Smc5/6 maintains stalled replication forks in a recombination-competent conformation

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The Smc5/6 structural maintenance of chromosomes complex is required for efficient homologous recombination (HR). Defects in Smc5/6 result in chromosome mis-segregation and fragmentation. By characterising two *Schizosaccharomyces pombe* smc6 mutants, we define two separate functions for Smc5/6 in HR. The first represents the previously described defect in processing recombination-dependent DNA intermediates when replication forks collapse, which leads to increased rDNA recombination. The second novel function defines Smc5/6 as a positive regulator of recombination in the rDNA and correlates mechanistically with a requirement to load RPA and Rad52 onto chromatin genome-wide when replication forks are stably stalled by nucleotide depletion. Rad52 is required for all HR repair, but Rad52 loading in response to replication fork stalling is unexpected and does not correlate with damage-induced foci. We propose that Smc5/6 is required to maintain stalled forks in a stable recombination-competent conformation primed for replication restart.

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Introduction

In eukaryotes, there are three conserved structural maintenance of chromosome (SMC) complexes each built around two SMC proteins. Each SMC protein has N- and C-terminal globular domains separated by two long coiled-coil regions with a central hinge at which the protein folds back on itself. The globular domains thus associate, forming a site for ATP binding and hydrolysis. SMC proteins heterodimerise through their hinges and the non-SMC subunits can bridge the two ATPase domains. Cohesin (Smc1/3) mediates sister chromatid cohesion. Condensin (Smc2/4) mediates chromosome condensation at mitosis (Losada and Hirano, 2005). The third complex, Smc5/6, is also essential and is required

for efficient homologous recombination (HR) (Murray and Carr, 2008).

The Smc5/6 complex contains six non-SMC subunits, Nse1–6. In *Schizosaccharomyces pombe*, Nse5 and Nse6 are non-essential, but mutants exhibit repair defects and slow growth (Pebernard *et al*, 2006). Of the four essential Nse proteins, Nse1 is predicted to be a ubiquitin ligase, Nse2 (MMS21) is a SUMO ligase, Nse3 is a member of the MAGE (Melanoma-antigen encoding gene) super-family and Nse4 (Rad62) resembles Kleisins and bridges Smc5 and 6 (Murray and Carr, 2008). In hypomorphic Smc5/6 complex mutants, HR-dependent DNA structures accumulate following replication fork collapse (Ampatzidou *et al*, 2006; Branzei *et al*, 2006). Consistent with this, these mutants show synthetic lethality/sickness with mutants in several HR regulators (Murray and Carr, 2008).

Homologous recombination is involved in bypass of some DNA damage during ongoing replication and exerts an effect to restart replication when forks break (Marians, 2000). The ability to restart stalled or broken replication forks is of particular importance when two converging forks stop. If a single fork stops and cannot be recovered, the problem is resolved by adjacent fork convergence. However, in regions of unidirectional replication (the rDNA array and telomeres), a single stopped fork must be recovered because no converging fork will be available (Murray and Carr, 2008). Both the rDNA and telomeres have served as potent readouts of recombination defects. For example, recombination regulators, such as the RecQ helicase and the Smc5/6 complex, are implicated in telomerase-independent telomere maintenance (Multani and Chang, 2007; Potts and Yu, 2007) and suppression of rDNA recombination (Sinclair *et al*, 1997; Fricke and Brill, 2003; Torres-Rosell *et al*, 2007).

Eukaryotic ribosomal DNA (rDNA) is organised as arrays containing 100–10 000 tandem repeats. rDNA is in the nucleolus where rRNA is synthesised and processed and the ribosomes are assembled (Nomura, 2001). Each rDNA repeat consists of 5.8S, 17S and 26S rDNA genes and a non-transcribed spacer (NTS) containing an autonomously replicating sequence (ARS) plus one or more polar replication fork barriers (RFBs). The RFBs prevent replication proceeding in the opposite direction to transcription (Figure 1A). *S. pombe* rDNA is heterochromatic and refractory to RNA polymerase II transcription. This silencing is dependent on the chromodomain protein Swi6^{HP1} (Huang, 2002). Such a large repetitive locus is a potent HR substrate, but rDNA recombination rates are significantly lower than expected, indicating that recombination within the array is suppressed (Petes, 1980). This negative regulation is important to prevent unequal recombination events that would cause gain or loss of repeats. It was recently suggested that the Smc5/6 complex exerts an effect as a negative regulator of HR in the rDNA, as budding yeast *smc5/6* mutants showed increased nucleolar Rad52 foci when a double strand break was induced within the rDNA array (Torres-Rosell *et al*, 2007).

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Figure 1 Rad52 association in response to both stalled and collapsed forks. **(A)** Schematic of *S. pombe* chromosomes 2 and 3 showing the loci analysed, position of the rDNA at both ends of chromosome 3 and an rDNA repeat unit showing transcripts and the non-transcribed spacer (NTS) containing ars3001 and replication fork barriers (RFB). **(B, a–c)** α GFP ChIP analysis of the loci indicated from *smc6⁺*, *smc6-X* and *smc6-74* mutant cells in a *rad52-GFP* background following arrest in 10 mM HU for 4 h with stable replication forks (*cds1⁺*). Increased rDNA enrichment is evident in *smc6⁺* and *smc6-X*, but not in *smc6-74* cells. **(B, d–f)** The equivalent experiment as in B1–3, with strains containing a *cds1* deletion. In this background, HU arrest results in collapsed replication forks. '10' and '20' indicate a control region 10 and 20 kb from the indicated ARS. **(C)** Representative examples of Rad52-GFP foci in *cds1⁺* and *cds1-d* cells blocked in HU. In the majority (95–98%) of *cds1⁺* (*smc6⁺* or *smc6* mutant cells) blocked in HU, Rad52-GFP showed a diffuse nuclear pattern that was excluded from the nucleolus. In *cds1-d* cells (*smc6⁺* or *smc6* mutant cells), defective in the S phase check point, stalled forks cannot be maintained and multiple Rad52-GFP foci are observed in virtually every cell arrested for 4 h in HU.

Here, we analyse two recessive *smc6* loss-of-function alleles in *S. pombe*: *smc6-X* and *smc6-74*. Both mutants are DNA damage sensitive and HR defective but are viable at all temperatures (Lehmann *et al*, 1995; Verkade *et al*, 1999; Fousteri and Lehmann, 2000). The *smc6-X* mutation (R706C) maps close to the hinge. The *smc6-74* mutation (A151T) maps within a highly conserved arginine finger close to the ATP-binding site of the N-terminal globular domain (Lammens *et al*, 2004). This suggests that *smc6-74* might affect DNA-dependent ATP binding/hydrolysis (ATP-binding site mutations are lethal (Verkade *et al*, 1999; Fousteri and Lehmann, 2000)).

smc6-74, but not *smc6-X*, can be suppressed by overexpression of a multi-BRCT domain protein, Brc1 (Verkade *et al*, 1999). This indicates that an Smc5/6 complex function is defective in *smc6-74*, but not in *smc6-X*, and that Brc1 can compensate for this *smc6-74*-specific function. Brc1 is homologous to *Saccharomyces cerevisiae* Esc4/Rtt107 and shares similarities with human PTIP, both of which have been implicated in stabilizing stalled replication forks (Jowsey *et al*, 2004; Rouse, 2004; Roberts *et al*, 2006). Brc1-mediated rescue of the *smc6-74* phenotypes requires a number of structure-specific nucleases (Sheedy *et al*, 2005; Lee *et al*, 2007).

We demonstrated previously that both *smc6-74* and *smc6-X* are defective in resolving HR-dependent intermediates that accumulate at collapsed forks (Ampatzidou *et al*, 2006). In budding yeast, Rad51/52-dependent DNA structures have similarly been observed to accumulate in an *smc5/6* complex mutant *mms21(nse2)* during MMS exposure (Liberi *et al*, 2005; Branzei *et al*, 2006). Thus, Smc5/6 complexes have a function processing HR intermediates generated at collapsed forks, the resolution of which is important for accurate chromosome segregation.

Here, we show that, when forks collapse, HR proteins are recruited to replication sites as expected and that this is not Smc5/6 dependent. However, we unexpectedly observed that Rad52 (encoded by *rad22* in *S. pombe*, but referred to here as Rad52) is rapidly loaded onto chromatin when replication forks are stably stalled, and that this is Smc5/6 dependent. Further, we also show that RPA loading to stable stalled replication forks is similarly Smc5/6 dependent. We thus identify an early HR function for Smc5/6 at stable replication forks and suggest that Smc5/6 is required to keep stalled forks in a conformation that allows RPA and Rad52 recruitment, and that this provides potential for replication restart.

Results

Smc6 is required for Rad52 chromatin association at stalled replication forks

Replication forks arrested in HU are stabilised by the Rad3-Cds1 checkpoint (Lindsay *et al*, 1998). In the absence of *cds1*, the replisome dissociates from the nascent DNA ends and DNA processing occurs, a process termed 'fork collapse' (Cobb *et al*, 2003; Katou *et al*, 2003). Checkpoint-defective cells treated with HU undergo genome-wide fork collapse, which rapidly recruits recombination proteins and Rad52 to microscopically visible foci (Lisby *et al*, 2004; Meister *et al*, 2005). In contrast, in wild-type cells treated with HU Rad52 foci are not induced. It is thus assumed that recombination

proteins do not associate with stalled (i.e., checkpoint stabilised) forks.

We reported previously that the dynamics of Rad52 foci in both *smc6* mutants upon exposure to HU is similar to that observed in wild-type cells (Ampatzidou *et al*, 2006). A focus represents the accumulation of multiple Rad52 molecules at a single random site within an individual cell. Foci are also thought to correlate with sites where repair is difficult or delayed. In contrast, chromatin immunoprecipitation (ChIP) data represents an average enrichment of protein at a defined DNA sequence in a population of cells. We thus examined Rad52 association with specific sequences (schematic Figure 1A) by ChIP in HU-arrested cultures. To distinguish between stably arrested and collapsed replication forks, we examined both *cds1*⁺ and *cds1-d* (checkpoint defective) backgrounds.

When *cds1* was absent, and forks collapse upon HU arrest, Rad52 was enriched at all loci investigated (Figure 1Bd–f). This is consistent with the multiple Rad52 foci observed in HU-arrested *cds1-d* and *smc6 cds1-d* double-mutant cells (Figure 1C and Meister *et al*, 2005; Ampatzidou *et al*, 2006). In contrast, in *cds1*⁺ cells, where stalled forks are stable and the majority of cells exhibit diffuse Rad52 nuclear staining (Figure 1C), we unexpectedly found *smc6*⁺, and *smc6-X* showed HU-dependent enrichment of Rad52 at the rDNA. In contrast, *smc6-74* did not exhibit this enrichment (Figure 1Ba–c). We conclude that Rad52 is chromatin associated at the rDNA locus when forks are arrested in a stable configuration and that this association is Smc6 dependent. When forks collapse, Rad52 is recruited to all loci in an Smc6-independent manner.

Smc6 is required for rDNA stability

The Smc5/6 complex has been shown to be required for rDNA segregation and stability and this most likely reflects its function resolving HR structures formed when replication forks collapse spontaneously. To assess if the Rad52 loading defect observed for *smc6-74* at stable stalled forks correlates with rDNA stability, we measured loss of a *ura4*⁺ marker integrated into one rDNA repeat (Thon and Verhein-Hansen, 2000) (Figure 2). In *smc6*⁺ (wild type) cultures, the *ura4*⁺ marker was lost from the rDNA once in 2.08×10^3 cells per generation, leading to ~2% of growing cells becoming *ura4*[−] (Figure 2A). As *polII* transcription is repressed in the rDNA by silencing (Huang, 2002), *ura4*⁺ loss was confirmed by Southern blotting (Supplementary Figure S1). *ura4*⁺ loss was elevated ~10-fold (1 in 1.94×10^2 cells per generation) in *smc6-X* cultures, confirming Smc5/6 complex as a negative regulator of rDNA recombination (Torres-Rosell *et al*, 2007). In fission yeast, rDNA repeats are located close to both telomeres of chromosome 3 (Figure 1A). Consistent with a requirement for HR to maintain this telomeric organisation, deletion of *rad22* (*RAD52*) led to ~50% marker loss (Supplementary Figure S2).

Despite the fact that *smc6-74* mutants show an equivalent HR defect to *smc6-X* mutants following HU treatment and DSBs induced by ionising radiation (Verkade *et al*, 1999; Ampatzidou *et al*, 2006), *ura4*⁺ loss in *smc6-74* cultures was equivalent to that observed in *smc6*⁺ cultures (1 in 2.73×10^3 cells per generation). Thus, *smc6-74* cells appear to be defective in a different aspect of rDNA HR repair compared

Figure 2 Recombination in the rDNA is regulated by Smc5/6. (A) Fluctuation analysis of rDNA marker loss in strains containing a *ura4*⁺ marker integrated into a single rDNA repeat (Thon and Verhein-Hansen, 2000). (B) *smc6-74* suppresses lethality associated with entry into stationary phase in *swi6-d* strains. The strains indicated were tested for the loss of viability during entry into stationary phase. Strains were grown in YES medium at 30°C from exponential phase to the stationary phase. (C) Cell density begins to plateau at ~20 h. Viability is calculated from the number of cells forming colonies when 2×10^3 cells were plated on rich media. Representative example of three independent experiments is shown.

with *smc6-X* cells, and this correlates with the reduced Rad52 association at stable stalled forks.

Smc6-74 regulates recombination in the rDNA in the absence of silencing

In the course of our experiments, we observed that *smc6-X* cells have a silencing defect (Supplementary Figure S3). To establish if this caused the increased marker loss, we repeated the experiments in a *swi6*-null background. *swi6* encodes heterochromatin protein 1 in *S. pombe*, and is absolutely required for heterochromatin formation. In *smc6+* cultures, the *ura4*⁺ loss rate was increased 7.3-fold when *swi6* was deleted (Figure 2A). Rate of loss in *smc6-X swi6-d* was increased 14.5-fold over the *smc6+ swi6+* (wt) strain. Thus, loss of silencing contributes to marker loss but is not the only cause of rDNA instability in *smc6-X* mutants. In contrast, *ura4*⁺ loss was significantly reduced in the *smc6-74 swi6-d* strain compared with the *smc6+ swi6-d* strain (0.5-fold lower ($P=0.0107$)). Thus, the *smc6-74* mutation represses the increased rate of marker loss in the rDNA in the absence of silencing.

We noted that *swi6-d* and *smc6-X swi6-d* strains, but not *smc6-74 swi6-d* strains, lost viability upon entry into stationary phase, a phenotype reported for the hyper-recombinogenic *fbh1* (F box DNA helicase)-null mutant (Morishita *et al*, 2005; Osman *et al*, 2005). We thus examined the survival of *swi6-d* strains transiting from logarithmic growth into stationary phase. *swi6-d* and *smc6-X swi6-d* cells lost viability in stationary phase (after 20 h), although more slowly than *fbh1-d* cells (Figure 2B and C). In contrast, *smc6-74 swi6-d* strains maintained viability under these conditions. This allele-specific rescue of the loss of viability of *swi6-d* cells entering stationary phase may suggest that recombination between repetitive sequences occurs less frequently in the *smc6-74* mutant background.

Rad52 chromatin association requires fork stalling and is not confined to the rDNA

The Smc6-dependent Rad52 chromatin association at the rDNA during HU treatment could either be a specific response to forks stalled by HU or could occur in unperturbed S phase and become apparent in HU-treated cultures because cells become synchronised in S phase. To distinguish between these two possibilities, we synchronised *smc6+* cells and assayed Rad52-GFP association in an unperturbed cell cycle (Figure 3A, left panel). Cell cycle progression was monitored by flow cytometry (Figure 3B) and by mitotic and septation indices (Figure 3C). No cell cycle-dependent enrichment of Rad52-GFP was observed either in the region of an early firing origin or at the rDNA locus. Thus, Rad52 chromatin association is dependent on fork stalling.

To determine the timing of Rad52 association upon fork stalling, we next compared the profile of Rad52 chromatin association with that of Mcm4 (*cdc21-GFP*) in HU-treated synchronised cells released into S phase (Figure 3A, right panel). Mini-chromosome maintenance proteins, Mcm2–7, are loaded onto origins of replication in G1 (pre-replication complex) and, upon initiation, form the replicative helicase that unwinds DNA at the fork (Forsburg, 2008). Thus, the loss of Mcm4 from an origin provides a measure of initiation.

We characterised Mcm4 association by ChIP. Cells were synchronised in late G2 and released into 10 mM HU. Cells progressed through mitosis, G1 and entered S phase, which in fission yeast is coincident with septation (Figure 3B). Mcm4 accumulated at the early origin, *ars2004*, between 30 and 90 min after release (G1 to early S phase) and is largely absent again at 120 min, indicative of early origin firing. However, as indicated by the 1C DNA peak (Figure 3B), replication stalls in HU after initiation. At a late firing origin, *ars2018*, Mcm4 similarly accumulated between 30 and 90 min, but remains

Figure 3 Rad52 is chromatin associated at unique origins when replication stalls. (A) qPCR analysis of Rad52 ChIP at early and late replicating and rDNA origins following synchronisation of Rad52-GFP cells in G2 by *cdc25-22* block and release. Parallel analysis of Mcm4 in Mcm4-GFP cells was used to monitor the timing of Rad52 association. Cells were monitored for cell cycle progression by FACS (B) and septation index (C). In an unperturbed cell cycle, G1 is very short and cytokinesis is coincident with S phase, giving rise to the 2-4C shoulder at 90 min (left). When cells are blocked in HU (right), cytokinesis proceeds as normal but S phase is slowed. This leads to uncoupling of septation and replication and the appearance of a 1C peak. Thus, the FACS profile of cells blocked in HU moves from a 2C to 1C peak with time. The 1C peak is maintained at 150 min, but by 210 min later, release has moved towards the 2C, consistent with replication being slowed but not completely stopped in HU. In the presence of HU (right-hand panels), Mcm4 (line) accumulated at early, late and rDNA origins as the cells progressed through G1, peaking at 90 min. It then declined at the early origin, indicative of origin firing but remained highly enriched at rDNA and late origins until 210 min, consistent with the repression of late firing origins. rDNA origins fire early, but only one in four fires and most Mcm4 is displaced by passive replication. In contrast to Mcm4, Rad52 levels (bars) declined, as cells progressed through G1 but increased at early and rDNA regions at 105 min and at the late origin at 210 min, when Mcm4 levels start to decline. No consistent increase in Rad52 association with any loci tested was observed in S phase in an unperturbed cell cycle (-HU, left-hand panel).

present at 120 min, consistent with late origin firing being delayed by the Rad3-Cds1 checkpoint (Patel *et al*, 2006; Hayashi *et al*, 2007). At the rDNA *ars3001*, Mcm4 is enriched between 30 and 90 min and remained significantly enriched at 120 min. *ars3001* is early firing (Kim and Huberman, 2001), but although every rDNA repeat contains an ARS element, only approximately one in four fire. Furthermore, active rDNA origins are clustered and thus interspersed with large domains where initiation is repressed (Pasero *et al*, 2002). In these regions, Mcm4 will be displaced by passive replication of repressed origins and so the association of Mcm4 in the rDNA will provide a measure of ongoing replication as opposed to origin firing.

We next examined Rad52 chromatin association in parallel *rad52-GFP* cultures (FACS shows an equivalent cell cycle profile (Supplementary Figure S4)). Rad52 levels decreased at all loci as the cells progressed through mitosis into G1. Rad52 levels then increased at the early firing origins (*ars2004* and *ars3001*) at 105 min after release into HU and were further increased at *ars3001* in the rDNA at 120 min. No increase was observed at the late origin at this time point. This indicates that Rad52 associates with chromatin as or after DNA replication forks have passed. In the presence of HU, replication is slowed but not completely stopped. In both budding and fission yeast, late origins do eventually fire (Meister *et al*, 2006; Alvino *et al*, 2007). By FACS analysis, the 1C DNA peak was maintained until ~150 min, but by 210 min the cells were starting to progress through S phase. Rad52 association with the late origin region was delayed until 210 min, when Mcm4 levels were starting to decline.

More Rad52 appeared to be associated with the rDNA than with either the early or late origins. This may reflect the fact that the region is repetitive, or that replication was ongoing within the array for longer (Mcm4 was still enriched at 240 min). However, Rad52 association with the DNA remained high throughout the time course both for the rDNA and the early origin region, and the degree of association did not reflect distance from the origin (not shown; the data for 3 kb from origins is representative). Taken together, these data demonstrate that Rad52 chromatin association following replication fork stalling occurs both at unique loci and in the rDNA but is more prevalent or easier to detect in the rDNA.

Smc6-dependent Rad52 chromatin association is reversible

When replication forks are collapsed by treating checkpoint-defective cells with HU, Rad52 foci form rapidly and persist for many hours following the release from HU. Rad52 foci are not observed in HU-arrested checkpoint-proficient cells (Figure 1 and data not shown). To establish if the association we observe by ChIP is reversible and thus distinct from that observed as foci, we examined Rad52 association at the rDNA repeats and euchromatic loci by ChIP in an HU block and release time course (Figure 4). We also wished to verify that the lack of Rad52 association observed in *smc6-74* did not reflect a difference in cell cycle progression in the presence of HU, and thus we examined *smc6-X* and *smc6-74* in parallel. Cell cycle progression was monitored by flow cytometry (Figure 4E) and cells scored for mitotic index and septation (Figure 4C).

Cell cycle progression in the *smc6* mutants was virtually identical to wild type both during the block and upon release. *smc6-X* has a slightly longer cell cycle, and mitosis is delayed about 10 min. Rad52-GFP immunoprecipitation of untreated *smc6⁺*, *smc6-X* and *smc6-74* extracts enriched all loci 3- to 5-fold compared with untagged control extracts (Figure 4B). In *smc6⁺* and *smc6-X* backgrounds, treatment with HU for either 2.5 or 4 h resulted in 10- to 20-fold enrichment of Rad52 at the rDNA RFB and *ars3001* loci, and 8- to 15-fold enrichment at the 17S rDNA locus. The unique non-rDNA loci showed a similar but less dramatic trend (for *smc6⁺* 2.5 h compared with asynchronous samples $P=0.017$, Student's *t*-test (paired)). Importantly, we reproducibly failed to observe HU-dependent enrichment at any loci in *smc6-74* extracts. As no differences in total Rad52 levels in the ChIP samples were observed (Figure 4D), this confirms that Smc6 is required to promote Rad52 chromatin association in response to replication fork stalling.

Following release from HU arrest, the level of Rad52 enrichment declined rapidly. At 0.5 h (G2) most enrichment was lost, and at 1.5 h (M-G1) enrichment was reproducibly less than that observed in asynchronous cultures (Figure 3B). Thus, Rad52 becomes chromatin associated during HU treatment but dissociates upon release as cells enter mitosis. It is important to note that this profile contrasts directly the kinetics of Rad52-GFP foci formation in HU-treated cells (Meister *et al*, 2005; Ampatzidou *et al*, 2006). Very few HU-arrested cells show Rad52-GFP foci and most have diffuse nuclear Rad52-GFP that is significantly reduced in the nucleolus (Figure 1 and Meister *et al*, 2005; Coulon *et al*, 2006). After release from HU, one or two foci become visible in ~25% of cells at 0.5 h, and this is true in *smc6⁺*, *smc6-X* and *smc6-74* strains (Ampatzidou *et al*, 2006). Thus, our ChIP data provide a distinct profile of Rad52 chromatin association that is dependent on Smc6. This is not representative of the repair events that occur at random collapsed forks, which are visualised by foci, and that are independent of Smc6.

Smc5/6 indirectly regulates Rad52 chromatin association

The association of Rad52 with stalled forks is unexpected. Recombination is temporally separated from replication, and recombination is proposed to reset collapsed and/or damaged forks but has not been assigned a function at stable stalled forks. Rad52 is required for Rad51-dependent HR and has Rad51-independent strand annealing activity that may be important to stabilise stalled forks. We therefore examined whether Rad51 association was reflecting Rad52 association (Figure 5A). In contrast to Rad52, Rad51 levels were not found to increase significantly when cells were treated with HU for 4 h. We conclude that Rad52 levels do not reflect Rad51-dependent HR at stable stalled forks. As rDNA marker loss is increased in *smc6-X* and suppressed in *smc6-74* when silencing is lost, we propose that Rad52 association allows a potential for recombination. This would be consistent with Rad51-dependent strand invasion events being tightly regulated by a number of helicases (Srs2, RecQ, Fbh1) (Branzei and Foiani, 2007).

Replication fork stalling leads to the accumulation of ssDNA, most likely due to transient uncoupling of the replicative helicases from DNA synthesis (Sogo *et al*, 2002). This ssDNA recruits RPA and activates the Rad3-Cds1 checkpoint

Figure 4 Smc6 is required for the association of Rad52 on replication inhibition. **(A)** Schematic of time course of HU block and release. **(B)** αGFP ChIP analysis of the loci indicated from *smc6*⁺, *smc6-X* and *smc6-74* mutant cells in a *rad52-GFP* background. Cells were arrested for 4 h in 10 mM HU and released into fresh media. Samples were taken at the times indicated in (A) and cell cycle progression was monitored by mitotic and septation indices and FACS (representative experiments (C) and (E)). **(C)** Proportion of aberrant mitotic cells. **(D)** Western blot analysis with α-GFP of whole cell extracts (ChIP input samples) shows that Rad52 levels do not vary significantly during HU treatment and release and are equivalent between *smc6*⁺ and *smc6* mutant strains. **(E)** FACS analysis of *smc6*⁺, *smc6-X* and *smc6-74* cells in 10 mM HU and on release shows that cell cycle progression is similar in *smc6*⁺ and *smc6* mutant strains. *S. pombe* is a haploid organism that spends ~70% of the time in G2. In asynchronous cultures (time 0), there is a strong 2C peak. By 2.5 h (generation time in rich media at 30°C), all the cells are arrested with a 1C DNA content. On release from HU at 4 h, the profile moves from 1C to 2C, showing that the cells complete S phase within 1 h of release. Two hours after release from HU, a 4C peak appears (coincident with the peak of septation), indicating that the cells have entered the next S phase.

that stabilizes the replication complex on the stalled fork (Forsburg, 2008). RPA-coated ssDNA is a substrate for Rad52 and thus we examined RPA association with stable stalled forks (Figure 5B). In *smc6*⁺, RPA (Rad11-GFP) was enriched at rDNA and unique loci after 2.5 h in HU and this declined at 4 h. In *smc6-X*, RPA was also enriched at 2.5 h at all loci (although to a lesser extent) and remained enriched at 4 h.

(*smc6-X* but not *smc6-74* has a slight growth defect with *rad11-gfp*). In contrast, RPA association was only slightly increased in *smc6-74* at 2.5 h and does not increase further. (RPA levels are unaltered in *smc6* mutants (Supplementary Figure S5)). These data suggest that in *smc6-74* the conformation of the stalled fork is altered, leading to a decrease in ssDNA and thus indirectly to a decrease in Rad52 recruitment.

Figure 5 Smc6 is required for the association of RPA on replication inhibition. Analysis of (A) Rad51 (encoded by *rhp51*) and (B) RPA (encoded by *rad11*) chromatin association in HU. (A) α GFP ChIP analysis of the loci indicated from *smc6*⁺, *smc6-X* and *smc6-74* mutant cells in an *rhp51-GFP* background following arrest in 10 mM HU for 4 h with stable replication forks. In contrast to Rad52 (Figure 1Ba–c), Rad51 chromatin association in HU was not increased over asynchronous levels. (B) α GFP ChIP analysis of the loci indicated from *smc6*⁺, *smc6-X* and *smc6-74* mutant cells in a *rad11-GFP* background following arrest in 10 mM HU for 2.5 or 4 h with stable replication forks. In *smc6*⁺ cells, RPA chromatin association increased at all loci as cells blocked in HU and declined at 4 h. In *smc6-X*, RPA association also increased, peaking at 4 h, consistent with a slower generation time. In contrast, in *smc6-74*, RPA chromatin association was decreased but not completely abolished.

Although RPA association with stalled forks is decreased in *smc6-74*, we have reported previously that checkpoints are activated normally (Verkade *et al*, 1999) and that replication intermediates are stable in HU (Ampatzidou *et al*, 2006). RPA-coated ssDNA recruits both the checkpoint sensors (Forsburg, 2008) and triggers PCNA ubiquitylation (Davies *et al*, 2008). In *S. pombe*, PCNA is ubiquitylated in HU-arrested cells (Frampton *et al*, 2006) and thus we examined PCNA ubiquitylation in *smc6-74* cells (Figure 6). *smc6*⁺ and *smc6-74* cells were synchronised in G2 and released into a HU block. In both strains, PCNA ubiquitylation was coincident with septation, a marker for S phase (Figure 6A and B) but Rad52 again associated with rDNA and the early origin only in *smc6*⁺ (Figure 6C). This indicates that, despite the lack of Rad52 recruitment, there is sufficient ssDNA to both initiate checkpoint responses and modify PCNA for post-replication repair in *smc6-74* cells.

Discussion

The analysis of *smc6-X* and *smc6-74* mutants has uncovered a separation of function that defines a requirement for the Smc5/6 complex for Rad52 chromatin association in response to replication forks that are stalled and stabilised by the S-phase checkpoint. This 'early' function for Smc5/6 can be

genetically separated from the previously described requirement for Smc5/6 during late stages of HR following the collapse or breakage of replication forks (Murray and Carr, 2008).

Two functions for Smc5/6 in response to replication perturbation

Following replication fork arrest by nucleotide depletion, most forks are stabilised by the S-phase checkpoint. Stochastically, however, some forks break or collapse and are recovered by HR. A defect in Smc5/6 complex mutants in the resolution of HR-dependent structures and the resulting mis-segregation of physically linked chromosomes following replication perturbation has been analysed extensively (Murray and Carr, 2008). Our analysis suggests that this 'late' function in HR is defective in both the *smc6-X* and the *smc6-74* alleles, but that *smc6-74* is specifically defective in a second function. The second defect results in an altered architecture at HU-stalled replication forks that are stabilised by the S-phase checkpoint. This is manifest as a decrease in RPA association and loss of Rad52 association upon HU treatment.

When we analysed marker loss from the rDNA as a read-out of rDNA recombination, we observed that, in *smc6-X* cells (defective only in the 'late' function and in which RPA and

Figure 6 Smc6 is not required for PCNA ubiquitylation. (A) Western blot analysis with α -PCNA or α -Cdc2 (loading control) on whole cell extracts, (B) mitotic and septation indices, (C) qPCR analysis of Rad52 ChIP following synchronisation of *smc6⁺* or *smc6-74 rad52-GFP* cells in G2 by centrifugal elutriation and release into 10 mM HU. Samples were taken at the indicated times. Mono-, di- and tri-ubiquitylated forms of PCNA were detected at about 75 min, coincident with the peak of septation and S phase in both *smc6⁺* and *smc6-74*, but Rad52 chromatin association only increased in *smc6⁺*.

Rad52 are recruited to stably stalled forks), marker loss from the rDNA was increased. This is consistent with previously reported rDNA segregation and recombination defects. In contrast, in *smc6-74* cells (defective in both the 'late' function and for the association of RPA and Rad52 with stalled forks), rDNA marker loss was not increased. This suggests that lack of Rad52 association results in reduced initiation of rDNA recombination, counteracting the increased marker loss due to the defect in the 'late' function of resolving HR intermediates.

Loss of silencing (*swi6-d*) also increases rDNA recombination, but this is suppressed in *smc6-74* and we further noticed that cell death observed when *swi6-d* cells enter stationary phase is specifically reduced by the *smc6-74* mutation. In budding yeast, increased rDNA origin firing has been observed in silencing defective mutants, which also exhibit a reduced lifespan (Pasero *et al*, 2002). Thus, the regulation of origin firing has been proposed to be important for the suppression of replication-dependent rDNA recombination and longevity. The suppression of both marker loss and

swi6 mutant-dependent cell death in stationary phase by *smc6-74* correlates with a reduction of RPA and lack of Rad52 recruitment when replication forks are stalled in HU, possibly because there is less potential for aberrant recombination in these circumstances.

Genome-wide functions for Smc5/6 complex

The link between Smc5/6, replication and recombination is not specific to the rDNA (Morishita *et al*, 2002; Torres-Rosell *et al*, 2005). We reported previously that Smc6 is enriched equally at rDNA and unique loci, although it can be visualised in the nucleolus by indirect immunofluorescence (Ampatzidou *et al*, 2006). We and others have shown that chromatin fragmentation upon loss of Smc5/6 function is not confined to the rDNA (Verkade *et al*, 1999; Morishita *et al*, 2002; Harvey *et al*, 2004; Torres-Rosell *et al*, 2005). It is most likely that the penetrance of the rDNA instability and mis-segregation phenotypes (Torres-Rosell *et al*, 2005) reflect the intimate linkage between replication and recombination

within the rDNA, rather than an rDNA-specific function for Smc5/6 (Murray and Carr, 2008).

Throughout the genome, replication fork stalling generates regions of ssDNA which, bound by RPA, recruit S-phase checkpoint sensors to stabilise replication complexes (Forsburg, 2008). Although the *smc6-74* mutant exhibited decreased RPA association in response to fork stalling, sufficient ssDNA must be revealed upon fork arrest because the mutant cells activate the DNA replication checkpoint normally (Verkade *et al*, 1999), and we show here that PCNA modification is similarly unaffected. Our data are consistent with the following model (Figure 7): the Smc5/6 complex is required to coordinate processes at the stalled fork, including transient mobile events, such as fork regression and restoration, that expose ssDNA and subsequently load RPA and Rad52. Such conformational changes may facilitate replication restart by template switching, and this is most likely to be important at a subset of stalled forks where replication cannot be reinitiated directly or rescued by an incoming fork. If the forks collapse (i.e., replicative enzymes dissociate from the nascent DNA), then Rad52 and Rad51 associate with the damaged chromatin independently of Smc5/6. However, the 'late' function Smc5/6 is subsequently required to process intermediates generated by the ensuing HR repair/replication restart. This late function is consistent with the function in completion of HR-mediated repair of double strand breaks (Lehmann *et al*, 1995; Verkade *et al*, 1999; De Piccoli *et al*, 2006). In both early (stalled fork) and late (post strand invasion) situations, mobile branched DNA structures are present and thus the function of the Smc5/6 complex (as distinct from the cohesin, condensin and MRN complexes) could be to hold such structures in a flexible conformation to

facilitate repair or replication restart. While *in vitro*, a number of proteins, such as the RecQ helicases, have been shown to carry out branch migration and strand displacement activities on naked DNA, *in vivo*, these processes are most likely to require additional regulation to limit the activities and coordinate with other processes. Consistent with this, fork regression has not been visualised in checkpoint-proficient eukaryotic cells (Lopes *et al*, 2001).

We observe that Rad52, but not Rad51, accumulates at stable stalled replication forks. It is possible that Rad52 assists to maintain DNA integrity at such forks, perhaps through its strand-annealing function. Stabilisation of stalled replication forks is required genome-wide, but we observe relatively more Rad52 enrichment at the rDNA locus. Replication takes longer in the rDNA, and this region is replicated unidirectionally; fork arrest in such a region would have severe consequences because a single stalled fork that does not restart is potentially lethal as there is no incoming fork to complete replication. If Rad52 recruitment in response to stalled forks facilitates a recombination protein-dependent replication restart without fork collapse, then Smc6-dependent Rad52 loading may be particularly important to both protect and rescue stalled forks when the problem cannot be resolved by a converging fork. Thus, the nucleolus may have adapted a genome-wide phenomenon to be more prevalent. In this scenario, in both *smc6-X* (that loads Rad52 but cannot resolve the resulting recombination intermediates) and *smc6-74* (that does not load Rad52), replication would be incomplete. Thus, the two mutants would, as we have reported previously (Ampatzidou *et al*, 2006), exhibit similar levels of aberrant mitosis because the DNA cannot properly segregate. However, in *smc6-X* cells, the aberrant recombination would increase marker loss, whereas in *smc6-74* cells recombination would occur less frequently and thus marker loss would be less pronounced.

Figure 7 Model for Smc5/6 complex function. Cds1^{Chk2} activity stabilises stalled replication forks and Smc5/6 keeps the stalled fork in a recombination-competent conformation that facilitates Rad52 association and replication restart (early function, left). In *smc6-74*, the stalled fork is not in the right conformation to enable Rad52 loading, and in *smc6-X*, the stalled fork is less stable, and hence more prone to collapse. Smc5/6 is not required for Rad52 association with the damaged chromatin at collapsed forks (replication complexes dissociated) (right) but the complex is required at a later stage for HR repair and/or restoration of replication (late function).

Potential mechanisms for the 'early' function of Smc5/6

The *smc6-74* mutation (A151T) maps close to the ATP-binding site of the N-terminal globular domain in an 'arginine finger' (R-loop) that is highly conserved between SMC proteins. This motif is proposed to bind DNA and allosterically regulate DNA-dependent ATP binding/hydrolysis (Lammens *et al*, 2004). Mutation of the R-loop in Cohesin caused increased chromosome instability and delayed Cohesin loading (Lengronne *et al*, 2006). However, we observed only a small increase in Smc6 chromatin association in response to HU treatment (Ampatzidou *et al*, 2006) and this was not significantly affected by *smc6* mutation (data not shown). Thus, an alternative explanation may be that ATP hydrolysis is required to keep the stalled fork in a conformation that permits RPA and Rad52 loading. This would be consistent with the observation that viability of *smc6-74* but not of *smc6-X* cells during low-dose MMS treatment is dependent on Slx1, a structure-specific nuclease required for stability of the rDNA (Sheedy *et al*, 2005; Coulon *et al*, 2006). It will be interesting to determine how the DNA-dependent ATP binding/hydrolysis activity of the Smc5/6 complex affects replication fork stability.

The allele-specific suppression of the DNA damage sensitivity of *smc6-74* by overexpression of the multiple BRCT-domain protein Brc1 (Verkade *et al*, 1999) depends on Slx1/4

and on a range of other structure-specific nucleases (Mus81/Eme1, Apn2 and Exo1) (Sheedy *et al*, 2005; Lee *et al*, 2007). All hypomorphic mutants in the Smc5/6 complex are synthetically lethal with *brc1* loss (Verkade *et al*, 1999; Morikawa *et al*, 2004), and Brc1 is required for DNA repair during DNA replication (Sheedy *et al*, 2005). This has been interpreted to suggest that the Smc5/6 and Brc1 pathways share overlapping functions in the processing of aberrant DNA structures that arise during S phase (Verkade *et al*, 1999). As over-expression of Brc1 does not restore Rad52 loading in *smc6-74* (Supplementary Figure S6), we speculate that the reduced Rad52 loading observed in *smc6-74* cells may provide a window of opportunity for Brc1-directed processes to initiate alternative Smc5/6-independent repair, thus bypassing the requirement for Smc5/6 to resolve HR-dependent DNA structures that would accumulate. This would be consistent with the *S. cerevisiae* homologue, Rtt107/Esc4, being involved in the stabilisation of stalled replication forks (Roberts *et al*, 2008).

Our data suggest that Smc5/6 has two functions: an early function identified here to maintain stalled forks in a recombination-competent conformation primed for restart; the second function exerts an effect at a late stage of recombination to resolve as yet unidentified DNA structure(s). The late function explains the DNA repair defects, chromosome mis-segregation and rDNA instability observed in Smc5/6 complex hypomorphic mutants in both yeasts. The conservation of the Smc5/6 complex from yeast to man suggests that it is most likely to perform similar functions in higher organisms.

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Materials and methods

Strains (Supplementary Table S1) were constructed and cell cycle analysis were carried out using standard techniques (Moreno *et al*, 1991). HU (10 mM) was used to inhibit replication. Survival analyses have been previously described (al-Khodairy *et al*, 1994). Stationary phase-associated cell death assays were performed as described by Morishita *et al* (2005). *ura4* loss assay: three separate experiments, each with 11 independent single colonies for each strain, were inoculated into 10 ml YES medium and grown to stationary phase. A total of 1×10^3 cells were plated on YES, grown for 5 days at 30°C and colonies replica-plated to $\bar{u}$ racil medium. The rate of *ura4* loss per cell per generation was determined with the method of the median (Lea and Coulson, 1949). Live cell imaging of Rad52-GFP foci was carried out in minimal media (EMM2) at room temperature. Detection of PCNA modification was done as described by Frampton *et al* (2006). ChIP and qPCR analysis were performed as described by Ampatzidou *et al* (2006). Each experiment was performed in triplicate, and values are an average of at least two independent experiments (error bars; standard error) or a representative time course shown. α -GFP (Molecular Probes) was used 1:200 to immunoprecipitate Rad52-GFP (Rad22-gfp), Mcm4-GFP (Rad21-gfp), Rad51-GFP (Rhp51-GFP) or Rpa-GFP (Rad11-gfp). The expression of tagged proteins and efficiency of IP was confirmed by western blot analysis of input and IP samples.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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