A role for chromatin remodellers in replication of damaged DNA

Atsuko Niimi, Anna L. Chambers, Jessica A. Downs* and Alan R. Lehmann*

Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton BN1 9RQ, UK

ABSTRACT

In eukaryotic cells, replication past damaged sites in DNA is regulated by the ubiquitination of proliferating cell nuclear antigen (PCNA). Little is known about how this process is affected by chromatin structure. There are two isoforms of the Remodels the Structure of Chromatin (RSC) remodelling complex in yeast. We show that deletion of *RSC2* results in a dramatic reduction in the level of PCNA ubiquitination after DNA-damaging treatments, whereas no such effect was observed after deletion of *RSC1*. Similarly, depletion of the BAF180 component of the corresponding PBAF (Polybromo BRG1 (Brahma-Related Gene 1) Associated Factor) complex in human cells led to a similar reduction in PCNA ubiquitination. Remarkably, we found that depletion of BAF180 resulted after UV-irradiation, in a reduction not only of ubiquitinated PCNA but also of chromatin-associated unmodified PCNA and Rad18 (the E3 ligase that ubiquitinates PCNA). This was accompanied by a modest decrease in fork progression. We propose a model to account for these findings that postulates an involvement of PBAF in repriming of replication downstream from replication forks blocked at sites of DNA damage. In support of this model, chromatin immunoprecipitation data show that the RSC complex in yeast is present in the vicinity of the replication forks, and by extrapolation, this is also likely to be the case for the PBAF complex in human cells.

INTRODUCTION

DNA damage interrupts the progress of replication forks, as replicative DNA polymerases are unable to accommodate most damaged bases in their active sites. In order to deal with this problem, all organisms have evolved a series of specialized DNA polymerases that have lower stringency than replicative polymerases and are able to replicate past different damaged bases (1,2). This is achieved by virtue of their more open active site and the lack of an associated 3′-5′ exonuclease 'editing function'. Replication past damaged sites—translesion synthesis (TLS)—may take place either at the stalled fork or, following replication restart beyond the lesion, behind the fork (2–4). The sliding clamp accessory protein, β-clamp in *Escherichia coli* or proliferating cell nuclear antigen (PCNA) in eukaryotes, plays a crucial regulatory role in mediating the switch from the replicative polymerase blocked at a lesion to the specialized TLS polymerase. In eukaryotes, single-stranded regions exposed at the blocked fork trigger the ubiquitination of PCNA on lysine-164 (5,6). This is brought about by the E2 ubiquitin-conjugating enzyme Rad6 and the E3 ubiquitin ligase, Rad18, the latter being activated by single-stranded DNA coated with the single-strand DNA binding protein Replication Protein A (RPA) (7). Most of the TLS polymerases belong to the Y-family (1,8) and all members of this family have ubiquitin-binding motifs, as well as PCNA-binding motifs (9,10) close to their C-termini. Ubiquitination of PCNA thereby, increases the affinity of these polymerases for the PCNA molecules at the blocked forks, providing a mechanism for the switch from replicative to TLS polymerase (6).

Recent work with chicken DT40 cells suggested that TLS at the replication fork was mediated by Rev1, whereas TLS in gaps left behind the replication fork was stimulated by PCNA ubiquitination (11). Further work from the same group revealed that when Rev1 was absent, the transmission of histone modification patterns from parental to daughter nucleosomes was disturbed (12). These results implicated a connection between replication of damaged DNA and the maintenance of chromatin structure.

Chromatin structure is modulated by two classes of proteins. Histone modifiers alter chromatin structure and regulate association of chromatin binding proteins by acetylating, methylating, phosphorylating or ubiquitinating histones (13). Chromatin remodellers use ATP

---

hydrolysis to move, slide or alter the composition of histones within nucleosomes. This is brought about by an ATPase component present in each of the different remodelling complexes (14).

Several reports have implicated chromatin remodelling complexes in responses to DNA damage. For example, both the Remodels the Structure of Chromatin (RSC) and INO80 complexes are recruited to double-strand breaks in yeast (15–19), and in human cells evidence has been provided for a role for INO80 in recruitment of XPC during nucleotide excision repair (20) and for the SWI/SNF complexes (related to budding yeast SWI/SNF and RSC) in phosphorylation of H2AX in response to DNA damage (21–23). INO80 and ISW2 have also been implicated in promoting replication fork progression, particularly at times of replication stress (24–27).

In order to gain further insight into how chromatin structure impacts on replication of damaged DNA, we have investigated the effects of deleting chromatin remodelling genes from *Saccharomyces cerevisiae* or depleting their mRNAs from human cells. Our data show a major involvement of one of the RSC chromatin remodelling isoforms in ubiquitination of PCNA in yeast and a similar role for the corresponding complex (Polybromo BRG1 (Brahma-Related Gene 1) Associated Factor) or SWI/SNF-B) in human cells.

MATERIALS AND METHODS

Antibodies

The yeast α-PCNA antibody was a kind gift from H. Ulrich. The anti-human polη antibody was raised in rabbit against the full-length protein (28). Other antibodies used in this work are as follows: yeast ubiquitin (P4D1; Cell Signaling Technology), human BAF180 (Bethyl), human PCNA (PC10; Cancer Research, UK), human Rad18 (Abcam), human RPA (RPA70-9; Calbiochem), human histone H3 (Abcam), BrdU (BD) and Myc (9E10; Cancer Research UK).

Yeast strains and plasmids

Yeast cells were cultured in standard YPD (Yeast extract-Peptone-Dextrose) media at 30°C. $^{His}POL30$ and $^{His}POL30\ K164R$ have been described previously (29,30). $rsc1\Delta$ and $rsc2\Delta$ were created in the same background. For complementation, pRS413-Rsc1 (JD580) or pRS413-Rsc2 (JD579) plasmids were transformed into $rsc2\Delta$ cells and cultured in standard Synthetic Dextrose (SD) (-His) media. For ChIP, a C-terminal 13Myc-tag was introduced in frame with the *INO80* or *RSC2* gene in BY4741.

Detection of yeast PCNA ubiquitination

Following by treatment with methyl methanesulfonate (MMS), hydroxyurea (HU) or ultraviolet light (UV), $^{His}PCNA$ was isolated by Ni-NTA under denaturing conditions and detected by western blotting as described previously (7). The dilutions of antibodies were as follows: α-yeast PCNA (1:2500) and α-yeast ubiquitin (1:1000).

Yeast chromatin fractionation

Where indicated, 50 ml mid-log phase cultures were irradiated with $100\ Jm^{-2}$ and incubated for a further hour at 30°C before being harvested and fractionated. Fractionation involved treatment with zymolyase to create spheroplasts, which were then resuspended in 1 ml lysis buffer (0.4 M sorbitol, 150 mM potassium acetate, 2 mM magnesium acetate, 20 mM HEPES/KOH pH 6.5, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, 0.5 µg/ml leupeptin). Spheroplasts were washed three times in lysis buffer, with centrifugation at 4200 rpm for 3 min between each wash. Triton X-100 was added to 1% final concentration and the insoluble fraction was pelleted at 14 000 rpm for 15 min at 4°C. The chromatin pellet was resuspended in 1 x loading buffer and was analysed by western blot.

Cell culture

SV40-transformed MRC5V1, XP12RO (XP-A) and XP30RO (XP-V) fibroblasts were grown in Eagle's MEM (Invitrogen) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 15% foetal calf serum (PAA laboratories).

RNA interference

MRC5V1, XP12RO and XP30RO cells were plated at 5×10^4 cells per 3.5-cm dish and transfected with 20 nM BAF180 siRNA or 20 nM non-targeting control (ON-TARGET plus SMART pool, Dharmacon), and then again with the same siRNAs 24 h later using Hiperfect transfection reagent (Qiagen). UV ($10\ Jm^{-2}$), MMS (1 mM for 1 h followed by 3-h incubation with fresh medium) and HU (1 mM, 24 h) treatments were performed 72 h after the first transfection.

Western blotting

Cells were washed in PBS and Laemmli buffer added directly to the plates. Triton X-100 extraction of cells was carried out as described previously (6). Cells were harvested by scraping and sonicated to shear the DNA and then loaded onto SDS–PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (GE Healthcare) and immunoblotted with α-BAF180 (1:500), PC10 (1:2500), α-Rad18 (1:2500), α-RPA (1:1000), α-polη (1:1000) or α-histone H3 (1:5000). For quantification, the signal was detected by ImageQuant LAS4000 (GE Healthcare).

FACS

Cells were labelled with 40 µM BrdU (SIGMA Aldrich) for 30 min, trypsinized, washed in PBS and fixed in 70% ethanol. Fixed cells were denatured in 2 M HCl–0.5% tritonX-100 for 30 min, then neutralized in 0.1 M NaB_4O_7 (pH 8.5). Cells were incubated with mouse antibodies to BrdU (1:200) and Fluorescein isothiocyanate-labelled secondary antibodies (1:500), then incubated with 5 µg/ml propidium iodide (Fluka) and analysed in a FACScanto flow cytometer (BD).

Immunolabelling of DNA fibre spreads

Cells were labelled with $20\,\mu M$ CldU (SIGMA Aldrich) for 20 min and then UV-irradiated ($20\,Jm^{-2}$). Cells were incubated with $200\,\mu M$ IdU (SIGMA Aldrich) for 20 min. The cells were trypsinized and resuspended in ice-cold PBS at 5×10^5 cells/ml. Cells ($2.5\,\mu l$) were spotted onto glass slides and lysed with $7.5\,\mu l$ spread buffer (200 mM Tris–HCl pH 7.5, 50 mM EDTA, 0.5% SDS) for 8 min at room temperature. Slides were tilted by 10–20° for the DNA to run down slowly. They were then air-dried and fixed in 3:1 MeOH/acetic acid. The DNA spreads were rinsed with water and denatured with 2.5 M HCl for 1 h. The slides were washed in PBS, then in blocking buffer (1% BSA, 0.1% Tween20 in PBS) and incubated in blocking buffer for 1 h, then with mouse anti-BrdU (1/500, Becton Dickinson) for 2 h at 37°C, washed in PBS and fixed in 4% paraformaldehyde for 10 min. Next they were washed in PBS and blocking buffer, then incubated with rat anti-BrdU (1/1000, Abcam) overnight at 4°C. After rinsing in 50 mM Tris–HCl pH 8.0, 0.5 M NaCl, 0.5% tween20 for 10 min, slides were washed in PBS and blocking buffer, then incubated with Alexa Fluor 488-labelled chicken anti-rat IgG (1/500, Molecular Probes) and Alexa Fluor 546-labelled goat anti-mouse IgG (1/500, Molecular Probes) for 2 h at 37°C. Finally, the slides were washed in PBS and blocking buffer, then PBS again. The signal was analysed using a zeiss microscope and the fibre signal lengths of 150 replication forks per sample were measured using ImageJ.

Cell survival assays

To measure cell survival, with yeast cells, 5-fold serial dilutions of mid-log phase cultures were spotted onto YPAD (Yeast extract-peptone-adenine-dextrose) media and irradiated with the indicated dose of UV.

With human cells, the MRC5V1 cells were treated with siRNA for 72 h, trypsinized and plated for 6 h before UV irradiation with different doses. Colonies were counted after 10 days of further incubation.

Yeast chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described (31), with the following modifications: after α-factor arrest, cells were released into 0.2 M HU and samples were fixed with 1% formaldehyde every 20 min. Immunoprecipitation was performed with anti-myc antibody using Protein G Sepharose 4B (Sigma Aldrich). Real-time PCRs were performed using Mx3005P (Stratagene). Primers that amplify regions in the genome correspond to ARS607, the site 4 kb away from ARS607 and a late replicating Tel VI-R region.

RESULTS

RSC and INO80 complexes and PCNA ubiquitination in yeast

The RSC complex is one of the SWI/SNF complex subfamilies. *In vitro*, it can mediate nucleosome sliding and remove histones from chromatin. The yeast complex contains 17 subunits, some of which are essential (32,33). Strains with deletions of genes encoding non-essential subunits are sensitive to many DNA damaging agents [e.g. Refs (18,34)]. There are two isoforms of the complex, differing only in whether they contain the Rsc1 (10%) or Rsc2 (90%) subunits (35). Rsc1 and Rsc2 have similar structures including two bromodomains and a BAH (Bromo-adjacent homology) domain. Cells in which either subunit is deleted, are viable, whereas deletion of genes encoding both subunits is lethal (35).

We examined the effect of deleting different remodelling genes on PCNA ubiquitination in *S. cerevisiae*. For these experiments, we used a strain expressing a His-tagged *POL30 (PCNA)* gene incorporated into the genome as the only source of PCNA (generously provided by H Ulrich) (30). Cells were treated with different agents, incubated for appropriate periods and PCNA was extracted from cell lysates using NiNTA agarose beads. Proteins adhering to the beads were separated on SDS–PAGE gels, which were then immunoblotted with anti-PCNA and anti-ubiquitin antibodies. The former detects unmodified, mono-ubiquitinated and sumoylated PCNA, whereas the latter detects di- and tri-ubiquitinated forms. Treatment of cells with 0.02% MMS for 30 min resulted in robust ubiquitination of PCNA in wild-type cells (Figure 1A, lane 2), but not in a strain in which PCNA was mutated at lysine 164, confirming this as the sole site of ubiquitination of PCNA (Figure 1A, lane 4). Whereas deletion of *RSC1* had no effect on PCNA ubiquitination (Figure 1A, lane 6), deletion of *RSC2* resulted in a substantial reduction (Figure 1A, lane 8). A similar reduction was found after UV-irradiation (100 Jm^{-2}, 1 h post-UV incubation) (Figure 1B, lanes 2 and 5) or treatment with 100 mM hydroxyurea for 30 min (Figure 1B, lanes 3 and 6). A time course for PCNA ubiquitination after MMS treatment showed substantial reduction in ubiquitination at all times (Figure 1C). The level of unmodified PCNA was not affected by any of the treatments. Overexpression of *RSC1* in the *rsc2Δ* strain failed to restore normal levels of PCNA ubiquitination (Figure 1D, lanes 6 and 8), whereas as expected, expression of *RSC2* did restore normal levels (Figure 1D, lanes 10 and 12). We conclude that an intact Rsc2 but not Rsc1 is required for normal levels of PCNA ubiquitination.

Figure 1E shows that *rsc2Δ* cells were sensitive to UV-irradiation, whereas *rsc1Δ* cells were not. This is consistent with our findings on the effects of these deletions on PCNA ubiquitination, but we interpret these data with some caution, as deletion of *RSC* genes may affect other UV repair processes as well.

The INO80 complex is another chromatin remodeller, with 15 subunits in budding yeast (36), that has the ability to replace H2AZ/H2B dimers with H2A/H2B dimers in nucleosomes (37). Consistent with a previous report (27), we found that deleting the gene encoding the Arp8 subunit of INO80 also reproducibly attenuated PCNA ubiquitination in response to MMS treatment (Figure 1F, compare lanes 2 and 6). However, in contrast to the effect of *RSC2* deletion, effects seen after UV-irradiation were marginal and not reproducible
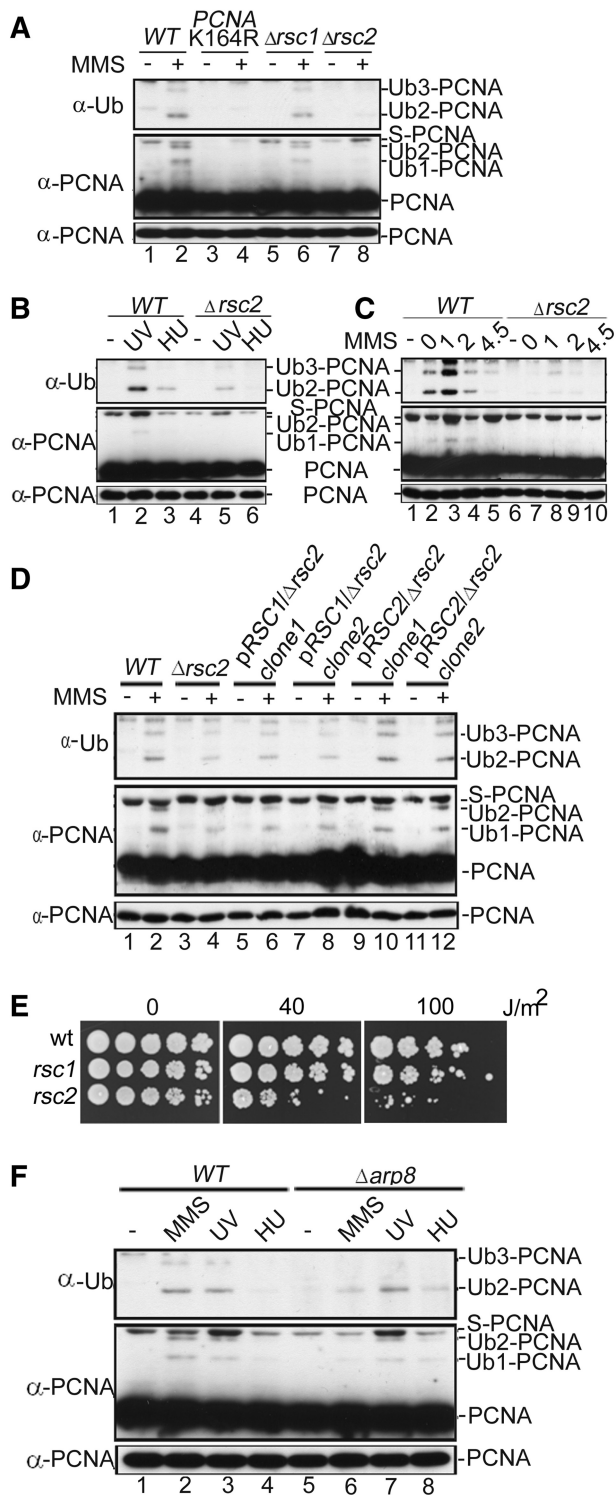
Figure 1. Deletion of *RSC2* results in reduced PCNA ubiquitination in *S. cerevisiae*. (A–E) Yeast cells in which His-tagged *POL30 (PCNA)* gene was incorporated into the genome were treated with different damaging agents, lysed and the PCNA recovered on nickel-agarose. The adhering PCNA was analysed by SDS–PAGE and immunoblotting with antibodies to ubiquitin (top panels) or PCNA (high exposure, middle panels; low exposure, bottom panels). S-, Ub1-, Ub2- and Ub3-PCNA indicate PCNA that is modified with SUMO, mono-, di- and tri-ubiquitin. (A) Different yeast strains were treated with or without 0.02% MMS for 30 min. (B) wt or *rsc2*Δ cells were treated with 100 Jm^{-2} UVC and incubated for 1 h, or with 100 mM HU for 30 min. (C) wt or *rsc2*Δ cells were treated with 0.02% MMS for 30 min

(Figure 1F, compare lanes 3 and 7) and there was no effect after HU treatment (Figure 1F, lanes 4 and 8) .

RSC complex and PCNA ubiquitination in human cells

There is only a single orthologue of Rsc1/Rsc2 in human cells, a protein-designated BAF180 (or Polybromo), which appears to be a fusion protein of Rsc1, Rsc2 and a third RSC protein, Rsc4. BAF180 contains six bromodomains, followed by two BAH domains and an HMG domain close to the C-terminus (38). BAF180 is a unique subunit of the PBAF (or SWI/SNF-B) remodelling complex, in which BAF180 and two other PBAF-specific subunits (BAF57 and BAF200) are associated with the SWI/SNF core complex (39,40). The PBAF complex affects the transcription of many genes (39,41,42). BAF180 knock-out mice are embryonic lethal (43), and, interestingly, many human cancers have mutations in the *PBRM1* gene encoding BAF180 (44,45).

We depleted BAF180 from MRC5 cells using siRNA and measured PCNA ubiquitination by immunoblotting of whole cell lysates with anti-PCNA antibody. The top panels in Figure 2A and B show that the knock-down was effective and the lower panel in Figure 2A shows a substantial reduction of PCNA ubiquitination at different times after UV-irradiation (10 Jm^{-2}), and even in untreated cells. MMS is a relatively weak inducer of PCNA ubiquitination in human cells (Figure 2B, bottom panel), but there was less ubiquitination after treatment with MMS or with HU in cells depleted of BAF180. These results were similar to those of *RSC*-depleted yeast cells.

In order to eliminate the possibility that the decreased PCNA ubiquitination might be connected in some way with nucleotide excision repair, we repeated the experiments with the nucleotide excision-repair-defective XP-A fibroblasts XP12RO-SV40. The results were very similar to those obtained with MRC5 cells (Figure 2C). Similar results were also obtained with XP30RO cells, defective in DNA polymerase η (Figure 2D). These findings indicate that the deficient PCNA ubiquitination in BAF180-depleted cells is not dependent on NER nor on TLS by polη.

Decreased chromatin association of PCNA and Rad18 in BAF180-depleted cells

In order to explore the possible cause of the decreased ubiquitinated PCNA under conditions of BAF180 depletion, we examined the amounts of chromatin-bound proteins involved in PCNA ubiquitination by immunoblotting extracts of MRC5 cells following extraction with buffer containing 0.2% Triton X-100. As with whole cell extracts, the level of ubiquitinated PCNA in the triton-extracted lysates was much less in BAF180-depleted cells than in the non-targeting siRNA

and then incubated in the absence of MMS for the indicated times. (D) The indicated strains were incubated with 0.02% MMS for 30 min. (E) Spot tests of different *rsc* strains exposed to the indicated doses of UV. (**F**) wt or *arp8*Δ cells were treated with 0.02% MMS for 30 min, UV-irradiated (100 Jm^{-2} and incubated for 1 h) or treated with 100 mM HU for 30 min.
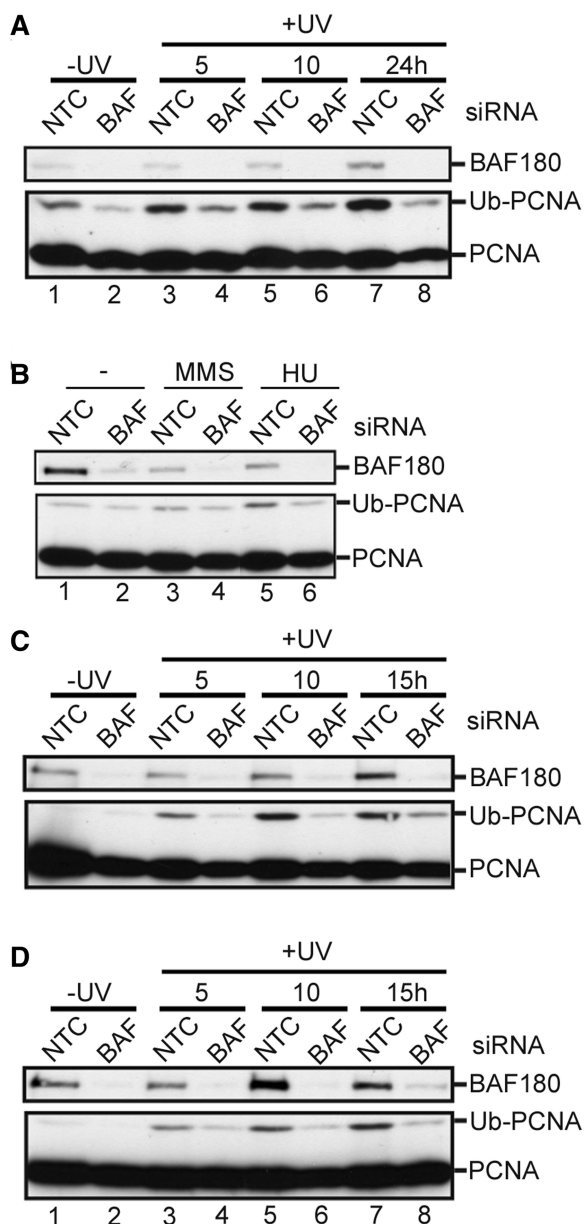
Figure 2. Depletion of BAF180 results in reduced PCNA ubiquitination in human cells. Cells were treated with a non-targeting control siRNA (NTC), or BAF180 siRNA (BAF) and subsequently treated as indicated, prior to harvesting the cells, running whole cell lysates on SDS–PAGE and immunoblotting with antibodies to BAF180 (upper panels) or PCNA (lower panels). (**A**) MRC5 cells UV-irradiated (10 Jm^{-2}) and incubated for the indicated times. (**B**) MRC5 cells treated with 1 mM MMS for 1 h and incubated for 3 h after removal of the MMS, or with 1 mM HU and incubated for 24 h, (**C**) XP12RO (XP-A) and (**D**) XP30RO (XP-V) cells treated as in (A).

controls (Figure 3A, second panel). Remarkably however, the level of unmodified PCNA in the chromatin fraction was also drastically reduced in the BAF180-depleted samples, though the amount in whole cell lysates was unaffected (e.g. compare with Figure 2A). Furthermore, the amount of chromatin-bound Rad18, the E3 ligase for PCNA ubiquitination, was also markedly reduced (Figure 3A, third panel). Rad18 is recruited to blocked replication forks by virtue of its affinity to RPA-coated

single-stranded DNA (7). We therefore measured the chromatin-bound RPA in BAF180-depleted cells, but found only a minor reduction in levels, insufficient to account for the substantial reduction in Rad18. Levels of histone H3 appeared to be unaffected by BAF180 depletion, so, in order to quantitate the effects that we have observed, we expressed the levels of protein of interest, using histone H3 as an internal control and normalized the data to the levels in unirradiated cells in the control samples (Figure 3B). It can be seen that, following UV-irradiation, the amounts of chromatin-bound unmodified (b) and ubiquitinated PCNA (c), Rad18 (d) and RPA (e) all increased substantially after UV-irradiation, albeit to different extents relative to unirradiated cells, and for all except RPA, much of this increase was ablated if BAF180 was depleted. The increase in chromatin-bound RPA was relatively unaffected. We also included polη in several of these experiments and, although there was quite significant variation between experiments, there was no substantial reproducible reduction in chromatin-associated polη [Figure 3B (f)].

We carried out similar analyses following treatment of cells with 1 mM HU for increasing periods of time. The blots are shown in Figure 3C and quantitated in Figure 3D. As with UV treatment, there was an increase in chromatin-bound PCNA and Rad18 upon HU treatment, which was reduced in BAF180-depleted cells. The reduction in unmodified and ubiquitinated PCNA and Rad18 was less than with UV-irradiated cells, but was also observed with RPA.

Using yeast, the data of Figure 1 show that the total amount of PCNA was similar in wild-type and *rsc2Δ* cells. Figure 3F shows, however, that the level of chromatin-associated PCNA was substantially lower in the *rsc2Δ* cells.

Cell cycle effects

Loading of PCNA onto chromatin and PCNA ubiquitination are generally DNA replication-associated processes confined to S-phase. We were concerned that the reduced levels of chromatin-bound proteins in BAF180-depleted cells might be the result of a distortion in cell cycle kinetics. Figure 4A shows FACS analysis of the cell cycle distribution of unirradiated cells pulse-labelled with BrdU. The proportion of cells in different phases of the cycle was very similar in the BAF180-depleted and control cells (Figure 4B). Following UV-irradiation, there was, as expected, an increase in the proportion of S-phase cells in both cultures, but this was actually slightly higher in the BAF180-depleted cells (Figure 4B). Thus, we cannot attribute the decreased levels of chromatin-bound proteins to a decreased proportion of S-phase cells.

Failure to ubiquitinate PCNA results in a defect in post-replication repair and substantial UV sensitivity (46). Therefore, to examine the biological effects of the decrease in PCNA ubiquitination brought about by depletion of BAF180, we measured the rate of fork progression using DNA fibre analysis. Cells were labelled for 20 min with chlorodeoxyuridine (CldU), UV-irradiated (0 or 20 Jm^{-2}) and incubated for a further
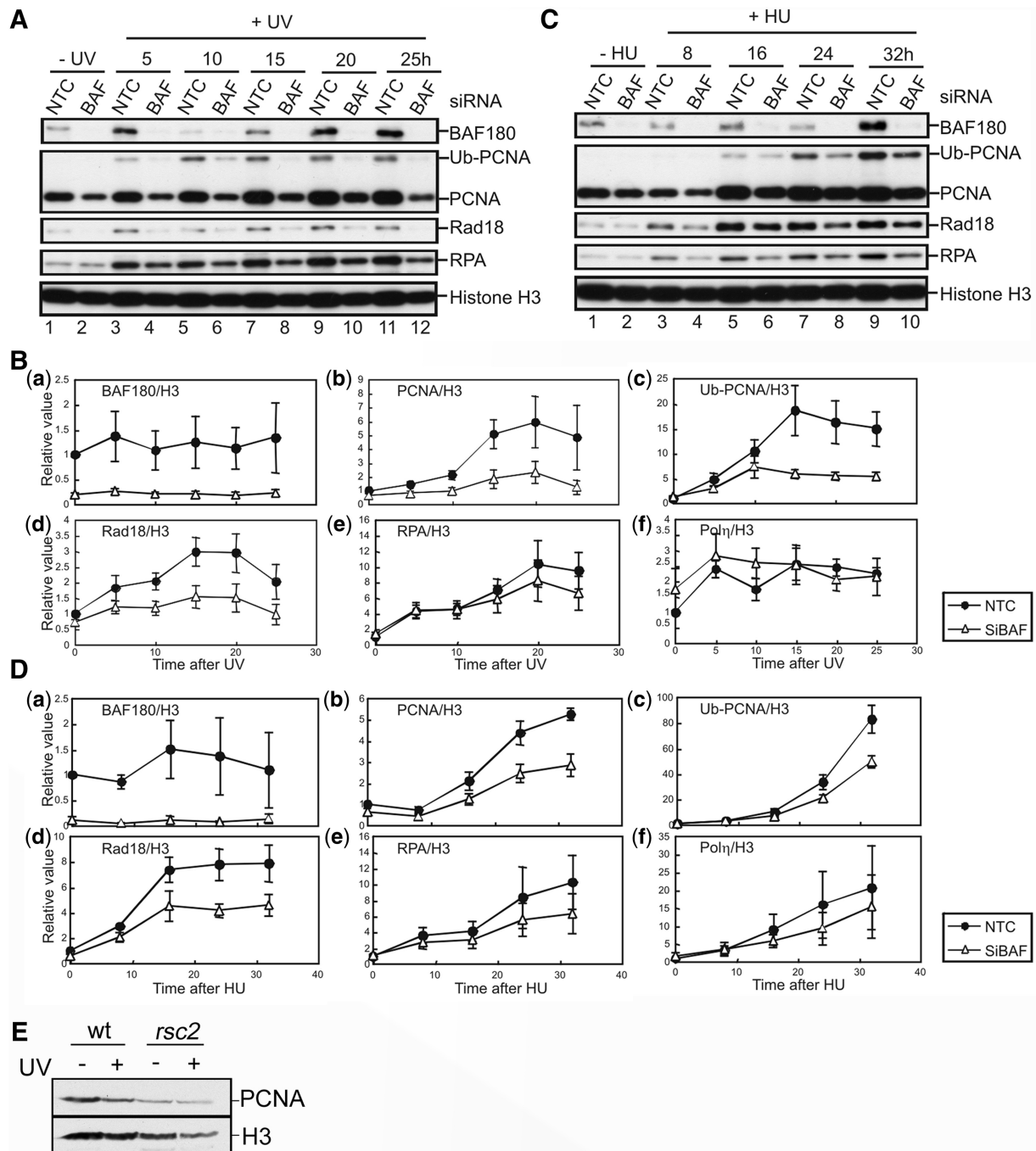
Figure 3. Effect of BAF180 depletion on proportion of nuclear proteins associated with the chromatin-fraction. (**A**, **B**) MRC5 cells, treated with non-targeting or BAF180 siRNA were UV-irradiated (10 Jm^{-2}) and incubated for the indicated times. They were then extracted with Triton X-100 and the Triton-extracted cells were analysed by SDS–PAGE and immunoblotting with antibodies to the indicated nuclear proteins. (**A**) Typical experiment. (**B**) The intensities of the signals relative to those from histone H3 (which remained constant under all conditions), were quantified by chemiluminescence. Results are normalized to the signal immediately after UV in cells treated with the non-targetting control. The results show the means ± standard deviation of six experiments. (**C**, **D**) As (A, B), except that cells were treated with 1 mM HU for the indicated times instead of UV. Results in (D) are means ± standard deviation of three experiments. Solid symbols, non-targeting control; open symbols BAF180 siRNA. (**E**) Chromatin-bound PCNA in yeast wild-type and *rsc2Δ* cells either unirradiated or UV-irradiated (50 Jm^{-2}).

20 min with iododeoxyuridine (IdU). The distribution of the ratio of CldU to IdU track lengths is shown in Figure 4C (Top panel, unirradiated; bottom panel irradiated). In unirradiated cells, there was no difference in this ratio between the BAF180-depleted cells and those treated with a non-targeting control. In contrast, in UV-irradiated cells, there was a small but reproducible increase in this ratio in the BAF180-depleted cells, compared with the controls. In three experiments the median value of the CldU to IdU ratio was
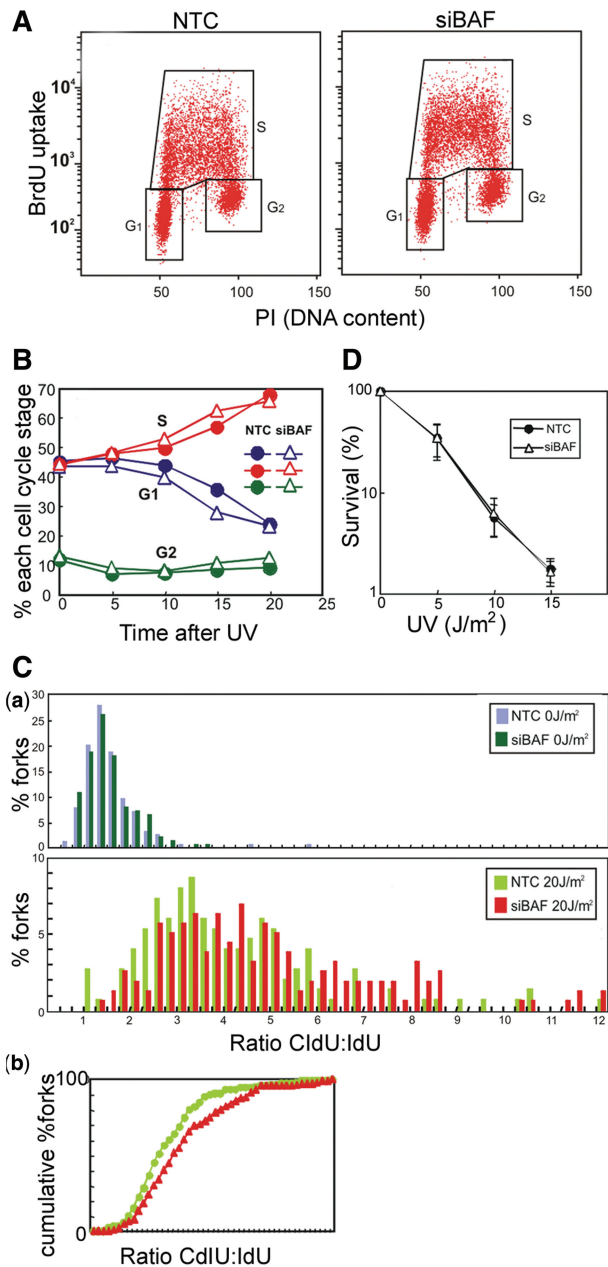
Figure 4. Biological consequences of BAF180 depletion. (**A**) MRC5 cells were treated with non-targetting or BAF180 siRNAs. After 72 h, the cells were pulse-labelled for 30 min with BrdU, harvested and analysed by flow cytometry. (**B**) At different times after UV-irradiation, cells were analysed by flow cytometry and the proportions of cells in different phases of the cell cycle were estimated from the FACS profiles. Closed symbols, non-targetting controls, open symbols, BAF180-depleted. (**C**) Analysis of fork progression. (a) Frequency distribution of ratio of CldU to IdU in the same fibre in unirradiated cells (top) and UV-irradiated cells (20 Jm^{-2}—bottom). (b) shows the cumulative frequency distribution of the data. (**D**) Cell survival after treatment with the indicated UV doses. Means ± standard deviation of three experiments.

1.37($\pm$0.20)-fold greater in the BAF180-depleted cells than in the controls. Applying the Kolmogorov–Smirnov test to our data confirmed that the two sets of data were significantly different (*P*-value of 0.000 for the null hypothesis that the distributions were the same). We

conclude that fork progression after DNA damage is less efficient in the absence of BAF180.

Despite the substantial effects described in the preceding sections, the survival of BAF180-depleted and control cells following UV-irradiation was very similar (Figure 4D).

Association of Rsc2 with sites of replication

Ubiquitination of PCNA takes place at the sites of blocked replication forks. Our data implicate a role for Rsc2/BAF180 in this process. Although Rsc2 or BAF180 may remodel chromatin at sites of replication, it is also possible that they are functioning indirectly by facilitating the transcription of genes involved in this pathway. In order to determine whether RSC is present at the replication fork, we have carried out ChIP of myc-tagged Rsc2 in yeast cells synchronized by α-factor and released into HU to maintain them in early S-phase. Figure 5A (left panel) shows some Rsc2 associated with chromatin at three different loci in G1 (compare with untagged cells in middle panel), in keeping with previous reports of chromatin association across the genome. However, when cells were in early S-phase, there was a 3-fold increase in the association of Rsc2 with the early-replicating ARS607 locus (left panel, black and grey bars). The level of association represented an ∼30-fold enrichment over untagged controls. In contrast, the enrichment of Rsc2 at a late-replicating telomere locus did not change significantly over the time course of the experiment (Figure 5A, white bars). The enrichment of Rsc2 was substantially greater than that found with myc-tagged Ino80 (Figure 5A, right panel— see also Refs 24–26) and indicates that the RSC complex is indeed associated with the replication fork.

DISCUSSION

In this article, we have shown that deletion of the *RSC2* gene in yeast or depletion of the orthologous BAF180 in human cells results in a pronounced decrease in the ubiquitination of PCNA following DNA damage or HU treatment relative to undepleted controls. In human cells, we have been able to attribute this reduction to a decreased concentration of the E3 ligase Rad18 in chromatin and remarkably to a decrease in chromatin-associated unmodified PCNA as well. All of these reductions are ∼3- to 5-fold. None of these effects results from distortions of the cell cycle in the absence of BAF180. We can draw two major conclusions from our findings. First, BAF180 and by implication the PBAF complex, plays an important role in loading PCNA onto chromatin and enhancing its ubiquitination following DNA damaging treatment. It is of interest to note that two recent papers report on the role of other chromatin modifications/chromatin proteins on recruitment of PCNA to chromatin in human cells. Mono-methylation of histone H3 on lysine 56 and the HMGN1 protein are both important for recruitment of PCNA to chromatin to facilitate DNA replication (47,48). Our second conclusion is that, whereas the reduced levels of unmodified and ubiquitinated
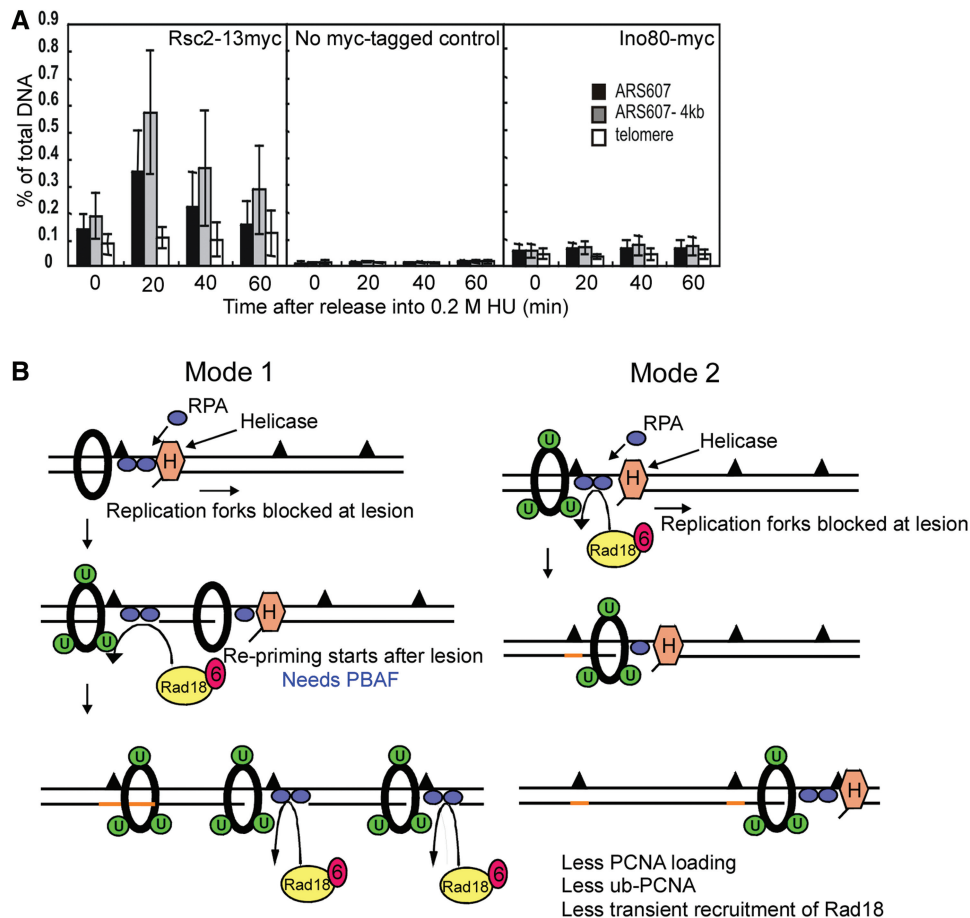
Figure 5. Role of RSC/PBAF in DNA replication. (**A**) Yeast cells in which Rsc2, no protein or INO80 was myc-tagged, were synchronized using α-factor and then released into 200 mM HU for the indicated times. Cells were treated as described in 'Materials and Methods' section, followed by immunoprecipitation with anti-myc antibody. The immunoprecipitates were deproteinized and the DNA amplified with primers specific for the ARS607 locus (black), a site 4 kb away from this locus (grey) and for a telomeric locus (white). (**B**) Model for the role of PBAF in replication of UV-damaged DNA. In mode 1, repriming of DNA synthesis downstream of a blocked fork occurs prior to TLS. We envisage that PBAF facilitates the repriming step. On this model, a new molecule of PCNA is loaded at each repriming event and is ubiquitinated when the fork is blocked. In mode 2, in the absence of PBAF, repriming is slower and TLS occurs, obviating the need for repriming, so that a single molecule of ubiquitinated PCNA can be used to replicate past several lesions. H, replicative helicase; U, ubiquitin; 6, Rad6.

PCNA result in a modest effect on fork progression, they have no effect on UV survival in human cells.

We previously showed that replacement of PCNA in MRC5 cells with mutant PCNA-K164R that cannot be ubiquitinated, resulted in a substantial reduction in UV survival (46). Our current data suggest that, following DNA damage, cells can quite happily dispense with 70–80% of both unmodified and ubiquitinated PCNA with no significant effect on cell survival. (It should be noted that the absence of polη in XP variant cells confers only minor sensitivity to UV-induced cell killing, despite polη being a central player in TLS.) Gong *et al.* (49) found that a human cell line lacking the Swi–Snf component Brg1 was sensitive to UV-irradiation. However, there are two Swi–Snf complexes in human cells with several components in common, including Brg1. Hence, lack of Brg1 would result in both complexes being defective. In our work, we depleted BAF180, which is a specific component of only one of the complexes, leaving the other one intact. Our data are therefore not directly comparable with those of Gong *et al.* (49).

A current model for replication at sites of DNA damage is that the PCNA-associated replication machinery is blocked at the site of damage, but the replicative helicase continues to unwind ahead of the fork, exposing single-stranded DNA at the fork. Two events then occur. One of these is the repriming of synthesis downstream of the lesion, using a new PCNA trimer. The other event is the recruitment of Rad18 by the single-stranded DNA to ubiquitinate PCNA. This in turn recruits a TLS polymerase to bypass the damage. Let us first consider the situation in which repriming occurs before TLS (Figure 5B, mode 1). The reprimed replication machinery will be blocked at the next lesion and a further repriming event will take place. Each repriming event requires a new PCNA molecule and each blockage at a lesion results in a new ubiquitination event. If repriming occurs in this way, TLS will take place behind the fork. In an alternative mode (Figure 5B, mode 2), TLS occurs at the fork, the blocked fork is then released to continue with the same PCNA trimer, and repriming may not be necessary. Since this PCNA is already ubiquitinated, another

ubiquitination event is not required at the next damaged site encountered. Note that replicative polymerases operate equally well irrespective of whether PCNA is ubiquitinated or not (50), and we have previously shown that PCNA remains ubiquitinated for many hours after UV-irradiation (46).

In this scenario, we propose that PBAF is required to mobilize nucleosomes to allow repriming to take place. This involvement may be either to assist the helicase in unwinding the DNA ahead of the fork or to help the repriming event itself. The latter is perhaps less likely, as repriming takes place on single-stranded DNA, which might be expected to be free of nucleosomes. Several reports have shown that UV-blocked replication forks can restart efficiently by repriming (mode 1) under normal circumstances (51–53). In the absence of PBAF, we suggest that there is a shift in the balance from mode 1 to mode 2, which results in reduced PCNA loading, less recruitment of Rad18 and decreased ubiquitination of PCNA. There is no intrinsic requirement for the involvement of more polη molecules in either mode. If the time interval between repriming and TLS in mode 1 is relatively short, the effect on fork progression need not be very dramatic, and there would not necessarily be any deleterious effect of shifting the balance from mode 1 to mode 2. This would correlate with our finding of a modest decrease in fork progression rate. The model is most easily applicable to events on the leading strand. We note however, that the decrease in chromatin-associated PCNA and ubiquitinated PCNA is >50% when PBAF is depleted, implying a role for PBAF in events on the lagging strand as well. The exact nature of this role must await further experimentation. Our model is highly speculative, but it does provide a satisfactory explanation for our otherwise apparently self-contradictory findings. It posits a role for PBAF/RSC in repriming beyond stalled forks, and predicts that the chromatin remodeller is located at the site of replication. This is consistent with our ChIP data showing that Rsc2 is indeed associated with chromatin in the vicinity of replication forks in yeast cells. In an elegant recent study, Cohen *et al.* (54) showed that BRG1, a core component of Swi–Snf complexes in mammalian cells, is localized at sites of DNA replication on chromatin fibres and is required for efficient fork progression. This work does not indicate which of the two mammalian Brg1-containing complexes, only one of which (PBAF) contains BAF180, is located and required at the replication forks. We did not find any effect of depleting BAF180 on fork progression in unirradiated cells. However, their results are consistent with our finding that Rsc2 is associated with the replication fork in yeast.

Our data demonstrate that RSC/PBAF has a major effect on PCNA ubiquitination in response to a range of DNA lesions, and that the complex is localized to sites of DNA replication *in vivo*. We also find, consistent with previous reports, that INO80 facilitates PCNA ubiquitination (55) and is localized to sites of DNA replication. INO80 contributes to PCNA ubiquitination only after MMS-induced DNA damage, but these results still raise the possibility that there are redundant functions of the two chromatin remodellers at stalled replication forks.

However, RSC promotes nucleosome repositioning *in vivo* (19,56), whereas INO80 removes H2A.Z/H2B dimers from nucleosomes and replaces them with H2A/H2B dimers (37). This difference in remodelling activity strongly suggests that the two complexes will promote PCNA ubiquitination in response to MMS by distinct mechanisms. This is consistent with a recent report showing that mutation of the INO80 subunits *ies3* or *nhp10* together with a *rsc4*-K25R mutation in RSC results in greater sensitivity to MMS than mutation of either complex alone (57). Importantly, our data suggest that RSC/PBAF impacts on PCNA loading and ubiquitination more broadly in response to DNA damage.

REFERENCES

1. Yang,W. and Woodgate,R. (2007) What a difference a decade makes: insights into translesion DNA synthesis. *Proc. Natl Acad. Sci. USA*, **104**, 15591–15598.
2. Lehmann,A.R., Niimi,A., Ogi,T., Brown,S., Sabbioneda,S., Wing,J.F., Kannouche,P.L. and Green,C.M. (2007) Translesion synthesis: Y-family polymerases and the polymerase switch. *DNA Repair*, **6**, 891–899.
3. Daigaku,Y., Davies,A.A. and Ulrich,H.D. (2010) Ubiquitin-dependent DNA damage bypass is separable from genome replication. *Nature*, **465**, 951–955.
4. Karras,G.I. and Jentsch,S. (2010) The RAD6 DNA damage tolerance pathway operates uncoupled from the replication fork and is functional beyond S phase. *Cell*, **141**, 255–267.
5. Hoege,C., Pfander,B., Moldovan,G.-L., Pyrolowakis,G. and Jentsch,S. (2002) *RAD6*-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, **419**, 135–141.
6. Kannouche,P.L., Wing,J. and Lehmann,A.R. (2004) Interaction of human DNA polymerase η with monoubiquitinated PCNA; a possible mechanism for the polymerase switch in response to DNA damage. *Mol. Cell*, **14**, 491–500.
7. Davies,A.A., Huttner,D., Daigaku,Y., Chen,S. and Ulrich,H.D. (2008) Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein A. *Mol. Cell*, **29**, 625–636.
8. Ohmori,H., Friedberg,E.C., Fuchs,R.P.P., Goodman,M.F., Hanaoka,F., Hinkle,D., Kunkel,T.A., Lawrence,C.W., Livneh,Z., Nohmi,T. *et al*. (2001) The Y-family of DNA polymerases. *Mol. Cell*, **8**, 7–8.
9. Bienko,M., Green,C.M., Crosetto,N., Rudolf,F., Zapart,G., Coull,B., Kannouche,P., Wider,G., Peter,M., Lehmann,A.R. *et al*. (2005) Ubiquitin-binding domains in translesion synthesis polymerases. *Science*, **310**, 1821–1824.
10. Plosky,B.S., Vidal,A.E., Fernandez de Henestrosa,A.R., McLenigan,M.P., McDonald,J.P., Mead,S. and Woodgate,R. (2006) Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin. *EMBO J.*, **25**, 2847–2855.
11. Edmunds,C.E., Simpson,L.J. and Sale,J.E. (2008) PCNA ubiquitination and REV1 define temporally distinct mechanisms for controlling translesion synthesis in the avian cell line DT40. *Mol. Cell*, **30**, 519–529.

12. Sarkies,P., Reams,C., Simpson,L.J. and Sale,J.E. (2010) Epigenetic instability due to defective replication of structured DNA. *Mol. Cell*, **40**, 703–713.

13. Bannister,A.J. and Kouzarides,T. (2011) Regulation of chromatin by histone modifications. *Cell Res.*, **21**, 381–395.

14. Saha,A., Wittmeyer,J. and Cairns,B.R. (2006) Chromatin remodelling: the industrial revolution of DNA around histones. *Nat. Rev. Mol. Cell Biol.*, **7**, 437–447.

15. Downs,J.A., Allard,S., Jobin-Robitaille,O., Javaheri,A., Auger,A., Bouchard,N., Kron,S.J., Jackson,S.P. and Cote,J. (2004) Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites. *Mol. Cell*, **16**, 979–990.

16. Morrison,A.J., Highland,J., Krogan,N.J., Arbel-Eden,A., Greenblatt,J.F., Haber,J.E. and Shen,X. (2004) INO80 and g-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. *Cell*, **119**, 767–775.

17. van Attikum,H., Fritsch,O., Hohn,B. and Gasser,S.M. (2004) Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair. *Cell*, **119**, 777–788.

18. Shim,E.Y., Ma,J.L., Oum,J.H., Yanez,Y. and Lee,S.E. (2005) The yeast chromatin remodeler RSC complex facilitates end joining repair of DNA double-strand breaks. *Mol. Cell Biol.*, **25**, 3934–3944.

19. Kent,N.A., Chambers,A.L. and Downs,J.A. (2007) Dual chromatin remodeling roles for RSC during DNA double strand break induction and repair at the yeast MAT locus. *J. Biol. Chem.*, **282**, 27693–27701.

20. Jiang,Y., Wang,X., Bao,S., Guo,R., Johnson,D.G., Shen,X. and Li,L. (2010) INO80 chromatin remodeling complex promotes the removal of UV lesions by the nucleotide excision repair pathway. *Proc. Natl Acad. Sci. USA*, **107**, 17274–17279.

21. Lee,H.S., Park,J.H., Kim,S.J., Kwon,S.J. and Kwon,J. (2010) A cooperative activation loop among SWI/SNF, gamma-H2AX and H3 acetylation for DNA double-strand break repair. *EMBO J.*, **29**, 1434–1445.

22. Ray,A., Mir,S.N., Wani,G., Zhao,Q., Battu,A., Zhu,Q., Wang,Q.E. and Wani,A.A. (2009) hSNF5/INI1, a component of the human SWI/SNF chromatin remodeling complex, promotes nucleotide excision repair by influencing ATM recruitment and downstream H2AX phosphorylation. *Mol. Cell Biol.*, **29**, 6206–6219.

23. Park,J.H., Park,E.J., Lee,H.S., Kim,S.J., Hur,S.K., Imbalzano,A.N. and Kwon,J. (2006) Mammalian SWI/SNF complexes facilitate DNA double-strand break repair by promoting gamma-H2AX induction. *EMBO J.*, **25**, 3986–3997.

24. Vincent,J.A., Kwong,T.J. and Tsukiyama,T. (2008) ATP-dependent chromatin remodeling shapes the DNA replication landscape. *Nat. Struct. Mol. Biol.*, **15**, 477–484.

25. Shimada,K., Oma,Y., Schleker,T., Kugou,K., Ohta,K., Harata,M. and Gasser,S.M. (2008) Ino80 chromatin remodeling complex promotes recovery of stalled replication forks. *Curr. Biol.*, **18**, 566–575.

26. Papamichos-Chronakis,M. and Peterson,C.L. (2008) The Ino80 chromatin-remodeling enzyme regulates replisome function and stability. *Nat. Struct. Mol. Biol.*, **15**, 338–345.

27. Falbo,K.B., Alabert,C., Katou,Y., Wu,S., Han,J., Wehr,T., Xiao,J., He,X., Zhang,Z., Shi,Y. *et al.* (2009) Involvement of a chromatin remodeling complex in damage tolerance during DNA replication. *Nat. Struct. Mol. Biol.*, **16**, 1167–1172.

28. Kannouche,P., Broughton,B.C., Volker,M., Hanaoka,F., Mullenders,L.H.F. and Lehmann,A.R. (2001) Domain structure, localization and function of DNA polymerase η, defective in xeroderma pigmentosum variant cells. *Genes Dev.*, **15**, 158–172.

29. Ulrich,H.D. and Jentsch,S. (2000) Two RING finger proteins mediate cooperation between ubiquitin-conjugating enzymes in DNA repair. *EMBO J.*, **19**, 3388–3397.

30. Stelter,P. and Ulrich,H.D. (2003) Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature*, **425**, 188–191.

31. Strahl-Bolsinger,S., Hecht,A., Luo,K. and Grunstein,M. (1997) SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev.*, **11**, 83–93.

32. Martens,J.A. and Winston,F. (2003) Recent advances in understanding chromatin remodeling by Swi/Snf complexes. *Curr. Opin. Genet. Dev.*, **13**, 136–142.

33. Wilson,B., Erdjument-Bromage,H., Tempst,P. and Cairns,B.R. (2006) The RSC chromatin remodeling complex bears an essential fungal-specific protein module with broad functional roles. *Genetics*, **172**, 795–809.

34. Chai,B., Huang,J., Cairns,B.R. and Laurent,B.C. (2005) Distinct roles for the RSC and Swi/Snf ATP-dependent chromatin remodelers in DNA double-strand break repair. *Genes Dev.*, **19**, 1656–1661.

35. Cairns,B.R., Schlichter,A., Erdjument-Bromage,H., Tempst,P., Kornberg,R.D. and Winston,F. (1999) Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. *Mol. Cell*, **4**, 715–723.

36. Morrison,A.J. and Shen,X. (2009) Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. *Nat. Rev. Mol. Cell Biol.*, **10**, 373–384.

37. Papamichos-Chronakis,M., Watanabe,S., Rando,O.J. and Peterson,C.L. (2011) Global regulation of H2A.Z localization by the INO80 chromatin-remodeling enzyme is essential for genome integrity. *Cell*, **144**, 200–213.

38. Goodwin,G.H. and Nicolas,R.H. (2001) The BAH domain, polybromo and the RSC chromatin remodelling complex. *Gene*, **268**, 1–7.

39. Mohrmann,L. and Verrijzer,C.P. (2005) Composition and functional specificity of SWI2/SNF2 class chromatin remodeling complexes. *Biochim. Biophys. Acta*, **1681**, 59–73.

40. Wilson,B.G. and Roberts,C.W. (2011) SWI/SNF nucleosome remodellers and cancer. *Nat. Rev. Cancer*, **11**, 481–492.

41. Burrows,A.E., Smogorzewska,A. and Elledge,S.J. (2010) Polybromo-associated BRG1-associated factor components BRD7 and BAF180 are critical regulators of p53 required for induction of replicative senescence. *Proc. Natl Acad. Sci. USA*, **107**, 14280–14285.

42. Hah,N., Kolkman,A., Ruhl,D.D., Pijnappel,W.W., Heck,A.J., Timmers,H.T. and Kraus,W.L. (2010) A role for BAF57 in cell cycle-dependent transcriptional regulation by the SWI/SNF chromatin remodeling complex. *Cancer Res.*, **70**, 4402–4411.

43. Wang,Z., Zhai,W., Richardson,J.A., Olson,E.N., Meneses,J.J., Firpo,M.T., Kang,C., Skarnes,W.C. and Tjian,R. (2004) Polybromo protein BAF180 functions in mammalian cardiac chamber maturation. *Genes Dev.*, **18**, 3106–3116.

44. Xia,W., Nagase,S., Montia,A.G., Kalachikov,S.M., Keniry,M., Su,T., Memeo,L., Hibshoosh,H. and Parsons,R. (2008) BAF180 is a critical regulator of p21 induction and a tumor suppressor mutated in breast cancer. *Cancer Res.*, **68**, 1667–1674.

45. Varela,I., Tarpey,P., Raine,K., Huang,D., Ong,C.K., Stephens,P., Davies,H., Jones,D., Lin,M.L., Teague,J. *et al.* (2011) Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature*, **469**, 539–542.

46. Niimi,A., Brown,S., Sabbioneda,S., Kannouche,P.L., Scott,A., Yasui,A., Green,C.M. and Lehmann,A.R. (2008) Regulation of proliferating cell nuclear antigen ubiquitination in mammalian cells. *Proc. Natl Acad. Sci. USA*, **105**, 16125–16130.

47. Yu,Y., Song,C., Zhang,Q., Dimaggio,P.A., Garcia,B.A., York,A., Carey,M.F. and Grunstein,M. (2012) Histone H3 lysine 56 methylation regulates DNA replication through its interaction with PCNA. *Mol. Cell*, **46**, 7–17.

48. Postnikov,Y.V., Kurahashi,T., Zhou,M. and Bustin,M. (2012) The nucleosome binding protein HMGN1 interacts with PCNA and facilitates its binding to chromatin. *Mol. Cell. Biol.*, **32**, 1844–1854.

49. Gong,F., Fahy,D., Liu,H., Wang,W. and Smerdon,M.J. (2008) Role of the mammalian SWI/SNF chromatin remodeling complex in the cellular response to UV damage. *Cell Cycle*, **7**, 1067–1074.

50. Garg,P. and Burgers,P.M. (2005) Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases eta and REV1. *Proc. Natl Acad. Sci. USA*, **102**, 18361–18366.

51. Lehmann,A.R. (1972) Postreplication repair of DNA in ultraviolet-irradiated mammalian cells. *J. Mol. Biol.*, **66**, 319–337.

52. Lopes,M., Foiani,M. and Sogo,J.M. (2006) Multiple mechanisms control chromosome integrity after replication fork uncoupling and restart at irreparable UV lesions. *Mol. Cell*, **21**, 15–27.
53. Elvers,I., Johansson,F., Groth,P., Erixon,K. and Helleday,T. (2011) UV stalled replication forks restart by re-priming in human fibroblasts. *Nucleic Acids Res.*, **39**, 7049–7057.
54. Cohen,S.M., Chastain,P.D. 2nd, Rosson,G.B., Groh,B.S., Weissman,B.E., Kaufman,D.G. and Bultman,S.J. (2010) BRG1 co-localizes with DNA replication factors and is required for efficient replication fork progression. *Nucleic Acids Res.*, **38**, 6906–6919.
55. Kato,D., Waki,M., Umezawa,M., Aoki,Y., Utsugi,T., Ohtsu,M. and Murakami,Y. (2012) Phosphorylation of human INO80 is involved in DNA damage tolerance. *Biochem. Biophys. Res. Commun.*, **417**, 433–438.
56. Wippo,C.J., Israel,L., Watanabe,S., Hochheimer,A., Peterson,C.L. and Korber,P. (2011) The RSC chromatin remodelling enzyme has a unique role in directing the accurate positioning of nucleosomes. *EMBO J.*, **30**, 1277–1288.
57. Charles,G.M., Chen,C., Shih,S.C., Collins,S.R., Beltrao,P., Zhang,X., Sharma,T., Tan,S., Burlingame,A.L., Krogan,N.J. *et al.* (2011) Site-specific acetylation mark on an essential chromatin-remodeling complex promotes resistance to replication stress. *Proc. Natl Acad. Sci. USA*, **108**, 10620–10625.