online data

Slx4 becomes phosphorylated after DNA damage in a Mec1/Tel1-dependent manner and is required for repair of DNA alkylation damage

Sonja FLOTT and John ROUSE[1]

MRC Protein Phosphorylation Unit, Medical Sciences Institute/Wellcome Trust Biocentre Complex, Dow St., University of Dundee, Dundee DD1 5EH, Scotland, U.K.

Members of the RecQ family of DNA helicases, mutated in several syndromes associated with cancer predisposition, are key regulators of genome stability. The *Saccharomyces cerevisiae SLX4* gene is required for cell viability in the absence of Sgs1, the only yeast RecQ helicase. *SLX4* encodes one subunit of the heterodimeric Slx1–Slx4 endonuclease, although its cellular function is not clear. Slx1–Slx4 was reported to preferentially cleave replication fork-like structures *in vitro*, and cells lacking *SLX4* are hypersensitive to DNA alkylation damage. Here we report that Slx4 becomes phosphorylated in cells exposed to a wide range of genotoxins. Even though it has been proposed that the role of Slx4 is restricted to S-phase, Slx4 phosphorylation is observed in cells arrested in G_1 or G_2 phases of the cell cycle, but not during an unperturbed cell cycle. Slx4 phosphorylation is completely abolished in cells lacking the Mec1 and Tel1 protein kinases, critical regulators of genome stability, but is barely affected in the absence of both Rad53 and Chk1 kinases. Finally we show that, whereas both Slx1 and Slx4 are dispensable for activation of cell-cycle checkpoints, Slx4, but not Slx1, is required for repair of DNA alkylation damage in both aynchronously growing cells and in G_2-phase-arrested cells. These results reveal Slx4 as a new target of the Mec1/Tel1 kinases, with a crucial role in DNA repair that is not restricted to the processing of stalled replisomes.

Key words: ataxia telangiectasia-mutated (ATM), ATM and Rad3-related (ATR), DNA damage, Mec1, Sgs1, Slx.

INTRODUCTION

Defects in DNA repair or in the ability of cells to slow cell-cycle progression after DNA damage can increase cellular mutation rates and cause genome instability. This often leads to a variety of debilitating diseases, heightened predispostion to cancer and, sometimes, organism death [1]. In eukaryotic cells, checkpoint control mechanisms postpone key cell-cycle transitions such as chromosome duplication and segregation, to allow time for DNA repair to occur. Different DNA lesions are repaired by different repair pathways, but most types of DNA damage, and stalling of DNA replication forks, can trigger a common signal-transduction pathway that regulates many aspects of the cellular DNA damage response, including cell-cycle arrest and increased DNA repair activity [2–4]. The principal regulators of this pathway are the ATM (ataxia telangiectasia-mutated) and ATR (ATM and Rad3-related) protein serine/threonine protein kinases that are vitally important for the maintenance of genome stability [3]. These kinases rapidly translocate to sites of DNA damage or to stalled DNA replisomes, where they are activated by mechanisms that are not yet clear. This causes phosphorylation of a range of proteins, including the BRCA1 and p53 tumour suppressors (in higher eukaryotes), and the conserved Chk1 and Chk2 protein kinases that, when activated by ATM/ATR, in turn phosphorylate effectors of cell-cycle progression [3]. It is likely that many substrates of ATM/ATR remain to be identified, and it is not yet fully clear, for example, how these kinases increase DNA repair activity, promote recovery from cell-cycle checkpoints, stabilize stalled DNA replisomes or inhibit firing of late origins of replication after replisome stalling. In this regard, the identification of new targets of ATM/ATR is critical to a full understanding of how these proteins guard genome stability. The importance of the ATM/ATR signalling pathway is underscored by its high degree of conservation in evolutionary terms. Tel1 and Mec1 are the budding yeast orthologues of ATM and ATR respectively [5–7].

Members of the RecQ family of DNA helicases are also involved in maintaining genome stability, at least in part by regulating stalled replication forks [8,9]. Three of the five human RecQ homologues [BLM (Bloom's-syndrome protein), WRN (Werner's-syndrome protein) and RECQL4 (mutated in Rothmund–Thompson syndrome)] are mutated in heritable diseases associated with genomic instability and a predisposition to cancer (reviewed in [10–12]). Budding yeasts have a single RecQ helicase, Sgs1, that appears to be required for efficient processing of stalled replication forks [13] and for DNA repair in G_2 phase [14]. Mutations in Sgs1 result in elevated levels of mitotic recombination [15,16] and in hypersensitivity to a variety of DNA-damaging agents [17,18]. Furthermore, Sgs1p contributes to activation of the intra-S-phase checkpoint response in yeast [19], and BLM in man is involved in activation and recovery from checkpoints induced by replication stress [20,21]. It was recently shown that the interaction of Sgs1 with Top3 (topoisomerase III) is important for MMS (methyl methanesulphonate)-induced recombination [14]. It is thought that 'chicken-feet' – Holliday-junction-like structures formed at sites of collapsed replication forks and possibly also at stalled forks – are resolved by Sgs1 in a non-recombinogenic manner. Despite these important cellular functions, *SGS1* is not an essential gene, most probably owing to functional redundancy with other genes, and a number of such genes have been identified [22,23]. One study

Abbreviations used: ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3-related; BLM, Bloom's-syndrome protein; CPT, camptothecin; DSB, double-strand break; HU, hydroxyurea; IonR, ionizing radiation; MMS, methyl methanesulphonate; 4-NQO, 4-nitroquinoline oxide; PFGE, pulsed-field gel electrophoresis; 3-PGK, 3-phosphoglycerate kinase; TAP, tandem affinity purification; WRN, Werner's-syndrome protein; UN, untreated; YPAD, yeast extract/peptone/dextrose medium + adenine.

[1] To whom correspondence should be addressed (email j.rouse@dundee.ac.uk).

identified six '*SLX*' genes required for viability in the absence of *SGS1* and whose products form three distinct heterodimeric complexes when over-expressed in yeast: *SLX2(MMS4)–SLX3(MUS81)*, *SLX5–SLX8* and *SLX1–SLX4* [24]. Mms4–Mus81 was subsequently described as a structure-specific endonuclease in different organisms with preference for 3′ flaps and replication-fork substrates [25–27] that has also been postulated to resolve Holliday-junction intermediates during recombination [26,28]. Little is currently known about Slx5–Slx8.

The budding yeast Slx1–Slx4 complex (expressed in *Escherichia coli*) has endonuclease activity *in vitro* towards branched DNA substrates distinct from that of Mms4–Mus81, with a preference for simple-Y, 5′-flap or replication-fork-like structures [29]. Slx1 is a member of a family of proteins that contains a URI (UvrC-intron excinuclease) domain and a PHD (plant homeodomain)-type zinc-finger domain, and recombinant Slx1 displays weak structure-specific endonuclease activity *in vitro* [29]. The activity of Slx1 co-expressed in bacteria with Slx4 is 500-fold greater than Slx1 expressed alone, and Slx4 alone also displays weak endonuclease activity [29]. Consistent with these data, the nuclease activity of endogenous Slx1 immunopurified from wild-type fission yeast extracts is much greater than when purified from extracts of cells lacking Slx4 [31], although, since the level of Slx1 protein in cells lacking Slx4 was not measured in that study, the possibility that Slx4 is required for Slx1 protein stability or folding could not be excluded. So while it is clear that Slx4 is required for efficient catalysis by Slx1, at least when assayed *in vitro*, the precise role of Slx4 is unclear. Since Slx4 does not possess any obvious catalytic motifs, the reported nuclease activity of Slx4 could be due to contaminating bacterial activities, and it more likely that Slx4 facilitates recognition of the DNA substrate by Slx1. Even though Slx1 and Slx4 interact physically, Slx4 is required *in vivo* for cellular resistance to DNA alkylation damage induced by MMS, whereas Slx1 is not [29]. This suggests that the nuclease activity of the complex is not important for resistance to MMS, and that Slx4 may have roles distinct from Slx1. Sgs1 and Slx4 do not appear to be required for bulk DNA synthesis, but play redundant roles in maintaining the structure of the ribosomal DNA repeats where replisomes stall at high frequency during DNA replication [30,31].

In the course of our studies we noticed that the electrophoretic mobility of budding yeast Slx4 is greatly decreased after exposure of cells to DNA damage, indicative of post-translational modification. Here we report that Slx4 is heavily phosphorylated after exposure of cells to a wide range of agents that damage DNA or cause replication fork stalling. Genotoxin-induced Slx4 phosphorylation requires both Mec1 and Tel1, and to a lesser extent Rad53 and Chk1, and occurs in all phases of the cell cycle. We also show that Slx4, but not Slx1, is required for efficient repair of alkylation damage in G_2-phase-arrested cells as well as in asynchronously growing cells, indicating that Slx4 function is not restricted to S-phase. Thus, our results reveal Slx4 as an important new target of the Mec1/Tel1 signalling pathway that plays an important role in DNA repair.

EXPERIMENTAL

Yeast strains

All strains used in this study are listed in supplementary Table 1 (http://www.BiochemJ.org/bj/391/bj3910325add.htm). Epitope tagging or disruption of yeast genes was verified by PCR or by Southern blotting. *S. cerevisiae* strains SFY001 and SFY002, in which *SLX4* bears a C-terminal MYC(13)-epitope tag, were constructed by a PCR-based method [32]. Strain SFY003, in which *SLX4* bears a C-terminal TAP (tandem affinity purification) tag was purchased from Open Biosystems (Huntsville, AL, U.S.A.). Strains SFY004, SFY005 and SFY006 were isolated as meiotic segregants from a cross between the strains SFY001 (W303 background) and strain DDY054 (*mec1*Δ*::TRP tel1*Δ*::HIS3 sml1-1*; also W303 background) [33]. Strain SFY007 is a meiotic segregant from a cross of SFY001 (W303 background) with the strain SFY011 (*chk1*Δ*::TRP1 rad53*Δ*::HIS3 sml1-1;* also W303 background). Haploids strains SFY008, SFY009, SFY010 and SFY012, lacking *SLX4*, *SLX1*, *SGS1* and *RAD52* respectively, were from EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis), Institute for Microbiology, Johann Wolfgang Goethe-University Frankfurt, Frankfurt, Germany.

Cell extracts and Western-blot analysis

Preparation of extracts for Western-blot analysis by the trichloroacetic acid-lysis method was performed as described previously [34]. Extracts were subjected to SDS/PAGE on Tris/glycine/SDS/4–12%-(w/v)-polyacrylamide gels and transferred to nitrocellulose membranes before Western blotting with anti-*c*-Myc monoclonal antibody (clone 9E10; Roche), anti-3-PGK [anti-(3-phosphoglycerate kinase) antibody; Molecular Probes] or with anti-Rad53 antibody (a mixture of yN-19 and yC-19; Santa Cruz).

Immunoprecipitation and phosphatase treatment

Protein extracts for immunoprecipitation were prepared from exponentially growing cells collected by centrifugation and resuspended in an equal volume (v/v) of detergent lysis buffer [50 mM Tris/HCl, pH 7.2, 0.27 M sucrose, 1 % Nonidet P40, 1 mM EDTA, 0.15 M NaCl, 0.1 % deoxycholic acid, 1 mM dithiothreitol and Complete EDTA-free Protease Inhibitor Mix (Roche)]. After addition of a 1:1 volume of acid-washed glass beads and bead beating (three times, 45 s each time), clarified protein extracts (2 mg) were incubated for 1 h at 4 °C with 20 μl of a 50 % (v/v) Protein G–Sepharose (Amersham Biosciences, Little Chalfont, Bucks., U.K.) slurry covalently linked to anti-*c*-Myc monoclonal antibody. Immunoprecipitates were washed three times with detergent lysis buffer, resuspended in 30 μl of phage λ-phosphatase buffer (50 mM Tris/HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01 % Brij 35 and 2 mM $MnCl_2$). After incubation for 30 min at 30 °C, with or without the addition of 200 units of λ-phosphatase (New England Biolabs) in the presence or absence of EDTA (100 mM), the beads were centrifuged and washed twice with PBS to remove λ-phosphatase. The beads were suspended in 20 μl of SDS/gel loading buffer and subjected to Western blotting with anti-Myc antibodies.

Cell-cycle arrest and release experiments

Cells in mid-exponential phase [attenuance (D_{600}) 1.0] were synchronized in G_1-phase by the addition of α-factor (5 μg/ml) until small unbudded cells accounted for more than 95 % of the cell population. To synchronize cells in G_2-phase, nocodazole (15 μg/ml) and DMSO (1 %) were added to mid-exponential-phase cells until large dumb-bell shaped cells accounted for more than 95 % of the cell population. To release cells from arrest, they were quickly filtered and washed extensively with YPAD (yeast extract/peptone/dextrose medium + adenine [34a]) before incubation in prewarmed medium. For FACS analysis, 1×10^7 cells were resuspended in 70 % (v/v) ethanol and fixed overnight at 4 °C. Cells were then washed in 50 mM sodium citrate, sonicated, treated with 0.25 mg/ml RNaseA (Roche) at 50 °C for 1 h, washed and propidium iodide was added to 50 μg/ml

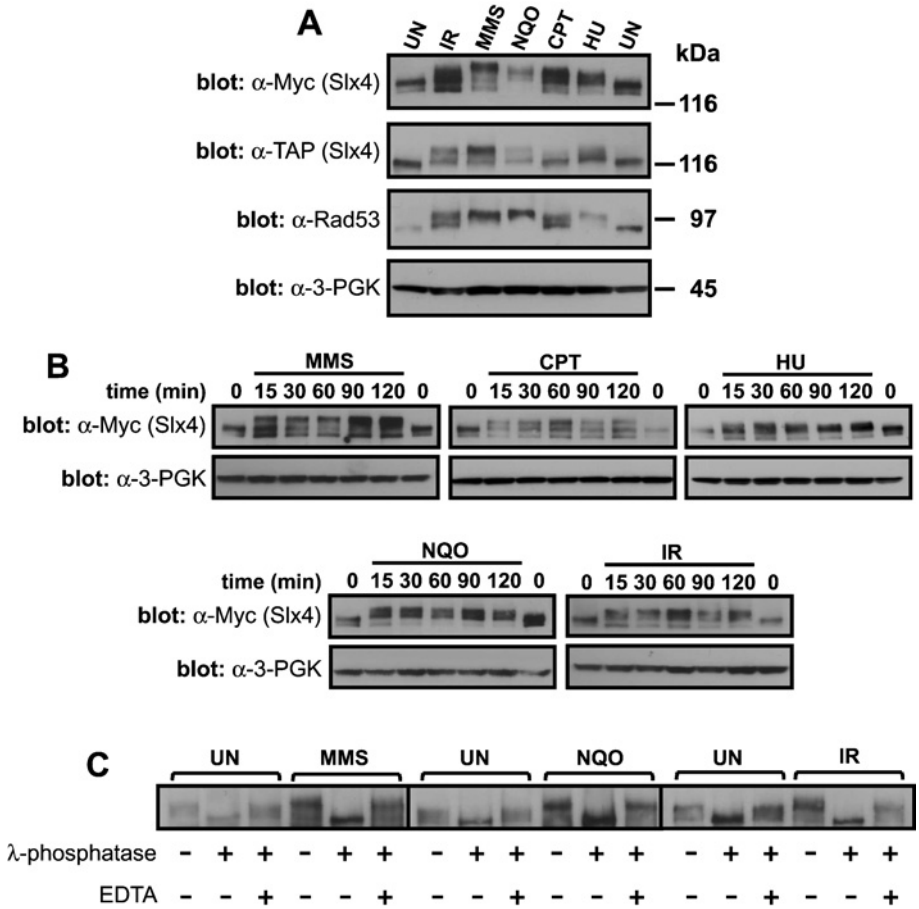

Figure 1 DNA damage triggers phosphorylation of Slx4

Strains SFY002 and SFY003 (**A**) were grown to mid-exponential phase in liquid culture and incubated in the presence of MMS (0.02 %), NQO (5 μg/ml), CPT (5 μg/ml), HU (0.2 M) or left untreated (UN) for 90 min (**A**) or for the times indicated (**B**). Alternatively, cells were exposed to IonR (IR; 150 Gy) and incubated at 30 °C for 50 min (**A**) or for the times indicated (**B**). Trichloracetic acid extracts were prepared and subjected to Western-blot analysis with anti-Myc, anti-3-PGK, and anti-Rad53 antibodies or with rabbit IgG (to recognize the Protein A moiety of TAP–Slx4, second panel in **A** only). (**C**) Strain SFY002 was grown to mid-exponential phase in liquid culture and incubated in the presence of MMS (0.02 %) or NQO (5 μg/ml) or left untreated (UN) for 90 min. Alternatively, cells were exposed to IonR (150 Gy) and incubated at 30 °C for 50 min. Native extracts were prepared and aliquots (2 mg) were subjected to immunoprecipitation with anti-Myc antibodies. The immunoprecipitates were washed extensively and either left untreated or incubated with λ-phosphatase (200 units) for 30 min at 30 °C in the presence or absence of EDTA (100 mM). Beads were then subjected to Western-blot analysis with anti-Myc antibodies.

for 30 min before FACS analysis using a Becton Dickinson FACSort machine.

Analysis of chromosomes by PFGE (pulsed-field gel electrophoresis)

Asynchronously growing cells (or cells arrested in G_2 phase) were grown to mid-exponential phase (D_{600} 2.0) in YPAD at 30 °C. After adding MMS for various times, depending on the experiment, cells were filtered, washed extensively with YPAD, containing 10 % (w/v) sodium thiosulphate, and incubated in YPAD at 30 °C (alternatively containing nocodazole). Sample preparation for the PFGE were performed as described previously [35].

RESULTS

DNA damage triggers phosphorylation of Slx4

A yeast strain was constructed in which the *SLX4* gene bears a Myc(13)-epitope tag. These cells were not hypersensitive to MMS, unlike *slx4*Δ cells, indicating that the Myc(13) tag did not affect Slx4 function (results not shown). Western blotting of extracts from exponentially growing cells with anti-Myc antibodies revealed a heterogeneous band (Figure 1A, top panel), not present in extracts of untagged cells (results not shown), corresponding to Slx4. Interestingly, exposure of cells to agents that cause different types of DNA damage caused a striking decrease in the electrophoretic mobility of Slx4–Myc (Figure 1A, top panel). Slx4–Myc in resting cells migrated as a doublet, whereas after DNA damage a third band appeared. The most pronounced shift in the electrophoretic mobility of Slx4–Myc was caused by MMS (Figure 1A, top panel), which potently impedes progression of DNA replication forks [36]. Slx4 mobility was also affected by exposure to CPT (camptothecin), which causes S-phase-specific DSBs (double- strand breaks) and was less weakly altered by HU (hydroxyurea), that stalls DNA replisomes by depleting cellular dNTP pools (Figure 1A, top panel). Exposure of cells to 4-NQO (4-nitroquinoline oxide), which mimics UV irradiation, or to ionizing radiation (IonR), which causes primarily DSBs, also changed the electrophoretic mobility of Slx4–Myc (Figure 1A, top panel). Similar results were obtained with TAP-tagged Slx4 (Figure 1A, second panel) and the mobility of an unrelated protein, 3-PGK, was unaffected (Figure 1A, fourth panel). Time-course experiments revealed that Slx4–Myc shifts

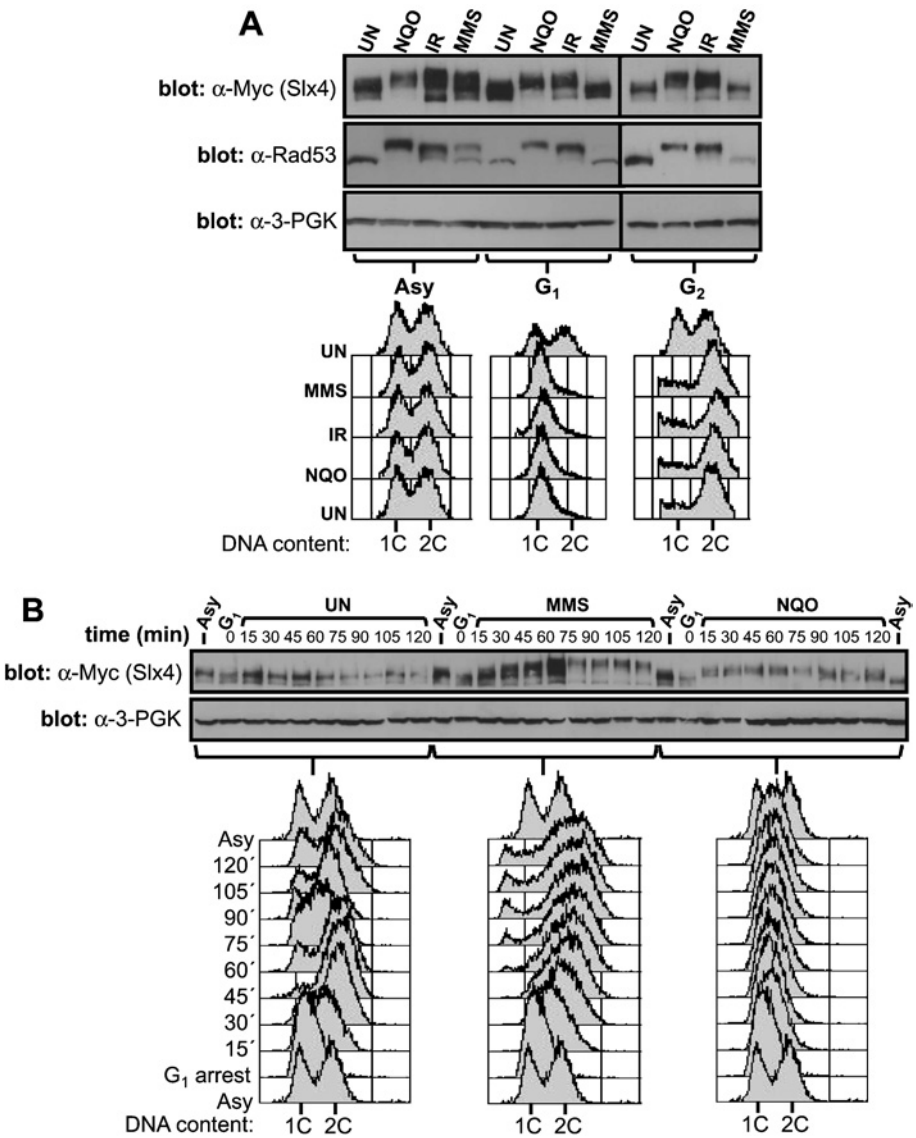

Figure 2 Slx4 is phosphorylated in response to DNA damage at all cell-cycle stages, but not during an unperturbed cell cycle

(**A**) Strain SFY002 was grown to mid-exponential phase, arrested in G_1-phase by the presence of α-factor or in G_2-phase by the addition of nocodazole and held there during treatment with MMS (0.02 %) or NQO (5 μg/ml) or without drug (UN) for 15 min; alternatively arrested cells were exposed to IonR (IR; 150 Gy) and incubated at 30 °C for 15 min before cell lysis. Trichloroacetic acid extracts were prepared and subjected to Western-blot analysis with antibodies against Myc, Rad53 or 3-PGK. In parallel, samples of cells (1×10^7) were fixed in ethanol and subjected to FACS analysis. (**B**) α-factor synchronized cultures were released from arrest at time zero into pre-warmed YPAD or into pre-warmed YPAD containing MMS (0.02 %), NQO (5 μg/ml) or no drug (UN). Samples were taken at the indicated times after release and analysed by FACS or subjected to Western-blot analysis with antibodies against Myc or 3-PGK.

rapidly (within 15 min) after exposure to genotoxins, and this is sustained for at least 120 min (Figure 1B).

The change in the electrophoretic mobility shift of Slx4 after DNA damage was similar to that of Rad53 (Figure 1A, third panel); the altered mobility of Rad53 is caused by Mec1/Tel1-mediated Rad53 phosphorylation [3]. To test whether the mobility shift of Slx4 after DNA damage was due to phosphorylation, Slx4–Myc was immunoprecipitated and subjected to treatment with λ-phosphatase. The mobility of Slx4–Myc isolated from untreated cells was increased by phosphatase treatment, suggesting that Slx4 is basally phosphorylated. The alteration in electrophoretic mobility of Slx4–Myc triggered by DNA damage was completely reversed by treatment of Slx4–Myc immunoprecipitates with λ-phosphatase and this was prevented by including inhibitors of λ-phosphatase such as EDTA (Figure 1C), microcystin-LR or sodium orthovanadate (results not shown). These data indicate that Slx4 becomes phosphorylated in response to DNA damage or when replication forks stall.

Slx4 is phosphorylated in response to DNA damage in a cell-cycle-independent manner

The synthetic lethal interaction of Slx4 with Sgs1, together with the observation that bacterially expressed Slx1–Slx4 preferentially cleaves replication-fork-like structures, suggested that Slx4 may function predominantly during S-phase. We tested whether Slx4 phosphorylation after DNA damage is restricted to S-phase. To do this, cells were arrested in the G_1- or G_2-phase of the cell cycle and treated with 4-NQO, MMS or IonR. As shown in Figure 2(A), Slx4–Myc was phosphorylated to the same

extent in G_1 or G_2-phase as in asynchronous cells after treatment with 4-NQO and IonR (Figure 2A), indicating that DNA-damage-induced phosphorylation of Slx4 is not restricted to a particular cell-cycle stage. Phosphorylation of Slx4–Myc in response to MMS was not observed outside of S-phase. Similar results were obtained with Rad53 (Figure 2A). These data are consistent with previous reports that, although MMS induces DNA damage that can be repaired at all cell-cycle stages, cells must be in S-phase for MMS to activate certain aspects of the DNA damage response [37].

We wished to exclude the possibility that DNA-damage-induced phosphorylation of Slx4 is simply due to arrest of the cell cycle at a point where Slx4 is normally phosphorylated. When cells were released from a G_1 arrest in the absence of genotoxin, no detectable change in the phosphorylation of Slx4–Myc was observed as the cells progressed through the cell cycle (Figure 2B). However, when cells were released into S-phase in the presence of MMS or 4-NQO, Slx4–Myc became highly phosphorylated (Figure 2B, upper panel). Maximal NQO-induced Slx4 phosphorylation was observed at the earliest time point (15 min) after release from arrest, before most cells had entered S-phase (Figure 2B, lower panel), whereas maximal MMS-induced Slx4 phosphorylation did not occur until most cells were in S-phase, 60 min after release from G_1 arrest (Figure 2B, lower panel). This is consistent with previous reports that MMS does not efficiently activate Mec1/Tel1 outside of S-phase [37]. The apparent increase in the intensity of the Slx4 signal after release of cells from G_1-phase into MMS was not reproducible and probably does not reflect a genuine increase in Slx4 protein levels under these conditions (results not shown).

Slx4 phosphorylation requires both Mec1 and Tel1

To investigate which kinase phosphorylates Slx4 in response to DNA damage/fork stalling, phosphorylation of Slx4–Myc was examined in cells lacking Mec1, Tel1 or in cells lacking both Mec1 and Tel1. Phosphorylation of Slx4 was very slightly reduced in *mec1*Δ cells and was barely affected in cells lacking Tel1, after exposure to IonR, MMS, NQO or CPT (Figures 3A and 3B). However, in cells lacking both Mec1 and Tel1, phosphorylation of Slx4–Myc after DNA damage was completely abolished (Figures 3A and 3B). In contrast, phosphorylation of Rad53 in response to MMS, NQO and CPT was abolished in cells lacking only Mec1 (Figure 3B). Time-course experiments showed that Slx4–Myc phosphorylation after genotoxic insult was not merely delayed but completely absent in the *mec1*Δ*tel1*Δ strain (Figures 3C and 3D, and results not shown). Interestingly, phosphorylation of Slx4 in response to HU was more pronounced in *mec1*Δ cells than in wild-type cells (Figure 3B).

As Mec1 and Tel1 activate Rad53 and Chk1 after DNA damage, Slx4 phosphorylation might be mediated by Rad53/Chk1. To test this hypothesis, yeast cells lacking Rad53 and Chk1 were exposed to genotoxic agents. In this strain, Slx4–Myc became phosphorylated in response to DNA damage, although not entirely to wild-type levels (Figure 3E). This indicates that phosphorylation of Slx4 after DNA damage is complex in nature and is probably mediated by several protein kinases.

Slx4 is required for efficient repair of DNA alkylation damage, but not for activation of the intra-S-phase checkpoint

It was previously demonstrated that cells lacking Slx4, but not Slx1, are hypersensitive to DNA alkylation damage induced by MMS [29,38], although the basis for this defect was not explored. To investigate this point further, the involvement of Slx4 in cell-cycle checkpoints and DNA repair was examined. Activation of Mec1 and Tel1 is required for phosphorylation and activation of Rad53, which is, in turn, important for cell-cycle checkpoints [39]. As shown in Figure 4(A), DNA-damage-induced phosphorylation of Rad53 occurred normally in the absence of Slx1, and was even more pronounced in the absence of Slx4 than in wild-type cells. Rad53 phosphorylation was severely decreased in cells lacking Mec1 (Figure 4A).

The major lesion (N^3-methyladenine) induced by MMS potently impedes replication fork movement [36] and activates the intra-S phase checkpoint; MMS does not efficiently activate Rad53 or cause cell cycle arrest outside of S-phase [37] (probably because DNA repair is so efficient). To test the potential involvement of Slx1–Slx4 in activation of the intra-S-phase checkpoint, cells were arrested in G1-phase, released from arrest in the presence or absence of MMS, and cell-cycle progression was monitored by FACS analysis. When wild-type cells were released into S-phase in the presence of MMS, DNA replication proceeded very slowly compared with cells incubated in the absence of this drug (Figure 4B). Cells lacking either Slx1 or Slx4 also proceeded very slowly through S-phase in the presence of MMS and were undistinguishable from wild-type cells in this regard (Figure 4B). In contrast, the intra-S-phase checkpoint was not evident in cells lacking Mec1 (Figure 4B). Thus Slx4 is not required for intra-S-phase checkpoint activation.

The involvement of Slx4 in DNA repair was next investigated using PFGE. Asynchronously growing cells were exposed to MMS and, after 45 min, MMS was removed and the cells were washed and allowed to recover. At various times, chromosomes were prepared from wild-type, *slx4*Δ, *slx1*Δ and *sgs1*Δ cells and separated by PFGE. These gels resolved the 16 yeast chromosomes into a characteristic ladder of bands, visualized by ethidium bromide staining (Figure 4C, upper panel; for example, lanes 1, 6, 11 and 16). Treatment of cells with MMS (0.1 %) for 45 min resulted in extensive chromosome fragmentation, as seen by the appearance of a 'smear' of low-molecular–mass DNA species (Figure 4C, upper panel, lanes 2, 7, 12 and 17). When wild-type cells were washed free of MMS and allowed to recover, these low-molecular-mass species disappeared and intact chromosomes reappeared, indicating that the chromosomal damage induced by MMS had been efficiently repaired (Figure 4C, upper panel, lanes 3–5). However, when *slx4*Δ cells were allowed to recover from exposure to MMS, intact chromosomes did not reappear (Figure 4C, upper panel, lanes 8–10), suggesting a defect in the repair of alkylation-induced DNA damage. Similar results were obtained with *sgs1*Δ cells (Figure 4C, upper panel, lanes 18–20), consistent with a previous report [14]. Interestingly, MMS-induced DNA damage was repaired in *slx1*Δ cells with similar efficiency to wild-type cells (Figure 4C, upper panel, lanes 13–15). Thus a correlation was observed between MMS hypersensitivity and defective recovery from MMS-induced DNA damage: *slx4*Δ cells but not *slx1*Δ cells are hypersensitive to MMS (Figure 4C, lower panel) [29]. Similar results were obtained in four independent experiments. Methylene Blue staining indicated that lack of DNA repair in *slx4*Δ cells was not a result of cell death, since more than 90 % of cells were still alive 5 h after recovery from MMS (results not shown).

It has been reported that chromosomes containing replication forks or bubbles do not enter pulsed-field gels [40], and thus failure to enter the gel may indicate a defect in the recovery of stalled forks as opposed to a defect in DNA repair. Thus it is possible that Slx4 is required for either DNA repair or recovery of stalled replication forks or for both. To investigate this further, PFGE analysis was carried out using cells arrested in the G_2-phase of the cell cycle, where there are no DNA replication forks. Cells were

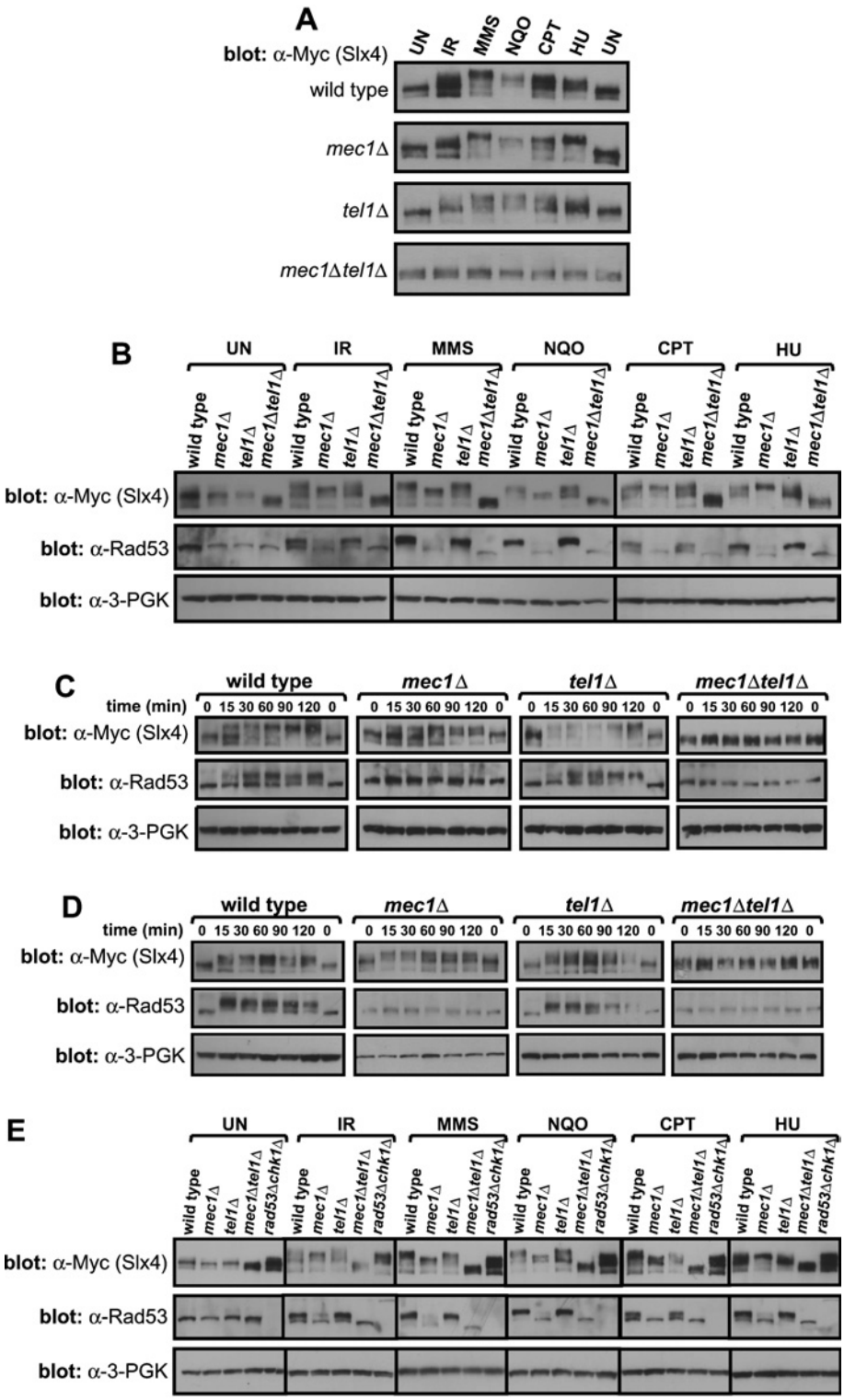

Figure 3 Slx4 phosphorylation is dependent on Mec1 and Tel1

Strains SFY001, SFY004 (*mec1*Δ), SFY005 (*tel1*Δ) SFY006 (*mec1*Δ*tel1*Δ) (**A**–**D**) and strain SFY007 (*rad53*Δ*chk1*Δ) (**E**) were grown to mid-exponential phase in liquid culture and incubated in the presence of MMS (0.02 %), NQO (5 μg/ml), CPT (5 μg/ml) or HU (0.2 M) or left untreated (UN) for 90 min (**A**, **B** and **E**) or incubated in MMS (0.02 %) for the times indicated (**C**). Alternatively, cells were exposed to IonR (IR; 150 Gy) and incubated at 30 °C for 50 min (**A**, **B** and **E**) or for the times indicated (**D**). Trichloroacetic acid extracts were prepared and subjected to Western-blot analysis with antibodies against Myc, Rad53 or 3-PGK.

synchronized in G_2-phase with nocodazole, treated with MMS (0.05 %) while still arrested, washed free of MMS and allowed to recover in the presence of nocodazole to maintain cells in G_2-phase. FACS analysis was used to confirm that G_2-phase arrest was maintained throughout (results not shown). In these experiments, 0.05 % MMS was used instead of the

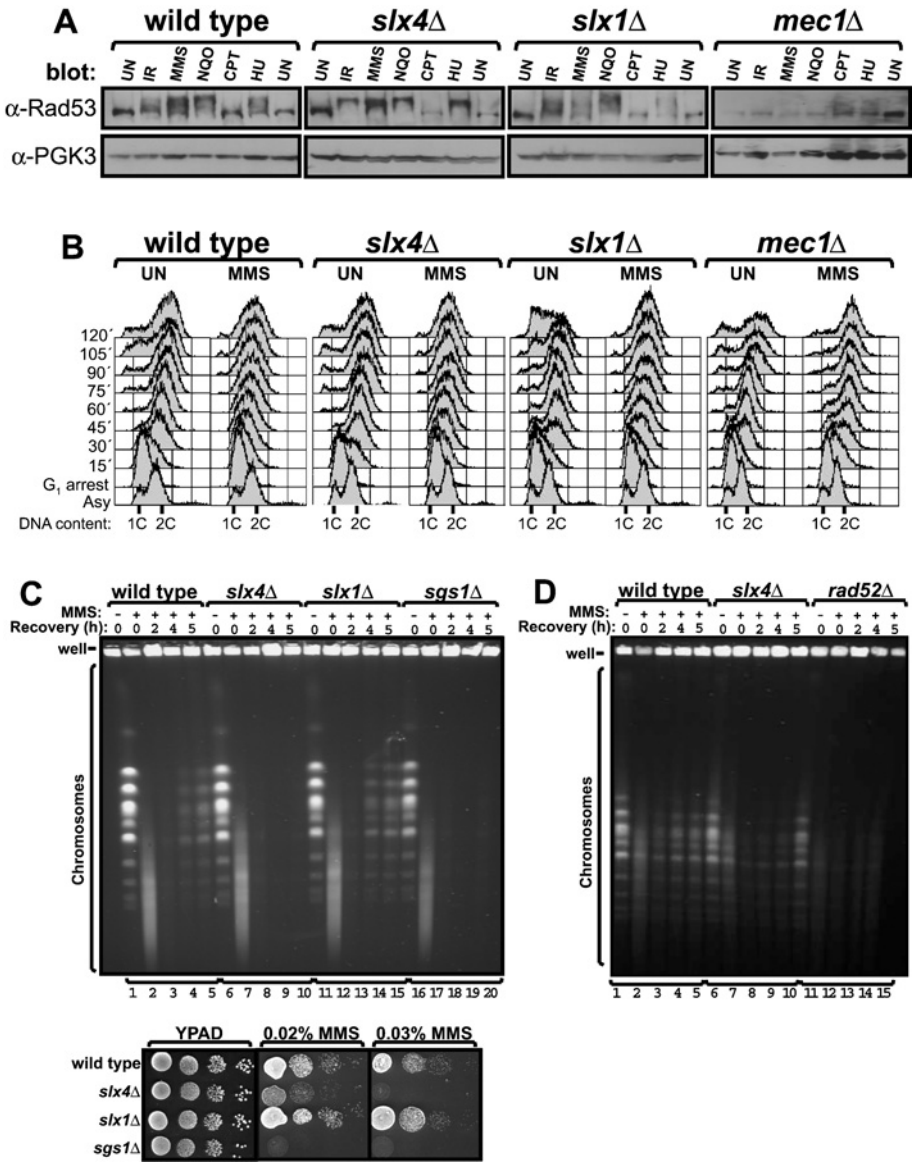

Figure 4 Slx4 is required for efficient repair of DNA alkylation damage but not for the intra-S-phase checkpoint

(**A**) Strains BY4741, SFY008, SFY009 and DDY053 were grown to mid-exponential phase in liquid culture and incubated in the presence of MMS (0.02 %), NQO (5 μg/ml), CPT (5 μg/ml), HU (0.2 M) or left untreated (UN) for 90 min. Alternatively, cells were exposed to IonR (IR; 150 Gy) and incubated at 30 °C for 50 min. Trichloroacetic acid extracts were prepared and subjected to Western-blot analysis with antibodies against Rad53 or 3-PGK. (**B**) Strains BY4741, SFY008, SFY009 and DDY053 were grown to mid-exponential phase and arrested in G_1-phase by the presence of α-factor. Cells were released from G_1-phase arrest into YPAD containing nocodazole either with or without MMS (0.033 %). FACS samples were taken at the indicated time points. (**C**) Strains BY4741, SFY008, SFY009 and SFY010 were grown to mid-exponential phase and treated with 0.1 % MMS for 45 min. Cells were filtered, washed extensively and incubated in YPAD at 30 °C for 5 h. Samples for the PFGE were taken before treatment with MMS (lanes 1, 6, 11 and 16) and at 0 (lanes 2, 7, 12 and 17), 2 (lanes 3, 8, 13 and 18), 4 (lanes 4, 9, 14 and 19) and 5 (lanes 5, 10, 15 and 20) h after cells were washed free of MMS. PFGE analysis was carried out as described in the Experimental section. (**D**) Same as (**C**), except that strains BY4741, SFY008 and SFY012 were grown to mid-exponential phase, arrested in G_2-phase and MMS (0.05 %) was added to the arrested cells. After 45 min cells were filtered, washed free of MMS and incubated at 30 °C in YPAD containing nocodazole to maintain G_2-phase arrest. Samples for the PFGE were taken before treatment with MMS (lanes 1, 6 and 11) and at 0 (lanes 2, 7 and 12), 2 (lanes 3, 8 and 13), 4 (lanes 4, 9 and 14) and 5 (lanes 5, 10 and 15) h after cells were washed free of MMS. PFGE analysis was carried out as described in the Experimental section.

0.1 % which was used in Figure 4(C). This is because although treatment of asynchronously growing wild-type cells with 0.1 % MMS had negligible effect on cell viability, DNA damage caused by treatment of G_2-phase-arrested cells with 0.1 % MMS could not be repaired and most cells died. In contrast, the viability of G_2-phase-arrested cells was barely affected by treatment with 0.05 % MMS. It is not clear why G_2-phase-arrested cells are more sensitive to MMS.

Treatment with MMS caused chromosome fragmentation in G_2-phase-arrested cells and, when wild-type cells were allowed to recover, DNA repair generated intact chromosomes which appeared between 2 and 5 h after MMS treatment (Figure 4D, lanes 3, 4 and 5). Repair of MMS-induced DNA damage was severely reduced in cells lacking Rad52 (Figure 4D, lanes 13, 14, 15), a major regulator of homologous recombination [41], the preferred mode of DNA repair in diploid cells. In cells lacking Slx4, repair of MMS-induced DNA damage did not occur as efficiently as in wild-type cells, although the defect was not as pronounced as in cells lacking Rad52 (Figure 4D, lanes 8, 9, 10). Similar results were obtained in four independent experiments.

These results suggest that Slx4 is required for efficient DNA repair at different cell-cycle stages.

DISCUSSION

Several observations suggest a role for the Slx1–Slx4 complex in processing stalled replisomes. First, their genes are synthetic lethal with Sgs1 [24]. Secondly, recombinant Slx1–Slx4 preferentially cleaves replication fork-like structures *in vitro* [29]. Thirdly, Slx4 is required for maintenance of the ribosomal DNA repeats where replication forks stall at high frequency [30,31]. However, it is possible that, in addition to processing stalled replisomes, Slx1–Slx4 plays a more general role in the cellular response to DNA damage. Although Sgs1 is important for the correct processing of stalled replication forks, it is also important for homologous recombinational DNA repair in G_2-phase-arrested cells [14]. In the present study we demonstrated that Slx4 becomes phosphorylated after inducing a range of DNA lesions and in response to DNA replisome stalling (Figure 1). Phosphorylation of Slx4 is not restricted to S-phase (Figure 2), suggesting that, like Sgs1, it has a more general function in the cellular DNA damage response. Consistent with this, Slx4 is required for efficient repair of DNA alkylation damage, not only in asynchronously growing cells but also in G_2-phase-arrested cells, where there are no DNA replisomes (Figure 4). The PFGE analysis in Figure 4(C) shows that exposure of cells to MMS caused chromosome fragmentation, probably due to the induction of DSBs. In asynchronously growing wild-type cells, although the MMS-induced 'smear' of low-molecular-mass fragments disappeared 2 h after MMS had been removed from the cells, intact chromosomes did not begin to appear for a further 2 h (Figure 4C). This lag might coincide with the resolution of intermediate structures that arise during repair and which prevent chromosomes from leaving the wells of the gels during PFGE. It is likely that this resolution process requires Slx4, since in cells lacking this protein, the MMS-induced smear disappears, but intact chromosomes do not reappear when MMS is removed from the cells. Since the presence of replisomes also prevents chromosomes from entering pulsed-field gels, it is possible that Slx4 is also involved in resolving stalled forks. However, this cannot explain the lack of reappearance of chromosomes on pulsed-field gels after MMS treatment of *slx4*Δ cells arrested in G_2 phase. Better assays are required to examine directly removal of alkylation damage.

The preferred mode of repair of DNA alkylation damage in diploid yeast cells is homologous recombination [42]; the presence of a sister chromatid after DNA replication provides a template for error-free DNA repair of a damaged chromatid. Thus Slx4 may be important for efficient homologous recombination, at least for the repair of alkylation damage, although at the present the molecular basis for this is unclear. Since Slx1 is not required for resistance to MMS, it is unlikely that the nuclease activity of the Slx1–Slx4 complex is required for DNA damage resistance, and it might be that the function of Slx4 in this regard involves proteins other than Slx1. It is possible that there are free pools of Slx4, not associated with Slx1, that have functions separate from those of the Slx1–Slx4 complex and, in this regard, it has not yet been determined what proportion of cellular Slx4 exists in or out of complex with Slx1.

Phosphorylation of Slx4 requires both Mec1 and Tel1 and, to a lesser extent, Rad53 and Chk1, and the pattern of Slx4 phosphorylation after DNA damage is complex in nature. In mammals, p53 and BRCA1 are also phosphorylated at different residues by ATM and by ATM-activated CHK2, with different effects on p53 function [3], and it may be that phosphorylation of Slx4 by different kinases has different effects on its function. The complex nature of Slx4 phosphorylation means that several approaches will be required to determine the sites of DNA-damage-induced phosphorylation. Slx4 contains 18 SQ/TQ motifs (potential Mec1/Tel1 phosphorylation sites) and four potential sites that might be targeted by Rad53/Chk1. We are currently in the process of mapping the DNA damage-induced phosphorylation sites on Slx4 *in vitro* and *in vivo*, and our preliminary analyses indicate that Slx4 phosphorylation is complex in nature and will take some time to resolve. Phospho-specific antibodies will be required to rigorously analyse Slx4 phosphoryaltion at different sites. At present the effects of phosphorylation on Slx4 function are unclear. It is possible, for example, that phosphorylation changes the catalytic activity or substrate specificity of Slx1–Slx4, or channels it into a particular mode of DNA repair. Alternatively phosphorylation may affect the interaction of Slx4 and Slx1 with each other or with other proteins or may change the cellular localization of the Slx1–Slx4 complex. It is noteworthy that, although Slx4 is required for efficient repair of alkylation damage in G_2-phase-arrested cells, Slx4 is not phosphorylated under these conditions (Figure 2A). Many more experiments are required to determine the precise roles of Slx1 and Slx4 in cells and to determine the contribution of Slx4 phosphorylation to Mec1/Tel1-mediated maintenance of genome stability. Orthologues of Slx1 are evident in a range of organisms including worms, frogs, flies and humans [24] and also occur in archaebacteria (results not shown). Searches of the available sequence databases failed, however, to reveal equivalent orthologues of Slx4, although a range of fungal counterparts were identified. However, all known regulators of DNA damage signalling and repair in yeasts have functional orthologues in mammals (results not shown), even though in many cases sequence similarity between orthologues is low. It is highly likely that this also applies to Slx4.

This work was funded by the Medical Research Council (MRC), U.K; S. F. is the recipient of an MRC Predoctoral Fellowship. We are grateful to our colleagues in the laboratory, especially Dr Paul Jowsey and Ms Mary Gardiner, for critical reading of the manuscript and for helpful suggestions. We are grateful to the DNA sequencing facility and to Dr Rosemary Clark for help with FACS analysis, all at WTB/MSI (Welcome Trust Biocentre/Medical Sciences Institute), University of Dundee, Dundee, Scotland, U.K.

REFERENCES

1 Nyberg, K. A., Michelson, R. J., Putnam, C. W. and Weinert, T. A. (2002) Toward maintaining the genome: DNA damage and replication checkpoints. Annu. Rev. Genet. **36**, 617–656
2 Rouse, J. and Jackson, S. P. (2002) Interfaces between the detection, signaling, and repair of DNA damage. Science **297**, 547–551
3 Shiloh, Y. (2003) ATM and related protein kinases: safeguarding genome integrity. Nat. Rev. Cancer **3**, 155–168
4 Bashkirov, V. I., King, J. S., Bashkirova, E. V., Schmuckli-Maurer, J. and Heyer, W. D. (2000) DNA repair protein Rad55 is a terminal substrate of the DNA damage checkpoints. Mol. Cell. Biol. **20**, 4393–4404
5 Abraham, R. T. (2004) PI 3-kinase related kinases: 'big' players in stress-induced signaling pathways. DNA Repair **3**, 883–887
6 Abraham, R. T. (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. **15**, 2177–2196
7 Carr, A. M. (2002) DNA structure dependent checkpoints as regulators of DNA repair. DNA Repair **1**, 983–994
8 Barbour, L. and Xiao, W. (2003) Regulation of alternative replication bypass pathways at stalled replication forks and its effects on genome stability: a yeast model. Mutat. Res. **532**, 137–155
9 Fabre, F., Chan, A., Heyer, W. D. and Gangloff, S. (2002) Alternate pathways involving Sgs1/Top3, Mus81/ Mms4, and Srs2 prevent formation of toxic recombination intermediates from single-stranded gaps created by DNA replication. Proc. Natl. Acad. Sci. U.S.A. **99**, 16887–16892

10 Hickson, I. D. (2003) RecQ helicases: caretakers of the genome. Nat. Rev. Cancer **3**, 169–178
11 Khakhar, R. R., Cobb, J. A., Bjergbaek, L., Hickson, I. D. and Gasser, S. M. (2003) RecQ helicases: multiple roles in genome maintenance. Trends Cell Biol. **13**, 493–501
12 Mankouri, H. W. and Hickson, I. D. (2004) Understanding the roles of RecQ helicases in the maintenance of genome integrity and suppression of tumorigenesis. Biochem. Soc. Trans. **32**, 957–958
13 Cobb, J. A., Bjergbaek, L., Shimada, K., Frei, C. and Gasser, S. M. (2003) DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1. EMBO J. **22**, 4325–4336
14 Ui, A., Seki, M., Ogiwara, H., Onodera, R., Fukushige, S., Onoda, F. and Enomoto, T. (2005) The ability of Sgs1 to interact with DNA topoisomerase III is essential for damage-induced recombination. DNA Repair **4**, 191–201
15 Gangloff, S., McDonald, J. P., Bendixen, C., Arthur, L. and Rothstein, R. (1994) The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase. Mol. Cell. Biol. **14**, 8391–8398
16 Watt, P. M., Hickson, I. D., Borts, R. H. and Louis, E. J. (1996) SGS1, a homologue of the Bloom's and Werner's syndrome genes, is required for maintenance of genome stability in *Saccharomyces cerevisiae*. Genetics **144**, 935–945
17 Mullen, J. R., Kaliraman, V. and Brill, S. J. (2000) Bipartite structure of the SGS1 DNA helicase in *Saccharomyces cerevisiae*. Genetics **154**, 1101–1114
18 Yamagata, K., Kato, J., Shimamoto, A., Goto, M., Furuichi, Y. and Ikeda, H. (1998) Bloom's and Werner's syndrome genes suppress hyperrecombination in yeast sgs1 mutant: implication for genomic instability in human diseases. Proc. Natl. Acad. Sci. U.S.A. **95**, 8733–8738
19 Frei, C. and Gasser, S. M. (2000) The yeast Sgs1p helicase acts upstream of Rad53p in the DNA replication checkpoint and colocalizes with Rad53p in S-phase-specific foci. Genes Dev. **14**, 81–96
20 Sengupta, S., Robles, A. I., Linke, S. P., Sinogeeva, N. I., Zhang, R., Pedeux, R., Ward, I. M., Celeste, A., Nussenzweig, A., Chen, J. et al. (2004) Functional interaction between BLM helicase and 53BP1 in a Chk1-mediated pathway during S-phase arrest. J. Cell. Biol. **166**, 801–813
21 Davies, S. L., North, P. S., Dart, A., Lakin, N. D. and Hickson, I. D. (2004) Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest. Mol. Cell. Biol. **24**, 1279–1291
22 Tong, A. H., Evangelista, M., Parsons, A. B., Xu, H., Bader, G. D., Page, N., Robinson, M., Raghibizadeh, S., Hogue, C. W., Bussey, H. et al. (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science **294**, 2364–2368
23 Gangloff, S., Soustelle, C. and Fabre, F. (2000) Homologous recombination is responsible for cell death in the absence of the Sgs1 and Srs2 helicases. Nat. Genet. **25**, 192–194
24 Mullen, J. R., Kaliraman, V., Ibrahim, S. S. and Brill, S. J. (2001) Requirement for three novel protein complexes in the absence of the Sgs1 DNA helicase in *Saccharomyces cerevisiae*. Genetics **157**, 103–118
25 Kaliraman, V., Mullen, J. R., Fricke, W. M., Bastin-Shanower, S. A. and Brill, S. J. (2001) Functional overlap between Sgs1-Top3 and the Mms4-Mus81 endonuclease. Genes Dev. **15**, 2730–2740
26 Doe, C. L., Ahn, J. S., Dixon, J. and Whitby, M. C. (2002) Mus81-Eme1 and Rqh1 involvement in processing stalled and collapsed replication forks. J. Biol. Chem. **277**, 32753–32759
27 Whitby, M. C., Osman, F. and Dixon, J. (2003) Cleavage of model replication forks by fission yeast Mus81–Eme1 and budding yeast Mus81–Mms4. J. Biol. Chem. **278**, 6928–6935
28 Boddy, M. N., Gaillard, P. H., McDonald, W. H., Shanahan, P., Yates, 3rd, J. R. and Russell, P. (2001) Mus81–Eme1 are essential components of a Holliday junction resolvase. Cell **107**, 537–548
29 Fricke, W. M. and Brill, S. J. (2003) Slx1-Slx4 is a second structure-specific endonuclease functionally redundant with Sgs1–Top3. Genes Dev. **17**, 1768–1778
30 Kaliraman, V. and Brill, S. J. (2002) Role of SGS1 and SLX4 in maintaining rDNA structure in *Saccharomyces cerevisiae*. Curr. Genet. **41**, 389–400
31 Coulon, S., Gaillard, P. H., Chahwan, C., McDonald, W. H., Yates, 3rd, J. R. and Russell, P. (2004) Slx1–Slx4 are subunits of a structure-specific endonuclease that maintains ribosomal DNA in fission yeast. Mol. Biol. Cell **15**, 71–80
32 Longtine, M. S., McKenzie, 3rd, A., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J. R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast **14**, 953–961
33 D'Amours, D. and Jackson, S. P. (2001) The yeast Xrs2 complex functions in S phase checkpoint regulation. Genes Dev. **15**, 2238–2249
34 Rouse, J. and Jackson, S. P. (2000) *LCD1*: an essential gene involved in checkpoint control and regulation of the *MEC1* signalling pathway in *Saccharomyces cerevisiae*. EMBO J. **19**, 5801–5812
34a Rose, M. D., Meluh, P. B. and Hieter, P. (1990) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
35 Rouse, J. (2004) Esc4p, a new target of Mec1p (ATR), promotes resumption of DNA synthesis after DNA damage. EMBO J. **23**, 1188–1197
36 Beranek, D. T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat. Res. **231**, 11–30
37 Tercero, J. A., Longhese, M. P. and Diffley, J. F. (2003) A central role for DNA replication forks in checkpoint activation and response. Mol. Cell **11**, 1323–1336
38 Chang, M., Bellaoui, M., Boone, C. and Brown, G. W. (2002) A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. Proc. Natl. Acad. Sci. U.S.A. **99**, 16934–16939
39 Lee, S. J., Schwartz, M. F., Duong, J. K. and Stern, D. F. (2003) Rad53 phosphorylation site clusters are important for Rad53 regulation and signaling. Mol. Cell. Biol. **23**, 6300–6314
40 Hennessy, K. M., Lee, A., Chen, E. and Botstein, D. (1991) A group of interacting yeast DNA replication genes. Genes Dev. **5**, 958–969
41 Krogh, B. O. and Symington, L. S. (2004) Recombination proteins in yeast. Annu. Rev. Genet. **23**, 233–271
42 Xiao, W., Chow, B. L. and Rathgeber, L. (1996) The repair of DNA methylation damage in *Saccharomyces cerevisiae*. Curr. Genet. **30**, 461–468

Received 10 May 2005/17 June 2005; accepted 23 June 2005
Published as BJ Immediate Publication 23 June 2005, doi:10.1042/BJ20050768