcn1-K164R* mutation confers greater MMS sensitivity than does the 3TLSΔ strain, and that *pcn1-K164R* cells are sensitive to UV-C irradiation in G2, whereas 3TLSΔ cells are not (FRAMPTON et al. 2006). We also note that the requirement for Rhp18 extends to the repair of lesions in cells irradiated in G2 (VERKADE et al. 2001). Therefore, this cryptic function for Rhp18 uncovered by the Brc1-mediated suppression of *smc6-74* is unlikely to be related to its tolerance function to promote the bypass lesions during DNA replication.

**AP endonuclease Apn2 is critical for survival of *smc6-74***

BER is a predominant pathway in the response to DNA alkylation in most eukaryotes (MARTI et al. 2002). In *S. pombe*, however, cells lacking the *mag1*-encoded DNA glycosylase that initiates the BER response to DNA alkylation show wildtype sensitivity to alkylation agents such as MMS, and genetic data implicates both NER and HR as options to repair alkylation damage (ALSETH et al. 2004; ALSETH et al. 2005; MEMISOGLU and SAMSON 2000; SUGIMOTO et al. 2005). With the HR defects in *smc6-74* mutants, we decided to investigate the contribution of BER to both the residual MMS resistance of *smc6-74* and to the suppression of MMS sensitivity by Brc1 overexpression.

Currently there are four genes known to function in BER in *S. pombe*: *mag1*, *nth1*, *apn2* and *rad2* (Fen1 homolog), although *rad2* is also required for other repair pathways (LIEBER 1997). Mag1 converts alkylated bases to abasic (AP) sites, which can
then be repaired by either short- or long-patch BER. Short-patch BER involves cleavage 3’ to the AP site by the AP lyase, Nth1. This lesion is further processed by a 5’ cleavage by the AP endonuclease Apn2, and the resulting gap filled by replication. In long-patch BER, Apn2 cleaves 5’ to the AP site, and the Rad2 flap endonuclease cleaves further 3’ to leave a longer gap that is also filled by replication. HR and NER can also repair alkylated bases not processed by Mag1, or intermediates in the Mag1 pathway. Deletion of mag1 reduces the MMS sensitivity of HR mutants as cells are unable to commit to BER and therefore are not reliant on HR to repair cleavage products (Alseth et al. 2005).

We constructed double mutants between smc6-74 and mag1Δ, nth1Δ and apn2Δ. rad2Δ has previously been shown to be epistatic with smc6-74 for MMS sensitivity. Although mag1Δ rescues the MMS sensitivity of HR mutants, mag1Δ smc6-74 strains were slightly more sensitive to MMS, though this was efficiently rescued by Brc1 overexpression (Fig 3). nth1Δ smc6-74 mutants were significantly more sensitive to MMS than either parent, though in this context nth1 was not required for Brc1-mediated suppression of smc6-74. However, apn2Δ smc6-74 mutants formed micro-colonies of highly aberrant cells that could not be propagated. An identical phenotype was observed in apn2Δ smc6-X double mutants (data not shown), and so was not allele specific. This synthetic lethality could be due to two possible scenarios. Firstly, Apn2 may have a function outside of the BER pathway. Secondly, Mag1-dependent commitment to Apn2-dependent long-patch BER in smc6-74 may need to be completed by replication rather than by shunting of cleavage products into an HR- or NER-dependent pathway. However, as Rad2 is required for long-patch BER and rad2Δ smc6-74 cells are fully viable, the
former is the most likely explanation. In keeping with this, from 30 dissected tetrads no viable mag1Δ apn2Δ smc6-74 colonies were obtained in a cross of mag1Δ smc6-74 x apn2Δ (data not shown).

We also investigated the relationship between brc1Δ and null alleles of the BER genes (Fig 4). As seen with HR mutants, mag1Δ brc1Δ cells were less sensitive to MMS than the brc1Δ parent (mag1Δ is not MMS sensitive). Conversely, apn2Δ, nth1Δ and rad2Δ increased the MMS sensitivity of brc1Δ. These data suggest that BER is important for the residual MMS resistance in brc1Δ cells, most likely due to a defect in recombination, as brc1Δ is epistatic to rhp51Δ (Sheedy et al. 2005).

**Exo1 is required for Brc1-mediated suppression of smc6-74**

MMR is another excision-based pathway that is essential in genome integrity to remove mismatched nucleotides that arise from replication errors or chemical modification (Marti et al. 2002). In *E. coli*, MutS recognizes the mismatch, and then together with MutL activates the MutH endonuclease. *S. pombe*, like many other eukaryotes, contains multiple MutS and MutL homologs, but lacks a gene with homology to MutH. The 5’-3’ exonuclease and endonuclease Exo1 is thought to substitute for MutH in MMR (Tran et al. 2004). We constructed double mutants between smc6-74 and null alleles for the major MutS (*msh2*) and MutL (*pms1*) homologs. Neither *msh2*Δ nor *pms1*Δ strains were significantly sensitive to MMS, nor did they enhance the MMS sensitivity of *smc6-74* (Table 1). Moreover, neither gene was required for Brc1-mediated suppression of *smc6-74*, nor enhanced the MMS sensitivity of brc1Δ (Supplementary Material). These data
suggest that mismatch repair is not required when Smc5/6 function is compromised or when Brc1 is absent.

In addition to its role in mismatch repair Exo1 is required for processing of DSBs in *S. pombe* (Tomita et al. 2003) and in *S. cerevisiae* Exo1 has been shown to have an overlapping function with the MRN complex for resection of a DSB for HR (Llorente and Symington 2004; Tsubouchi and Ogawa 2000). It has also been shown to process stalled replication forks (Cotta-Ramusino et al. 2005). Deletion of *exo1* results in only a minor increase in MMS sensitivity, but when combined with *smc6-74* resulted in a substantial enhancement of MMS sensitivity (Fig 5A). Importantly, this was not rescued by Brc1 overexpression on plates with >0002% MMS. Similarly, *exo1Δ* significantly enhanced the MMS sensitivity of *brc1Δ* cells (Supplementary Material). Together these data suggest Brc1 and Exo1 have overlapping and non-overlapping roles.

We also investigated whether Exo1 was required for Brc1 to suppress the UV-C sensitivity of *smc6-74* (Fig 5B). *exo1Δ* cells were only modestly sensitive to high doses of UV-C, but when *exo1Δ* was combined with *smc6-74*, the double mutant had an enhanced UV-C sensitivity compared to that of the *smc6-74* parent. However, the double mutant was not rescued by Brc1 overexpression. Thus, Exo1 is another nuclease that becomes essential for the repair of lesions when Smc5/6 function is compromised.

**Mutations in Nucleotide Excision Repair (NER) genes cause synthetic growth defects with *smc6-74***

Previous studies have investigated the relationship between Smc6, Brc1 and only one factor in NER, the *rad13*-encoded XPG nuclease (Lehmann et al. 1995; Sheedy et al.
2005). XPG nucleases remove the flap generated by the NER-dependent synthesis at sites of UV-induced lesions such as Cyclobutane dimers and 4-6 photoproducts. \textit{rad13Δ} enhances the DNA damage sensitivity of \textit{smc6-74}, though is not required for Brc1 to suppress this. As members of other excision pathways are required for either Brc1-mediated suppression of \textit{smc6-74} or indeed viability of this mutant, we considered whether there may be some redundancy for Rad13 function. We constructed double mutants between \textit{smc6-74} and null alleles for the NER genes \textit{swi10} (ERCC1/XPF/RAD10 homolog (\textit{HANG} \textit{et al.} 1996)), \textit{rhp41} (XPC/RAD4 (\textit{MARTI} \textit{et al.} 2003)) and \textit{rhp14} (XPA/RAD14 (\textit{HOHL} \textit{et al.} 2001)), involved in 5’ incision (\textit{swi10}) and lesion recognition (\textit{rhp41}, \textit{rhp14}). In each case, the growth of doubles was inhibited, particularly with \textit{swi10Δ smc6-74} and \textit{rhp41Δ smc6-74} strains. In each case, spontaneous suppressors of this slow growth arose, which made interpretation of \textit{smc6-74} epistasis studies and Brc1-mediated suppression unfeasible. Nevertheless, these observations do indeed show that NER becomes more important for processing of spontaneous lesion when Smc5/6 function is compromised, most likely as recombinational repair is significantly attenuated in this circumstance. Not surprisingly therefore, these mutations also enhanced the MMS sensitivity of \textit{brc1Δ} (Table 1 and Supplementary Material).

**Discussion**

The Smc5/6 complex is a key determinant of genome integrity. Since it is structurally related to cohesin and condensin, it is reasonable to hypothesize that the complex plays a fundamental role in chromosome organization, and the dysfunctional response to DNA damage is a consequence of this. Brc1 serves to activate a pathway that can bypass
defects in Smc5/6 mutants, and is required for viability when Smc5/6 function is compromised. The experiments described in this manuscript extend our previously published studies and provide four key findings. Firstly, Rhp18 has a function that is independent of Rhp6, its RING domain (and therefore E3 ligase activity), and of PCNA ubiquitination and lesion bypass. This can be seen by the requirement for Rhp18 in order for Brc1 to suppress the sensitivity of smc6-74 to lower doses of alkylation damage and to UV-C irradiation in G2. Secondly, the AP endonuclease Apn2 is critical for the survival of smc6 mutants, and this is independent of its role in BER. Exo1 is another nuclease required for Brc1 to suppress the MMS and UV-C sensitivity of smc6-74, although the exo1 null mutant itself is only slightly sensitive to these agents. Finally, Swi10 (XPF/RAD10) is yet another nuclease required for the viability of smc6-74, as are other components of the NER pathway.

Homologs of Rad18, such as S. pombe rhp18, are involved in the tolerance of lesions that impede DNA replication. To this end, Rad18 proteins mono-ubiquitinate PCNA, and this promotes the recruitment of the translesion polymerases to replicate past the lesion, sometimes in an error prone manner (FRIEDBERG 2005). However, S. pombe rhp18Δ strains synchronized at various points of the cell cycle are sensitive to DNA damaging agents (VERKADE et al. 2001), suggesting roles for Rhp18 outside the realm of DNA replication. This observation was corroborated by the finding that, while Rhp18 was required for Brc1 to suppress the UV-C sensitivity of G2 smc6-74 cells, the translesion polymerases were not. Here, we have shown that the bypass synthesis independent function for Rhp18 is not related to its E3 ubiquitin ligase activity. Recent studies have implicated Rad18 in HR in avian DT40 cells (SZUTS et al. 2006), and as HR
is required for Brc1 to suppress \textit{smc6-74} (\textsc{sheedy} \textit{et al.} 2005), we speculate that this may be relevant function for Rhp18 in our experiments. Thus, the current models of DNA damage tolerance are perhaps incomplete. We also note that the \textit{pcn1-K164R} mutation confers greater sensitivity to UV-C and MMS than the \textit{3TLS\Delta} strain. Thus, either there are additional bypass polymerases in \textit{S. pombe}, or modification of K164 on PCNA promotes additional repair or tolerance functions. Further, it is pertinent to note that the \textit{pcn1-K164R} sensitivity to UV-C irradiation was assayed in G2 cultures, and it was recently shown that PCNA is mono-ubiquitinated in \textit{S. pombe} in G2, even following ionizing radiation (\textsc{frampton} \textit{et al.} 2006). Thus, the ubiquitination of PCNA in G2 may define a function that is not related to lesion bypass, further highlighting the need to refine models for Rad18 function.

Deletion of \textit{apn2}, which encodes the major AP endonuclease in \textit{S. pombe}, is synthetic lethal with \textit{smc6-74} and \textit{smc6-X}. In principle, this effect could be via the AP endonuclease activity of Apn2, though it is not clear as to why \textit{smc6} mutants should accumulate more AP sites than wildtype cells, nor did we see such an effect with other BER mutants. Apn2 is a member of the exonuclease III family, which display the additional activities of a 3’-5’ exonuclease, 3’-phosphodiesterase, and 3’-phosphatase (\textsc{boiteux} and \textsc{guillet} 2004; \textsc{unk} \textit{et al.} 2001). Therefore, another possible function for Apn2 in this context is the processing of 3’-blocked ends to enable repair synthesis. In budding yeast, the Rad1-Rad10 nuclease (also known as ERCC1-XPF) has also been shown to process 3’-blocked ends (\textsc{guzder} \textit{et al.} 2004), as has the Mus81/Mms4 3’-flap endonuclease (\textsc{boiteux} and \textsc{guillet} 2004). The \textit{S. pombe} homologs, Rad16-Swi10, and Mus81/Eme1 also show strong genetic interactions with \textit{smc6} alleles; \textit{swi10\Delta smc6-74}
double mutants showed a strong synthetic growth defect (Fig 3), and mus81Δ smc6-74 double mutants are poorly rescued by Brc1 overexpression (Sheedy et al. 2005). These data suggest that 3’-blocked ends may be one structure that accumulates in smc6 mutants during incomplete HR, and given that brc1 becomes essential in smc6 mutants, increasing Brc1 levels may aid in the processing of blocked ends to enable repair.

The other nuclease identified here as being required for Brc1 to suppress smc6-74 is Exo1. Exo1 possesses 5’-3’ exonuclease activity, though it is also a 5’-flap endonuclease that is related to the structure-specific nucleases Fen1 and XP-G (Tran et al. 2004), encoded by rad2 and rad13 in S. pombe. Another 5’-flap endonuclease, Slx1/4, showed interactions with smc6 that are very reminiscent of Exo1; slx1Δ cells show wildtype sensitivity to MMS but greatly sensitize smc6-74 to MMS and are required for Brc1-mediated suppression (Sheedy et al. 2005). Similarly for UV-C, both exo1Δ and slx1Δ show little sensitivity themselves and yet enhance the sensitivity of smc6-74, and again are required for Brc1 to rescue smc6-74. These enzymes have the capacity to remove 5’-blocked ends, which may be rare in wildtype cells, explaining why these strains are not MMS sensitive, but become more abundant when Smc5/6 function is compromised. Rad2, and perhaps Rad13, could also carry out this function, though Rad13 is not required for Brc1 to suppress smc6-74, and rad2 mutants are epistatic with smc6.

An alternative explanation as to why these nucleases are required for Brc1 to suppress smc6-74 would be the requirement to excise 5’- and 3’-flaps. Epistasis analysis predicts that mutants of the Smc5/6 complex are defective in Rad51-dependent HR (De Piccoli et al. 2006; Lehmann et al. 1995) so flaps could arise from Rad51-independent
recombination, such as single-stranded annealing (SSA). The nature of the flap would be dependent on the polarity of the exonuclease activity that processes a particular DSB. However, the suppression of smc6-74 by Brc1 requires the recombination mediator complexes consisting of Rhp55/Rhp57 (homologs of Rad55/57) and Swi5/Sfr1 (Sheedy et al. 2005) and these proteins are not required for SSA. Thus, the majority of the lesions arising from HR defects in smc6-74 cells are likely to be processed back into Rhp51-dependent HR substrates by Brc1 and the nucleases. A direct requirement for Rhp51 in Brc1-mediated suppression of smc6-74 cannot be assayed due to epistatic nature of smc6-74 and rhp51Δ.

We have no evidence that Brc1 directly modifies the activities of the nucleases, or that it modifies the novel function for Rhp18. Indeed, where applicable, double mutants between these genes and brc1Δ are significantly more sensitive to DNA damage. Further, Brc1 appears to only be required for DNA damage responses during S-phase in wildtype cells, though clearly can rescue smc6-74 when DNA damage is inflicted during G2. It is possible, therefore, that Brc1 somehow stabilizes recombination intermediates in smc6 mutants to allow them to be modified by these nucleases, or perhaps aids in their recruitment to the sites of lesions.

The budding yeast homolog of Brc1, known as Esc4/RTT107, is similarly required for resistance to MMS (Rouse 2004), but its relationship to Smc5/6 is currently not known. The closest human homolog, PTIP, may function in DNA damage responses (Jowsey et al. 2004), but its expression in S. pombe is not able to rescue brc1Δ or smc6-74 (our unpublished data). However, members of the Smc5/6 complex are highly conserved, as are homologs of Rad18 and the nucleases discussed in this paper, and so it
is likely that the DNA damage response that we have uncovered in these studies is conserved. Molecular function(s) for the Smc5/6 are only beginning to emerge, and understanding how Brc1 can bypass defects in the function of this complex will be an important tool to decipher what appears to be a very complex series of events.

Acknowledgments

We thank Felicity Watts, Antony Carr, Jurg Kohli, Oliver Fleck and Magnar Bjoras for \textit{S. pombe} strains, Felicity Watts for the anti-PCNA antibody and Edgar Hartsuiker for advice with fluctuation tests. This work was supported by the National Cancer Institute/National Institutes of Health CA100076 (M. O'C), Cancer Research UK and the BBSRC (JM). K.M.L. is supported by National Institutes of Health/National Cancer Institute training grant T32 CA78207.

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Table 1. Interactions of *smc6* and *brc1* with excision repair genes.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene</th>
<th>Sc+ homolog</th>
<th><em>smc6-74</em></th>
<th><em>brc1Δ</em></th>
<th>Brc1 supp of <em>smc6-74</em></th>
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<tbody>
<tr>
<td>BER</td>
<td><em>mag1</em></td>
<td>MAG1</td>
<td>not epistatic&lt;sup&gt;1&lt;/sup&gt;</td>
<td>partial rescue&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>nth1</em></td>
<td>NTH1</td>
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<td>not epistatic&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>apn2</em></td>
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<td>SL&lt;sup&gt;1&lt;/sup&gt;</td>
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<tr>
<td></td>
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<td>RAD27</td>
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<td>not epistatic&lt;sup&gt;5&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>MSH2</td>
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<td>NA&lt;sup&gt;6&lt;/sup&gt;</td>
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</tr>
<tr>
<td></td>
<td><em>pms1</em></td>
<td>PMS1</td>
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<td>NA&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
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<td></td>
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<td>not epistatic&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Yes&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*S. cerevisiae*; *Phenotype in combination with *smc6-74* (for MMS and UV-C) or *brc1Δ* (for MMS). SL: synthetic lethal; SGD: synthetic growth defect. ND: not determined. NA: not applicable.</sup>

<sup>1Figure 3; 2(Lehmann et al. 1995); 3Figure 5; 4Figure 6; 5Figure 4; 6Supplementary Figure; 7(Sheedy et al. 2005)*/
Table 2. Strains used in this study. Genotypes of parental strains are shown. Strains with different auxotrophic markers and/or containing plasmids were derived from these.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>leu1</em>-32, <em>ura4-D18</em>, <em>ade6</em>-704 h-</td>
<td>(O'CONNELL et al. 1997)</td>
</tr>
<tr>
<td><em>smc6</em>-74 <em>leu1</em>-32, <em>ura4-D18</em>, <em>ade6</em>-704 h-</td>
<td>(VERKADE et al. 1999)</td>
</tr>
<tr>
<td><em>rhp18::ura4</em> <em>leu1</em>-32, <em>ura4-D18</em>, <em>ade6</em>-704 h-</td>
<td>(VERKADE et al. 2001)</td>
</tr>
<tr>
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<td>(VERKADE et al. 2001)</td>
</tr>
<tr>
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<td>this study</td>
</tr>
<tr>
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<td>(SHEEDY et al. 2005)</td>
</tr>
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<td><em>smc6</em>-74 <em>rhp18</em>-40 <em>leu1</em>-32, <em>ura4-D18</em>, <em>ade6</em>-704 h-</td>
<td>this study</td>
</tr>
<tr>
<td><em>smc6</em>-74 <em>rhp18</em>-1 <em>leu1</em>-32, <em>ura4-D18</em>, <em>ade6</em>-704 h-</td>
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<tr>
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<td>(FRAMPTON et al. 2006)</td>
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<td><em>eso1::kanMx6</em> <em>rev3::hphMx6</em> <em>dinB::bleMx6</em> <em>ura4-D18</em> <em>leu1</em>-32 <em>ade6</em>-</td>
<td>(SHEEDY et al. 2005)</td>
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<tr>
<td><em>smc6</em>-74 <em>eso1::kanMx6</em> <em>rev3::hphMx6</em> <em>dinB::bleMx6</em> <em>ura4-D18</em> <em>leu1</em>-32 <em>ade6</em>-</td>
<td>(SHEEDY et al. 2005)</td>
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<td><em>mag1::ura4</em> <em>ura4-D18</em> <em>leu1</em>-32 <em>ade6</em>-704 h+</td>
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</tr>
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<td>(RUDOLPH et al. 1998)</td>
</tr>
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</tr>
</tbody>
</table>
Legends to the figures

Figure 1. Brc1-mediated suppression of smc6-74 is independent of Rhp6-binding and the RING domain of Rhp18. (A) The indicated strains were transformed with vector (V) or pBrc1 (B), grown to $4 \times 10^6$ cells/ml, and 5µl of 10-fold serial dilutions were assayed at a range of MMS doses. Shown are control (0% MMS), 0.0025% MMS and 0.005% MMS. (B) Plates with lower concentrations of MMS are shown for rhp18Δ strains. Plates were photographed after 4 days at 30°C. (C) Epistasis analysis of MMS sensitivity between brc1Δ and alleles of rhp18. Refer to panel (A) for sensitivity of rhp18 alleles. UV-C survival data is normalized to unirradiated controls for rhp18-40 (D) and rhp18-1 (E). Open triangles move to the level of survival of open circles with suppression.

Figure 2. PCNA modification on K164 is not required for Brc1-mediated suppression of smc6-74. (A) Strains containing a lysine to arginine mutation at residue 164 of PCNA (pcn1-K164R) or lacking polymerases η, ζ, and κ (encoded by eso1, rev3 and dinB, 3TLSΔ) alone, or in combination with smc6-74 containing either empty vector or pBrc1 were assayed for MMS sensitivity as described in figure 1. In each case a range of MMS concentrations was assayed. Control (0% MMS), 0.002% MMS and 0.005% MMS are shown. Double mutants with smc6-74 were suppressed on 0.002% MMS. (B) UV-C survival data for the indicated strains show effective suppression of pcn1-K164R smc6-74 by pBrc1. (C) Ubiquitination of PCNA on K164 was assayed by Western-blotting with polyclonal anti-PCNA antibodies. Ubiquitination of PCNA is enhanced by treatment with
MMS or UV-C, but is absent in the *rhp18* mutants. Note that a non-specific band of ~47kD co-migrates with di-ubiquinted PCNA.

**Figure 3.** Synthetic lethality of *smc6-74* with *apn2Δ* but not other BER mutants.

*mag1* (A) and *nth1* (B) are not required for Brc1-mediated suppression of *smc6-74* for MMS sensitivity. In each case a range of MMS concentrations was assayed as described in figure 1. (C) Severe synthetic growth defect of *apn2Δ smc6-74*. Tetrads were dissected and grown on YES for 4 days at 30°C; squares indicate double mutants.

**Figure 4.** Epistasis of the MMS sensitivity of *brc1Δ* with BER mutants. Plate assays of MMS sensitivity of *brc1Δ* combined with: A. *mag1Δ* and *nth1Δ*; B. *apn2Δ* and *rad2Δ*. Control (0% MMS) and a range of MMS concentrations are shown. *mag1Δ brc1Δ* (A) shows reduced sensitivity compared to the *brc1Δ* parent. *nth1Δ brc1Δ* (A), *apn2Δ brc1Δ* and *apn2Δ brc1Δ* (B) synergize for MMS sensitivity, but the double mutants grow as wildtype cells on the control plate.

**Figure 5.** Exo1 nuclease is required for Brc1-mediated suppression of the MMS sensitivity of *smc6-74*. Survival assays carried out as described in figure 1. Note no suppression and enhanced sensitivity of the *exo1Δ smc6-74* double mutant for concentrations of MMS >0.002% (A), and no suppression of the UV-C sensitivity of the *exo1Δ smc6-74* double mutant (B).
Figure 6. Synthetic growth defects of *smc6-74* with the NER mutants *swi10Δ, rhp41Δ* and *rhp14Δ*. Tetrads were dissected and grown on YES for 4 days at 30°C; squares indicate double mutants.
A. Control  0.0025% MMS  0.005% MMS

- wildtype
- smc6-74
- rhp18-1
- rhp18-40

B. 0.001% MMS  0.0015% MMS

- rhp18Δ
- smc6-74
- rhp18-1

C. Control  0.0025% MMS

- brc1Δ
- brc1Δ rhp18-1
- brc1Δ rhp18-40
- brc1Δ rhp18Δ

D. % Survival vs UV-C (J/m²)

- wildtype
- rhp18-40
- smc6-74
- rhp18-1

E. % Survival vs UV-C (J/m²)

- wildtype
- rhp18-1
- smc6-74

Lee et al, Fig 1
A. Control 0.002% MMS 0.005% MMS

B. % Survival

C. wildtype pcn1-K164R rhp18Δ rhp18-1 rhp18-40

Lee et al, Fig 2
A. \( \text{mag1}\Delta \)

<table>
<thead>
<tr>
<th>Control</th>
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<th>0.005% MMS</th>
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<tbody>
<tr>
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<td>( B)</td>
<td>( V)</td>
</tr>
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</table>

B. \( \text{nth1}\Delta \)

<table>
<thead>
<tr>
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<th>0.0025% MMS</th>
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</thead>
<tbody>
<tr>
<td>( V)</td>
<td>( B)</td>
<td>( V)</td>
</tr>
</tbody>
</table>

C. \( \text{smc6-74} \times \text{apn2}\Delta \)

Lee et al, Fig 3
A. Control  0.0075% MMS  0.01% MMS
wildtype  brc1Δ  mag1Δ  nth1Δ  mag1Δ brc1Δ  nth1Δ brc1Δ

B. Control  0.0025% MMS  0.0075% MMS
wildtype  brc1Δ  apn2Δ  rad2Δ  apn2Δ brc1Δ  rad2Δ brc1Δ

Lee et al, Fig 4
Legends to the Supplementary Figures.

A. Epistasis analysis of MMS sensitivity of exo1Δ and brc1Δ.

B. msh2 is not required for Brc1-mediated suppression of smc6-74, and does not enhance the MMS sensitivity of smc6-74.

C. pms1 is not required for Brc1-mediated suppression of smc6-74, and does not enhance the MMS sensitivity of smc6-74.

D. msh2Δ and pms1Δ do not enhance the MMS sensitivity of brc1Δ.

E. pcn1-K164R significantly enhances the MMS sensitivity of brc1Δ, though pcn1-K164R itself is not sensitive to MMS.

F. NER mutants swi10Δ and rhp14Δ significantly enhance the MMS sensitivity of brc1Δ, though by themselves are not sensitive to MMS.
A. Control 0.001% MMS 0.0025% MMS 0.005% MMS
   Wildtype
   brc1Δ
   exo1Δ
   brc1Δ exo1Δ

B. Control 0.005% MMS
   V msh2Δ
   V smc6-74
   V msh2Δ
   V smc6-74

C. Control 0.005% MMS
   V pms1Δ
   V smc6-74
   V pms1Δ
   V smc6-74

D. Control 0.005% MMS 0.0075% MMS
   Wildtype
   msh2Δ
   brc1Δ
   brc1Δ msh2Δ
   Wildtype
   brc1Δ
   pms1Δ
   brc1Δ pms1Δ

E. Control 0.0025% MMS
   Wildtype
   brc1Δ
   pcn1-K164R
   brc1Δ pcn1-K164R

F. Control 0.0025% MMS 0.005% MMS
   Wildtype
   brc1Δ
   swi10Δ
   brc1Δ swi10Δ
   rhp14Δ
   brc1Δ rhp14Δ

Lee et al, Supplementary Figure