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Extensive RPA2 hyperphosphorylation promotes apoptosis in response to DNA replication stress in CHK1 inhibited cells

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ABSTRACT

The replication protein A (RPA)–ssDNA complex formed at arrested replication forks recruits key proteins to activate the ATR-CHK1 signalling cascade. When CHK1 is inhibited during DNA replication stress, RPA2 is extensively hyperphosphorylated. Here, we investigated the role of RPA2 hyperphosphorylation in the fate of cells when CHK1 is inhibited. We show that proteins normally involved in DNA repair (RAD51) or control of RPA phosphorylation (the PP4 protein phosphatase complex) are not recruited to the genome after treatment with CHK1 and DNA synthesis inhibitors. This is not due to RPA2 hyperphosphorylation as suppression of this response does not restore loading suggesting that recruitment requires active CHK1. To determine whether RPA2 hyperphosphorylation protects stalled forks from collapse or induction of apoptosis in CHK1 inhibited cells during replication stress, cells expressing RPA2 genes mutated at key phosphorylation sites were characterized. Mutant RPA2 rescued cells from RPA2 depletion and reduced the level of apoptosis induced by treatment with CHK1 and replication inhibitors however the incidence of double strand breaks was not affected. Our data indicate that RPA2 hyperphosphorylation promotes cell death during replication stress when CHK1 function is compromised but does not appear to be essential for replication fork integrity.

INTRODUCTION

DNA damage response pathways preserve genome integrity by recognizing replication errors and DNA damage to arrest cell cycle progression and activate repair. These pathways may also commit highly damaged cells to death. Work from a many laboratories has identified CHK1 as a key mediator of cell death following DNA replication inhibition or some forms of DNA damage (1–3). DNA replication stress triggers apoptosis in the absence of CHK1 function, particularly in tumour cells where oncogene activation may inappropriately drive DNA replication (4,5). This has led to renewed interest in the use of CHK1 inhibitors in therapies targeted to tumour cells (6–9). CHK1 is largely activated as a result of ssDNA formation that may be generated by the uncoupling of polymerase and helicase complexes following DNA replication inhibition (10) or by other pathways that process stalled replication forks (11). Replication protein A (RPA) rapidly coats ssDNA to form an RPA-ssDNA complex that recruits Ataxia telangiectasia mutated and Rad3 related (ATR) through a complex mechanism involving the ATR interacting protein (ATRIP) (12,13). ATR then activates CHK1 through phosphorylation of Ser345 and Ser317 (14,15) to coordinate cellular responses to replication stress. It slows S-phase progression by suppressing inappropriate firing of replication origins, helps maintain fork integrity, facilitates resolution of stalled forks, and triggers G2/M arrest (16–19).

RPA plays a wide role in DNA metabolism (20,21). It coats ssDNA to protect it from nucleolytic attack and remove secondary structure and interacts with a number of proteins during replication or repair. RPA is a heterotrimer consisting of 70, 32 and 14 kDa subunits. The 70 and 32 kDa subunits contain DNA binding motifs necessary for recruitment of the complex to ssDNA (22) while the 32 kDa subunit (RPA2) is the target of phosphorylation during normal G1/S transition at conserved cyclin-CDK phosphorylation sites (Ser23 and Ser29) (23,24). When DNA is damaged or replication is disrupted under some conditions other sites on RPA2 may be phosphorylated by PIK-like kinases including DNA-PK, ATM and ATR to produce a 'hyperphosphorylated' state (23–28). The role of hyperphosphorylated RPA2 in the response to replication fork stress has been extensively studied. The sites are not essential for RPA function in unstressed cells as nonphosphorylatable mutant RPA2 has no effect on normal cell growth (29,30) although initial reports suggested that RPA2 phosphorylation may

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enhance or inhibit replication or repair (30–33). More recent findings indicate that it mediates S-phase checkpoints and recovery from replication stress (28,33,34). In particular phosphorylation of Ser4/Ser8 by DNA-PK appears to be required for induction of S-phase checkpoints and regulation of replication fork restart after exposure to replication inhibitors (28,34,35). While RPA levels have been shown to be critical to prevent replication fork collapse following treatment with an ATR inhibitor (36), the role of RPA2 hyperphosphorylation is not known.

We previously showed that RPA2 hyperphosphorylation is enhanced in CHK1 depleted cells exposed to replication inhibitors relative to cells treated with replication inhibitors alone (37). Considering the potential impact of this protein modification on high levels of ssDNA generated at arrested DNA replication forks in tumour cells under these conditions (38,39), we investigated the relationship of RPA2 hyperphosphorylation to cell fate.

MATERIALS AND METHODS

Cell culture

The HCT116 and SW480 human colon cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). For experiments using thymidine, dialyzed FBS was used to remove deoxynucleosides in the serum that might interfere in the response to this agent. Replication inhibitors thymidine (TdR) and hydroxyurea (HU) were used at a concentration 2 mM although 4 mM thymidine was used for SW480. The chemical inhibitor of Chk1 activity (Gö6976, Calbiochem (40) or MK-8776, Selleckchem (41)) was added to cell cultures at a concentration of 1 μM 1h prior treatment with replication inhibitors. Stable transfected HCT116 cells were growing in DMEM supplemented with 10% FBS and 2 μg/ml puromycin.

RPA2 mutagenesis and plasmid transfection

RPA2 cDNA cloned into pOTB7RPA2 plasmid (IMAGE clone: 3538351, BC001630.1, Gene Service Ltd) was amplified and subcloned into pcDNA3.1/V5-His TOPO vector (Invitrogen, Life Technologies). The RPA2 serine to alanine mutations at amino acids 4, 8, 33, 29 and 23 were generated by four consecutive site-directed mutagenesis reactions. The primer sequences used were:

Ser33Ala forward 5′GCCACCATGTGGAACGGTGG ATTCGAAGGCTATGGC

5′GGCTTTGGATCGCCCGCACCTgCTCAAGCC GAAAAGAAATCAAGA, Ser33Ala reverse 5′TCTTGA TTTCTTTTCGGCTTGAGcAGGTGCGGGCGATC CAAAGCC,

Ser29Ala forward 5′CAGTCCCCGGGGGGCTTTG GAgCGCCCGCACCTTCTCAAGCCGAA, Ser29Ala r everse 5′TTCGGCTTGAGAAGGTGCGGGCGcTCC AAAGCCCCCCGGGGACTG, Ser23Ala forward 5′G GAGCCGGCGGCTACACGCAGgCCCCGGGGGGC TTTGGATCGCCC, Ser23Ala reverse 5′GGGCGATCC AAAGCCCCCCGGGGcCTGCGTGTAGCCGCCGG CTCC.

Ser4,8Ala forward 5′GCCACCATGTGGAACgGTGG ATTCGAAgGCTATGGC, Ser4,8Ala reverse 5′GCTAGC GACGTCGGCGCGCCCTCGAGTCGC.

The RPA2 wild type and mutant were subcloned into pCAG-Flox and HCT116 were stably transfected using Lipofectamine 2000 (Invitrogen, Life Technologies) in according to manufacturer's instructions. The cells that integrated the gene were selected using 2 μg/ml puromycin.

siRNA transfection

For CDC45 depletion a set of siRNAs were used (ON-TARGETplus CDC45 siRNA, L-003232, Dharmacon, GE Healthcare). For endogenous RPA2 depletion a pool of three different siRNAs targeted to 3′ untranslated gene region were used (ON-TARGETplus siRNA J-017058-09, J-017058-11, J-017058-12, Dharmacon, GE Healthcare). The corresponding sense sequences are AACAUGAAGU-UCUGCGGUA, GAGCAGGACCAGGGCGUUA and GGAAGUAGGUUUCAUCUAU. Control siRNAs, containing nonspecific sequences that do not have homology in human genome, were provided by Eurofins (UAAU-GUAUUGGAACGCAUA). siRNA duplexes were transfected into cells using Lipofectamine 2000 (Invitrogen, Life Technologies) according to manufacturer's instructions. The cells were then incubated for 24 h before further treatment.

Cell cycle and caspase3 analysis

Cells were collected after the treatment, fixed with 70% ice-cold ethanol and stored at −20°C. After fixation, cells were washed twice with PBS and incubated for 15 min in PBS-T (PBS, 0.1% BSA, 0.25% Triton X-100). After centrifugation, the cell pellet was suspended in the same buffer plus an antibody specifically recognizing cleaved-caspase3 (Cleaved Caspase-3 (Asp175) Antibody 9661, Cell Signalling) diluted 1:500 and incubated for 3 h at room temperature. The cells were then rinsed with PBS containing 0.25% Triton X-100 and incubated with the FITC-conjugated goat anti-rabbit immunoglobulins antibody (sc-2012, Santa Cruz Biotechnology) diluted at a ratio of 1:30 in PBS containing 1% BSA. After a 30 min incubation at room temperature in the dark, the cells were washed with PBS and stained with PI solution (PBS with 5 μg/ml PI and 100 μg/ml RNAse A), and cellular fluorescence was measured by flow cytometry (LSRII, Becton Dickinson, Oxford, UK) and analysed using FlowJo software.

Immunofluorescence

HCT116 cells were cultured on glass coverslips and treated as indicated. Cells were then fixed with 3% buffered paraformaldehyde for 15 min at RT, incubated in PBS containing 30 mM NH_4Cl for 5 min and permeabilized in PBS containing 0.5% Triton X-100 for 8 min at RT. Cells were stained using an anti-phosphoRPA2 Ser-4,8 (A300-245A) from Bethyl cells diluted 1:1000 for 45 min at RT and detected with Alexa 488-conjugated goat anti-rabbit IgG (A11008; Molecular Probes, Invitrogen) diluted 1:1000. Antibody dilutions and washes after incubations

were performed in PBS containing 0.5% BSA and 0.05% Tween 20. Coverslips were finally mounted in Vectashield mounting medium with DAPI (H-1500; Vector Laboratories, Burlingame, CA, USA). Resulting fluorescence was visualized using a Nikon Eclipse T200 microscope equipped with a Hamamatsu Orca ER camera and the Volocity 3.6.1 (Improvision, Cambridge, UK) software. Pictures were further processed using Image J software (http://rsbweb.nih.gov/ij/).

Protein extraction and Western blotting

Whole-cell extracts were prepared after cells were trypsinized, collected and lysed by incubation with Cell Lysis Buffer (#9803, Cell Signalling) on ice for 30 min followed by centrifugation at maximum speed (15 000 rpm) for 5 min at 4°C. Chromatin-bound proteins were isolated using a modification of protocol described by Mendez and Stillman (42). A total of 3×10^6 cells were washed with PBS, resuspended in 200 μl of solution A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF, phosphatase inhibitors [PhosStop, 04906837001 Roche] and protease inhibitors [Complete Protease Inhibitor Cocktail, Roche]) containing 0.1% Triton X-100, and incubated on ice for 5 min. Cytoplasmic proteins were separated from nuclei by low-speed centrifugation (1000 x g for 5 min at 4°C). Isolated nuclei were washed once with solution A and then lysed in 200 μl of solution B (10 mM HEPES at pH 7.9, 3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF and protease and phosphatase inhibitors) on ice for 30 min. Soluble nuclear proteins were separated from insoluble chromatin by centrifugation (1500 x g for 5 min at 4°C). Isolated chromatin was washed once with solution B and centrifuged at 1500 x g for 5 min. The final chromatin was resuspended in 200 μl of buffer A without glycerol and incubated with 100 U Benzonase Nuclease (Novagen) to release chromatin bound proteins for 1 h at room temperature. The samples were centrifugated at maximum speed (15 000 rpm) for 5 min at 4°C before quantification by Bradford method (Bio-Rad). Twenty microgram of protein from each sample were loaded.

Proteins were separated by SDS-PAGE and blotted onto nitrocellulose (Whatman Schleicher & Schuell). Primary antibodies used were: anti-γH2AX (#2577), anti-phosphoCHK1 Ser296 (#2349), anti-phosphoCHK1 Ser317 (#2344), anti-phosphoCHK1 Ser345 (#2348), anti-CHK1 (#2360) from Cell Signaling; anti-H2AX (ab11175), anti-RAD51 (ab213), anti-active caspase 3 (ab32042), anti-PP2CA (ab33537) from Abcam, anti-RPA1 (sc-14696), anti-CDC45 (sc-20685) from Santa Cruz Technology; anti-RPA2 (NA19L) from Calbiochem; anti-PP4R2 (A300–838A), anti-phosphoRPA2 Ser33 (A300-246A), anti-phosphoRPA2 Ser-4,8 (A300-245A), PALB2 (A301-246A) from Bethyl; or anti-β-actin (A-5060) from Sigma. Proteins were detected using the enhanced chemiluminiscence detection system (ECL, GE Healthcare) according to the manufacturer's recommendations and signals were recorded by means of LAS-3000 device (Fujifilm). Image J software (http://rsbweb.nih.gov/ij/) was used to process images and quantify protein band intensities on the western blots.

In vitro lambda protein phosphatase treatment

Protein extracts were prepared after cells were collected and lysed for 30 min incubation on ice in buffer 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Igepal CA-630, 0.5 mM PMSF and protease inhibitors. Protein samples were split in two (500 μg each), one of them was treated with 400 U/μl lambda protein phosphatase (P0753, New England BioLabs) plus 1 mM $MnCl_2$ at 30°C for 1 h, the other one (negative control) was only incubated with $MnCl_2$.

Detection of DSBs by pulsed-field gel electrophoresis

Cells were treated with 2 mM thymidine and/or Chk1 inhibitor Gö6976 for 24 h. Following treatments, 10^6 cells were embedded into agarose plugs. The agarose inserts were incubated in 0.5 M EDTA pH 8.0, 0.2% sodium deoxycholate, 1% *N*-laurylsarcosyl, and 1 mg/ml proteinase K at 50°C for 24 h and then washed four times in TE buffer, before loading onto a 0.8% agarose gel. Pulsed-field gel electrophoresis (120° angle, 60–240 s switch time, 4 V/cm; Bio-Rad) was then carried out for 24 h. The gel was subsequently stained with ethidium bromide.

RESULTS

Nuclear RPA2 is extensively hyperphosphorylated in CHK1 inhibited cells treated with replication inhibitors

In addition to the recruitment of RPA to stalled replication forks, RPA2 is heavily hyperphosphorylated in HCT116 cells after treatment with CHK1 and replication inhibitors (37). To determine the extent of this hyperphosphorylation, chromatin-bound proteins from HCT116 cells treated with the CHK1 inhibitor Gö6976 (40) after treatment with thymidine for various times were isolated and analysed by western blotting. Notably chromatin-bound hyperphosphorylated RPA2 could be detected as early as 2 h after treatment with the CHK1 inhibitor and thymidine (but not by either agent alone) as indicated by the reduced electrophoretic mobility of this protein (Figure 1A, Supplementary Figure S1) and antibodies specific for phosphorylated forms of RPA2 (Figure 1B). By 16h nearly 70% of chromatin-bound RPA2 was altered (Figure 1C). The sensitivity of this slowly migrating RPA to λ phosphatase treatment confirmed that the altered mobility was the result of phosphorylation (Figure 1D). Since immunofluorescence analysis showed that 70% of cells contain pSer4,8 RPA2 foci (Figure 1E) our data suggest that the hyperphosphorylation of chromatin bound RPA2 under these conditions is nearly complete. Like RPA2, increased loading of RPA1 onto chromatin was clearly evident by 2h however there was no detectable change in the electrophoretic mobility of this subunit (Figure 1F).

Similar levels of chromatin-bound hyperphosphorylated RPA2 were found in HCT116 cells treated with hydroxyurea (HU) and CHK1 inhibitor (Supplementary Figure S2) and in SW480 colon cancer cells treated with thymidine and the

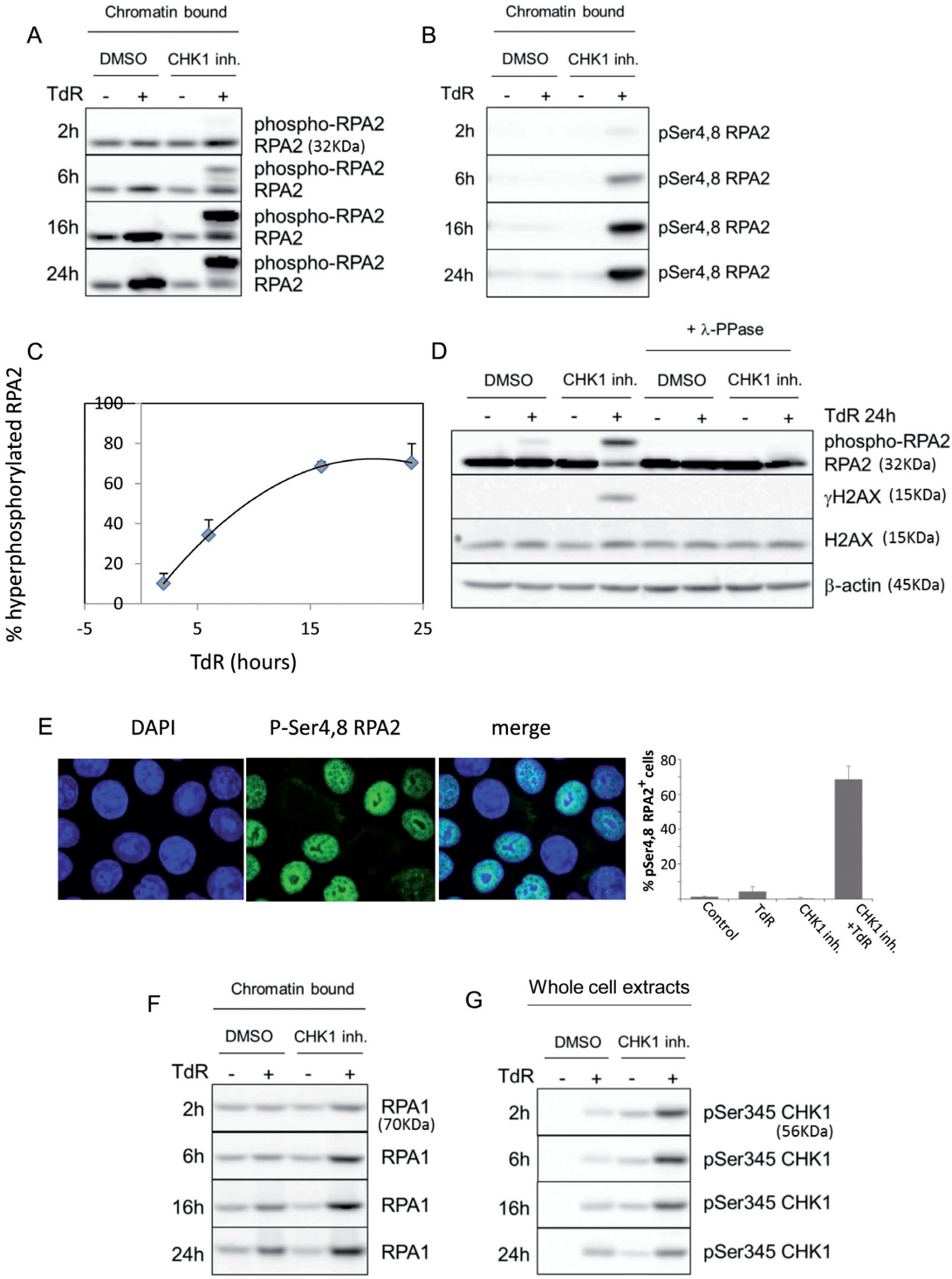

Figure 1. Nuclear RPA2 is extensively hyperphosphorylated in HCT116 cells treated with CHK1 inhibitor and thymidine. HCT116 cells treated or not treated with the CHK1 inhibitor Gö6976 in presence or absence of 2 mM thymidine were harvested at the indicated times and cytosolic, nuclear soluble or chromatin bound fractions were isolated. RPA2 (**A**) or pSer4,8-RPA2 (**B**) in the chromatin fractions were then analysed by western blotting. Densitometric measurements of phosphorylated RPA2 indicate that up to 70% of chromatin bound RPA2 is hyperphosphorylated (**C**). (**D**) Whole cell extracts obtained from cells treated with thymidine for 24 h in the presence or absence of the CHK1 inhibitor in the right lanes of this blot were treated with λ-phosphatase to confirm that the slower migrating band was the result of phosphorylation of RPA2. A blot of γH2AX is presented as a control to confirm the effectiveness of the phosphatase treatment. (**E**) Cultures of HCT116 cells treated for 24 h with Gö6976 in presence or absence of 2mM thymidine were fixed and stained for P-Ser4,8 RPA2 and the fraction of cells staining for this modified protein was determined by immunofluorescence. (**F**) Extracts prepared for panel A were also analysed for RPA1 content. (**G**) Whole cell extracts prepared from HCT116 cells treated as above were probed with an antibody for pSer345CHK1. Blots of all cellular fractions from this experiment are presented in Supplementary Figure S1. A western blot of those samples probed with an antibody for the origin binding protein ORC2 is also presented in this figure as a control for the cellular fractionation and gel loading.

CHK1 inhibitor (Supplementary Figure S3). The strong recruitment of RPA1 and 2 to chromatin in the colon cancer cell lines treated with CHK1 and replication inhibitors produced a noticeable decline in the cytosolic pools of these proteins at >16h although they were not fully depleted. Nuclear soluble pools did not appear to be greatly affected (Supplementary Figures S1–S3). Thus in contrast to previous studies using other tumour cell lines (36), our data suggest that the pools of RPA1 and 2 are not exhausted in these tumour cell lines by treatment with CHK1 and replication inhibitors.

This early hyperphosphorylation of RPA2 was accompanied by a robust phosphorylation of CHK1. ATR-mediated phosphorylation of CHK1 at Ser345 and Ser317 following DNA replication stress is essential for the activation of its checkpoint functions. In the presence of the CHK1 inhibitor, this phosphorylation would be expected to continue but the phosphorylated CHK1 would no longer be able to fulfill its role in the downstream response to DNA replication stress. pSer345 and pSer317 CHK1 markedly increased in CHK1 inhibited cells within 2h of thymidine treatment relative to cells treated with thymidine or CHK1 inhibitor alone (Figure 1G, Supplementary Figure S4). In contrast levels of autophosphorylated CHK1 (pSer296 CHK1) decreased in cells treated with the CHK1 inhibitor, indicating the effectiveness of the CHK1 kinase inhibitor (43) (Supplementary Figure S4). These data suggest that ATR (which phosphorylates both CHK1 and RPA2) is more strongly activated in CHK1 inhibited tumour cells during replication stress than in cells treated with a replication inhibitor alone, consistent with the enhanced level of ssDNA found in such cells (38,39).

Effects of CHK1 Inhibition on loading of replication and repair proteins

Since the fate of cells treated with replication and CHK1 inhibitors is likely to be influenced by the response of DNA repair and replication proteins, we next investigated the recruitment of such proteins to chromatin under these conditions. Given a report that RPA hyperphosphorylation suppressed the loading of RAD51 onto ssDNA (33), the levels of this protein bound to chromatin were first measured. Chromatin bound RAD51 increased in HCT116 cells treated with thymidine by 16h (Figure 2A, Supplementary Figure S5A). In contrast there was no change in chromatin bound RAD51 in cells treated with Gö6976 and thymidine, where hyperphosphorylation of RPA2 was nearly complete. It is also notable that the level of RAD51 in all cellular fractions was appreciably lower in cells treated with the CHK1 inhibitor, particularly in the presence of thymidine (Supplementary Figure S5A). Thus the amount of RAD51 bound to chromatin was much lower in CHK1 inhibited cells treated with thymidine where RPA2 was extensively hyperphosphorylated, however this decreased loading was accompanied by an overall decrease in the level of RAD51 in such cells (Supplementary Figure S2 and Figure 3A). Similar effects on RAD51 loading were found in SW480 cells treated with thymidine and Gö6976 however the overall level of RAD51 was not greatly reduced (Supplementary Figure S3).

The PP4 phosphatase complex (including PP4 catalytic and the PP4R2 regulatory subunits) has been shown to facilitate RAD51 binding to chromatin through its role in dephosphosphorylation of RPA2 (33). To determine whether this complex also influenced RPA2 hyperphosphorylation in CHK1 depleted cells, HCT116 cells were first analysed for the recruitment of the PP4R2 subunit to chromatin following treatment with thymidine in the presence or absence of Gö6976. In the absence of the CHK1 inhibitor PP4R2 binding was elevated by 16 h post thymidine treatment relative to control cells (Figure 2B, Supplementary Figure S5B). However, there was no corresponding increase in the level of chromatin bound PP4R2 following treatment with thymidine and the CHK1 inhibitor. Loading of PP4R2 onto chromatin was also suppressed in SW480 cells treated with thymidine and CHK1 inhibitor (Supplementary Figure S3). In contrast binding of PP2A, which has been implicated in the dephosphorylation of RPA2, γH2AX and CHK1 (44–46), was not altered in cells treated with thymidine in the presence or absence of the CHK1 inhibitor (Supplementary Figure S5C).

Given the role of CHK1 in controlling inappropriate origin firing during DNA replication stress and replication fork integrity, we next investigated whether CHK1 inhibition also affected the localization of CDC45 which is an essential helicase co-factor involved in replication initiation in both yeast and human cells (47–49) and PALB2 which has recently been shown to facilitate replication fork recovery (35). Extracts prepared from cells treated as before with Gö6976 and/or thymidine were fractionated and analysed for CDC45 and PALB2 (Figure 2C and D, Supplementary Figure S5D). PALB2 was recruited to chromatin following thymidine-induced replication stress in the presence or absence of the CHK1 inhibitor. In contrast chromatin bound CDC45 increased in cells as early as 2 h after treatment with thymidine and Gö6976 consistent with the role of this protein in the increased firing of origins that occurs in the absence of CHK1 function. Thus, the loading of CDC45 onto chromatin increased in cells under conditions where RPA2 is extensively hyperphosphorylated.

CDC45 depletion does not restore loading of PP4R2 or RAD51 in CHK1 inhibited cells

We previously reported that when inappropriate origin firing in the absence of CHK1 was suppressed by CDC45 depletion, both RPA2 hyperphosphorylation and the induction of caspase 3-dependent apoptosis were substantially reduced (37). To determine whether CDC45 depletion also restored RAD51 or PP4R2 loading onto chromatin, HCT116 cells depleted of CDC45 were treated with thymidine in the presence or absence of CHK1 inhibitor. We previously showed that CDC45 depletion slowed S-phase progression but had little effect on cell viability (37). Whole cell extracts or chromatin bound fractions prepared from these cells were analysed by western blotting. Consistent with previous results, both RPA2 hyperphosphorylation and caspase 3 activation were suppressed in CDC45 depleted cells treated with thymidine and CHK1 inhibitor (Figure 3A and B). In addition the elevated level of phosphorylated CHK1 found in cells treated with the CHK1 inhibitor was reduced to

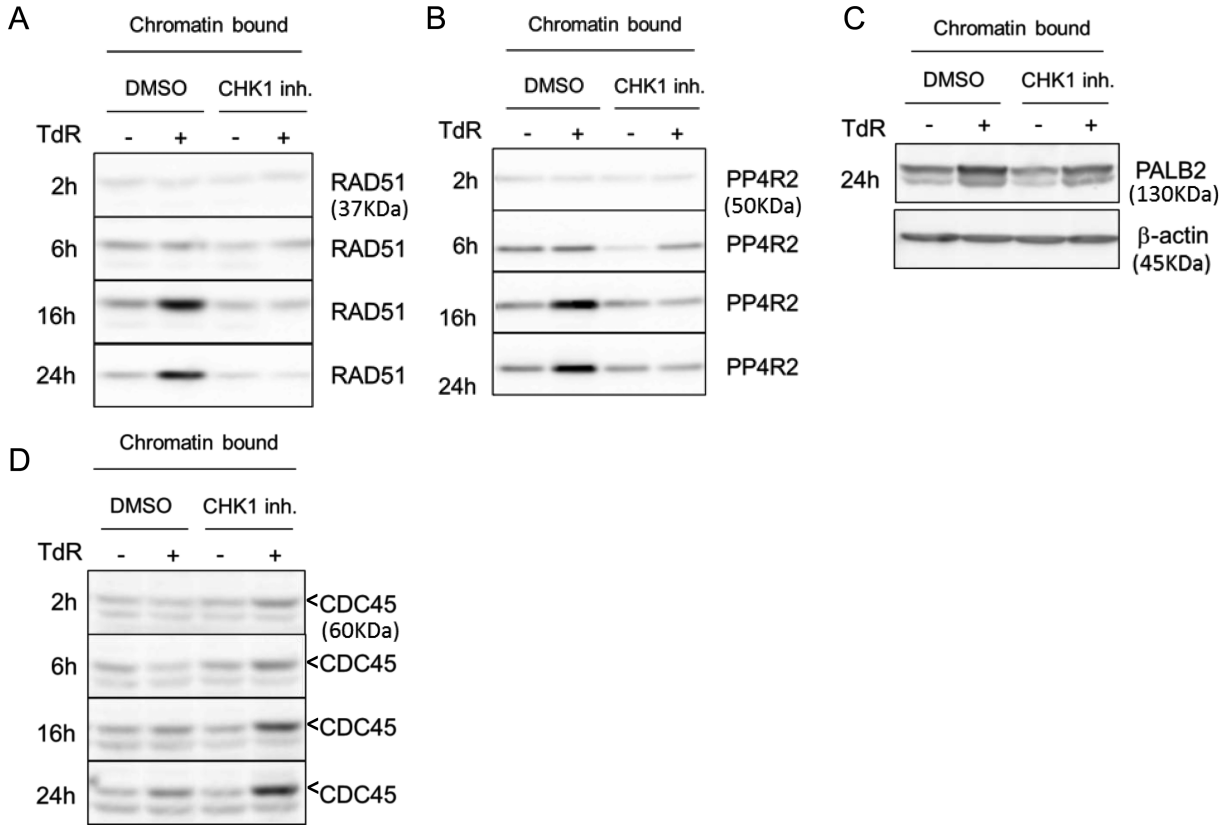

Figure 2. Recruitment of DNA replication and repair proteins to chromatin in HCT116 cells in the presence or absence of CHK1 inhibitor and thymidine. HCT116 cells treated or not treated with the CHK1 inhibitor Gö6976 in presence or absence of 2 mM thymidine were harvested at the indicated times and the chromatin bound fractions were analysed by western blotting. (**A**) RAD51, (**B**) PP4R2, (**C**) PALB2, (**D**) CDC45. Blots of all cellular fractions from this experiment are presented in Supplementary Figure S5.

that found in cells treated with thymidine alone. Despite the reduction of RPA2 hyperphosphorylation, neither RAD51 nor PP4R2 loading were restored (Figure 3B). Thus, RPA2 hyperphosphorylation alone is not responsible for the suppression of RAD51 or PP4R2 loading in the absence of CHK1 function.

Mutations of RPA2 phosphorylation sites fail to restore RAD51 or PP4R2 loading

To further investigate the cellular effects of RPA2 hyperphosphorylation, we isolated HCT116 strains ectopically expressing wild type RPA2 (called RW, Figure 4A), RPA2 mutated at CdK and ATR phosphorylation sites (Ser33->Ala, Ser29->Ala and Ser23->Ala, called RM), or RPA2 mutated at five serine residues contributing to hyperphosphorylation (Ser33->Ala, Ser29->Ala and Ser23->Ala, Ser8->Ala and Ser4->Ala, called R5M). To determine the effects of the constructs on cellular RPA2 content and phosphorylation, HCT116 and strains expressing wild type or mutant RPA2 were transfected with control siRNA or RPA2 siRNA targeted to the 3′ UTR of the endogenous RPA2 transcript to deplete endogenous RPA2 but not the ectopically expressed protein (Figure 4B&C). Cells were then treated with CHK1 inhibitor and/or thymidine for 24h for preparation of whole cell and chromatin extracts for analysis of RPA2 by Western blotting. RPA2 and RPA1 were substantially depleted and pSer4,8 RPA2 was reduced in parental HCT116 cells following transfection with the 3′ UTR siRNA (Figure 4B). In contrast the RW, RM, and R5M derivatives retained a high level of RPA2 and RPA1 after transfection (Figure 4C and D). Hyperphosphorylated RPA2 and pSer4,8 RPA2 were still detected in RW while they were reduced in RM cells treated with the RPA2 siRNA. However hyperphosphorylated RPA2 and pSer4,8 RPA2 were not evident in whole cell or chromatin extracts of R5M treated with CHK1 inhibitor, thymidine and the RPA2 siRNA (Figure 4C and D).

To investigate the relationship between RAD51 and PP4R2 loading and RPA2 hyperphosphorylation, chromatin isolated from the cells treated with thymidine in the presence or absence of the CHK1 inhibitor was analysed for RAD51 and PP4R2 content. Clearly RAD51 and PP4R2 loading onto chromatin were not regained in cells expressing the mutant RPA2 alleles where hyperphosphorylation was suppressed following treatment with thymidine and the CHK1 inhibitor (Figure 4D). Additionally RPA2 depletion in HCT116 cells did not affect the loading of the two proteins (Figure 4E). Thus, these data support our conclusion that the failure to load RAD51 or PP4R2 in CHK1-inhibited cells is not solely a consequence of RPA2 hyperphosphorylation or the level of chromatin bound RPA under these conditions. It is also interesting to note that the re-

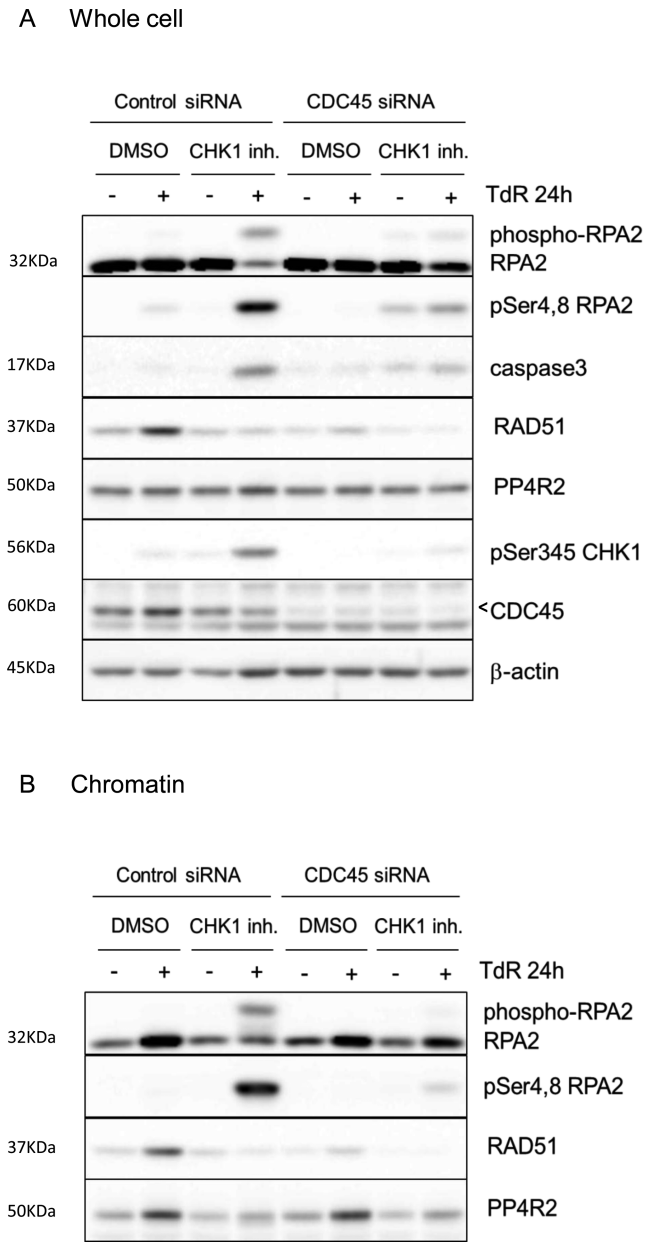

Figure 3. CDC45 depletion suppresses RPA2 and CHK1 phosphorylation and caspase 3 activation but fails to fully restore RAD51 or PP4R2 binding to chromatin. HCT116 cells transfected with control or CDC45 siRNAs in the presence or absence of the CHK1 inhibitor Gö6976 and/or 2 mM thymidine were harvested after 24 for Western blot analysis. Whole cell extracts (**A**) or chromatin bound proteins (**B**) from these cells were analysed for the indicated proteins by Western blotting.

cruitment of PALB2 to chromatin in HCT116 cells occurred in the presence or absence of RPA2 hyperphosphorylation.

Effects of mutant RPA2 on the fate of HCT116 cells treated with CHK1 and replication inhibitors

Tumour cells treated with CHK1 and replication inhibitors have been shown to suffer a number of fates including the induction of apoptosis (1–3) and extensive induction of DSBs (38). To determine whether RPA2 hyperphosphorylation contributed to any of these outcomes, we next determined the effects of expression of mutant and wild type RPA2 alleles. To investigate effects on the apoptotic response, HCT116, RW, RM and R5M cells were treated with RPA2 3′ UTR siRNA to suppress the expression of the endogenous wild type RPA2. After 48 h cells were harvested and analysed for cell cycle distribution by flow cytometry. When parental HCT116 was treated with RPA2 siRNA, cells accumulated in late S and G2/M consistent with the central role played by RPA in DNA replication and many other DNA transactions (Figure 5A). In contrast, as reported previously (29,30), the ectopically expressed wild type or mutant RPA2 constructs were able to support the growth of HCT116 cells after depletion of the endogenous wild type RPA2 as RW, RM and R5M cells show a normal cell cycle distribution (Figure 5A) with the exception of a significant decrease in the level of cells with a subG1 DNA content in the R5M cells (Figure 5A and C). Thus, both wild type and mutant RPA2 are able to rescue cells transfected with RPA2 siRNA from S-G2/M arrest.

RW, RM, or R5M cells treated with the RPA2 3′ UTR siRNA were then exposed to CHK1 inhibitors (Gö6976 or MK8776) in the presence or absence of thymidine for 48 h before harvest and analysis of DNA content by flow cytometry. Like HCT116, the fraction of RW cells with a subG1 content increased following co-treatment with CHK1 inhibitors and thymidine relative to cells treated with either inhibitor alone (Figure 5B and C). In contrast the frequency of similarly treated RM or R5M cells with a subG1 DNA content was reduced. In parallel with the reduction of cells with a subG1 content the fraction of S-phase cells increased in RM and R5M cultures treated with CHK1 inhibitors and thymidine. The fractions of cells containing activated caspase 3 were also measured in these experiments (Figure 5D and E). Consistent with the reduction in the level of subG1 cells, the fractions of cells containing activated caspase 3 were reduced in both RM and R5M compared to RW after treatment with CHK1 inhibitors and thymidine.

RPA2 phosphorylation is not required to protect HCT116 cells from DSBs induced by CHK1 and replication inhibitors

Similarly some tumour cell lines show extensive DSB induction after treatment with CHK1 inhibitor as a single agent or in combination with a replication inhibitor (38,50). However, the level of DSBs is low in HCT116 cells treated with these inhibitors singly or in combination (37). To determine whether expression of the mutant RPA2 affected the frequency of DSBs, HCT116, RW, RM or R5M cells treated the CHK1 inhibitor and/or thymidine were harvested and analysed for DSBs by pulsed field gel electrophoresis (Figure 6). DSB formation increased in HCT116 cells depleted of RPA2, again consistent with its role in DNA metabolism. The level of DSBs increased in RPA2-depleted cells treated with Gö6976 although the level of breaks was lower in cells treated with both CHK1 and replication inhibitors. In contrast expression of wild type or mutant RPA2 proteins strongly reduced DSBs induced in HCT116 after siRNA-mediated depletion of the endogenous RPA2 in the presence or absence of the inhibitors. Thus, the mutant RPA2 that lacks key phosphorylation sites is still able to protect

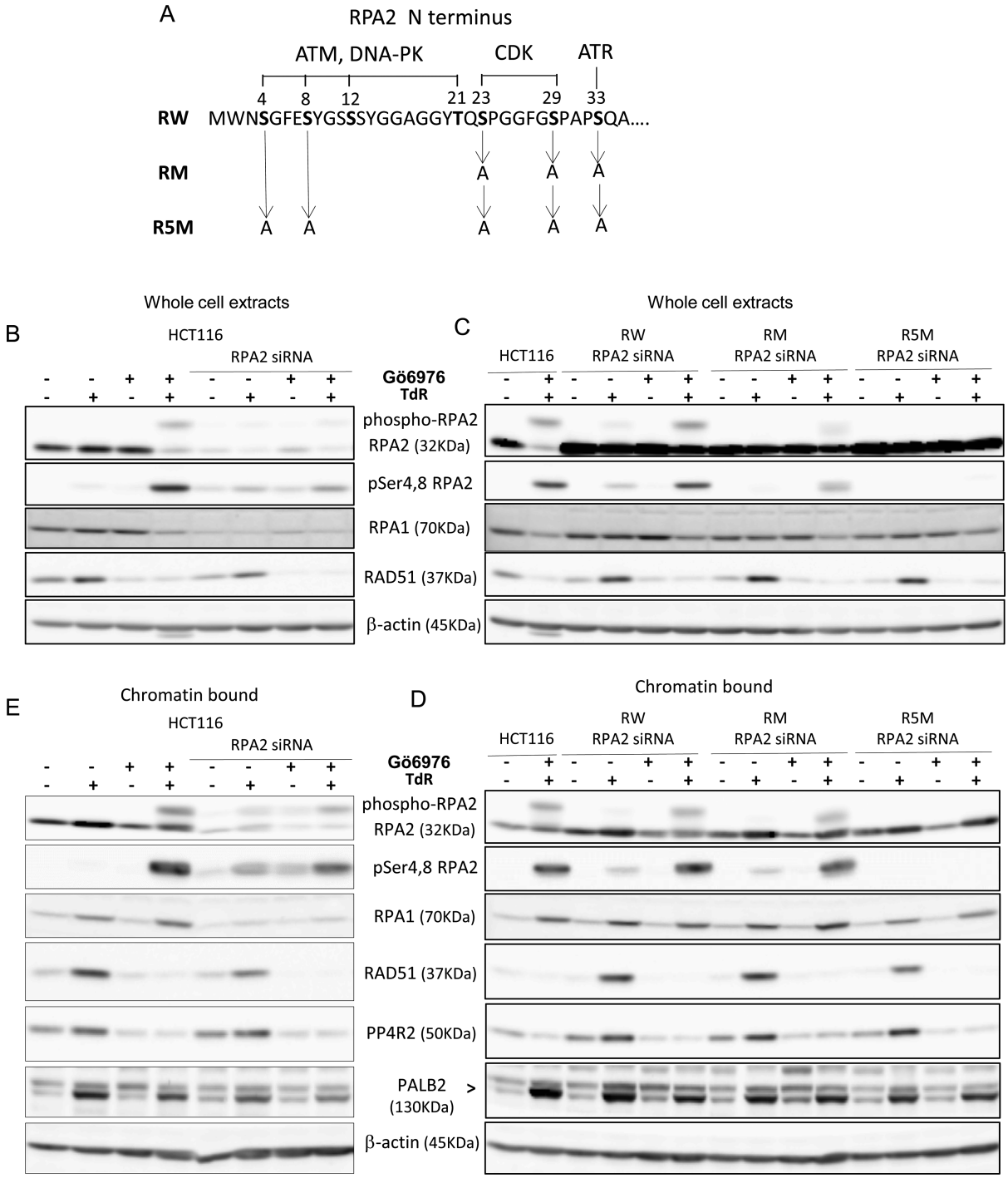

Figure 4. Expression of a nonphosphorylatable RPA2 does not restore loading of RAD51 or PP4R2 onto chromatin after treatment with CHK1 and replication inhibitors. (**A**) Mutant RPA2 cDNAs containing alterations of the indicated phosphorylation sites transfected into HCT116 cells used for the isolation of strains stably expressing the mutant constructs. (**B**) Parental HCT116 cells transfected with control or RPA2 siRNAs (specific for the 3′ UTR of the endogenous RPA2 transcript) were treated or not treated with CHK1 inhibitor Gö6976 in the presence or absence of 2mM thymidine. Cell free extracts prepared from these cells were analysed for the indicated proteins by western blotting. (**C**) Protein extracts from parental cells and HCT116 cells ectopically expressing wild type or mutant RPA2 treated as in panel B were analysed by Western blotting for the indicated proteins. RPA2 levels in RW and RM cells are about 50% greater than those in the HCT116 as determined by densitometry. The images presented here were over-exposed to detect residual hyperphosphorylated RPA2. The 3′ UTR siRNA effectively depleted parental HCT116 cells of RPA2 but did not greatly affect the overall level of RPA2 in RW or RM cells. Importantly hyperphosphorylated RPA2 was substantially reduced in the R5M cells (ectopically expressing the nonphosphorylatable mutant RPA2) treated with thymidine and the CHK1 inhibitor. These cells also showed substantially reduced levels of pSer4,8-RPA2. (**D**) Western blot analysis of indicated chromatin proteins prepared from parental HCT116 and strains ectopically expressing mutant and wild-type RPA2 as in Panel C. Notably the suppression of RPA2 hyperphosphorylation failed to restore RAD51 or PP4R2 binding to chromatin in HCT116 cells expressing mutant RPA2. (**E**) Western blot analysis of indicated chromatin proteins prepared from parental HCT116 cells depleted or not depleted of RPA2. Notably chromatin binding of RAD51 and PP4R2 are not affected by RPA2 depletion.

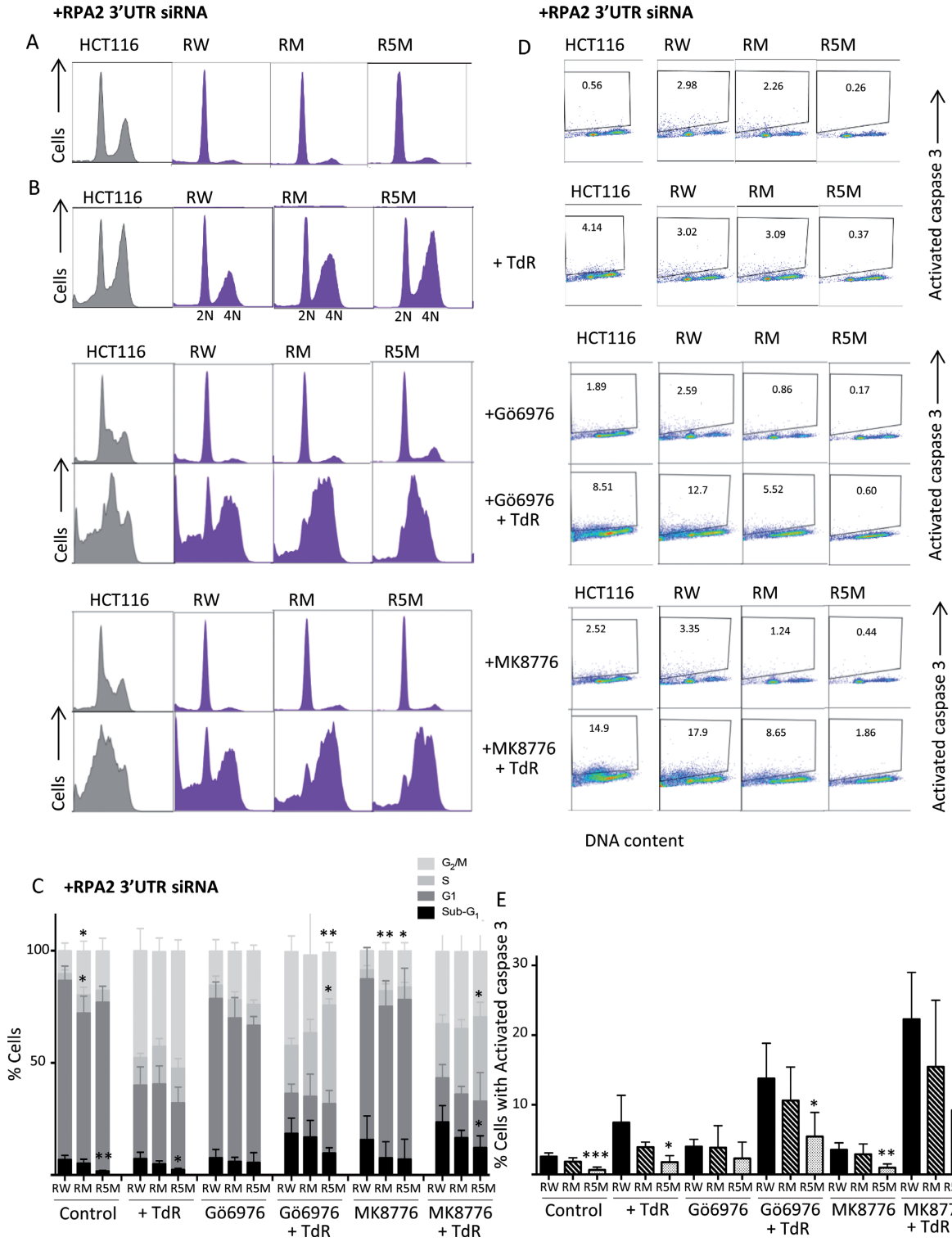

Figure 5. Mutant RPA2 suppresses the accumulation of cells with a subG1 DNA content and caspase 3 activation after treatment with CHK1 and replication inhibitors. (**A**) Representative cell cycle profile for HCT116, RW, RM or R5M cells transfected with RPA2 siRNA, harvested after 72 h, and analysed for DNA content by flow cytometry. (**B**) Representative profiles for HCT116, RW, RM and R5M cells transfected with RPA2 siRNA and treated with the CHK1 inhibitors Gö6976 (1 μM) or MK8776 (1 μM) in the presence or absence of 2 mM thymidine for 48 h before harvest and analysis of DNA content by flow cytometry. (**C**) Summary of flow cytometry analyses as above. The data presented are the means of three independent experiments and error bars represent standard deviations. The *P* values for the indicated measurements are relative to the results obtained for RW cultures treated in the same manner. $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$. (**D**) The same cultures were analysed for caspase 3 activation by flow cytometry and the percentages of RW, RM or R5M cells showing caspase 3 activation after these treatments are presented in (**E**). The data presented are the means of three independent experiments and error bars represent standard deviations. The *P* values for the indicated measurements are relative to the results obtained for RW cultures treated in the same manner. $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$.

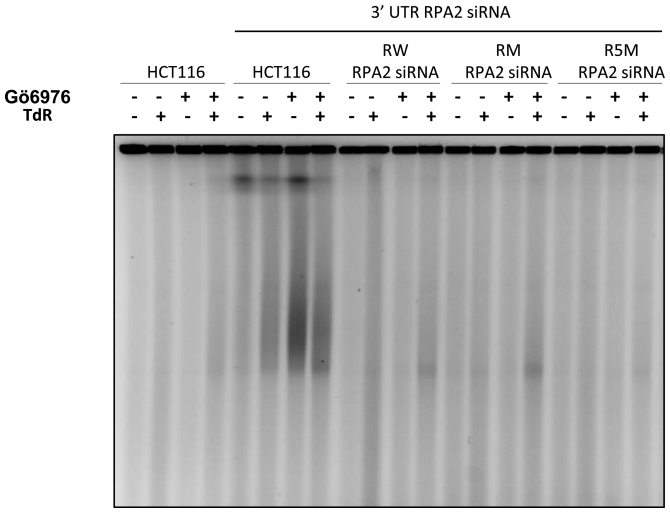

Figure 6. DSB analysis of HCT116 and strains expressing wild type and mutant RPA2. Parental HCT116 and strains ectopically expressing wild type or mutant RPA2 were depleted of endogenous RPA2 by transfection of the 3′ UTR siRNA before a 24 h treatment with 1 μM Gö6976 and 2 mM thymidine. Cells were then harvested for analysis of DSBs by pulsed field gel electrophoresis. RPA2 depletion triggers DSB formation in HCT116 but this is rescued by ectopic expression of mutant or wild type RPA2. Neither thymidine nor CHK1 inhibitors alone or in combination enhance DSB formation in RPA2 depleted cells ectopic expression of mutant RPA2s is still able to rescue HCT116 from DSB formation.

HCT116 cells from DSBs induced by RPA2 depletion or by CHK1 and replication inhibitors.

DISCUSSION

When DNA synthesis is arrested proteins required for repair of blocking lesions and replication restart are recruited to stalled forks and there is compelling evidence that the ATR-CHK1 signalling pathway plays a key role in coordinating this response (51). In the absence of ATR-CHK1 signalling these responses are disrupted, potentially leading to a loss of replication fork integrity and cell death. Consistent with many previous studies we show that one of the key proteins recruited to DNA when DNA replication is arrested, RPA2, is extensively hyperphosphorylated following treatment with DNA replication and CHK1 inhibitors. Hyperphosphorylation commences early after induction of replication stress and is accompanied by increased phosphorylation of H2AX (52). RPA2 phosphorylation occurs at stalled replication forks (53) and suppresses the loading of other proteins (such as RAD51) essential for replication restart (33,54). Consequently RPA2 phosphorylation is normally carefully controlled, in part by protein phosphatase complexes that are also recruited to DNA upon replication arrest (33). Here we show that the loading of RAD51 together with a component of the PP4 protein phosphatase complex (PP4R2) are suppressed by CHK1 inhibition and the failure to activate these repair pathways is likely to underlie the inability of cells treated with replication and CHK1 inhibitors to reenter and complete S-phase after release (52). However our data suggest that hyperphosphorylation of RPA2 is not directly responsible for the failure to load these proteins as suppression of this phosphorylation by CDC45 depletion

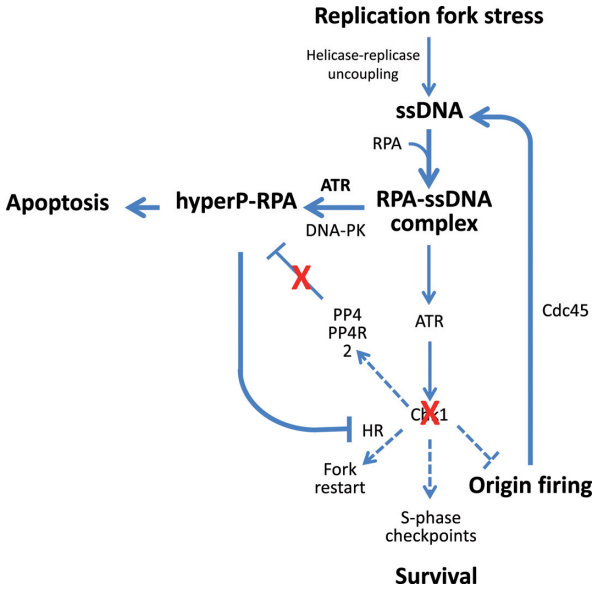

Figure 7. Model for the induction of apoptosis in cells lacking CHK1 function during replication stress. In normal cells DNA replication fork stress generates ssDNA as a result of uncoupling of replication and helicase complexes. RPA coated ssDNA triggers activation of the ATR-CHK1 pathway to enhance survival by suppressing origin firing and apoptosis and stimulating S-phase checkpoints and HR-dependent replication fork restart. Our results indicate that CHK1 is also required for the loading of the PP4 protein phosphatase complex onto chromatin to control phosphorylation of RPA2. When CHK1 is inhibited or depleted, cells fail to trigger S-phase checkpoints or fork restart and fail to load the PP4 complex onto chromatin. Cells lose control of CDC45-dependent origin firing leading to the accumulation of RPA-coated ssDNA and enhanced activation of ATR. This together with the failure to load the PP4 protein phosphatase complex and activation of DNA-PK leads to extensive hyperphosphorylation of RPA2. Depletion of CDC45 substantially reduces ssDNA formation, RPA2 hyperphosphorylation, ATR activation, and apoptosis while suppression of RPA2 hyperphosphorylation alone reduces the level of apoptosis. While these data are consistent with the proposal that ssDNA formation driven by inappropriate firing of replication origins in the absence of CHK1 function commit cells to apoptosis through RPA2 hyperphosphorylation, it is also likely that other cellular responses to the high level of ssDNA may also contribute to the induction of apoptosis.

or by mutation of RPA2 phosphorylation sites failed to restore RAD51 or PP4R2 loading. Thus, these observations suggest that CHK1 itself is the primary mediator of these protective pathways. A previous report that CHK1 affects RAD51 function through post-translational phosphorylation (19) supports this conclusion. Post-translational phosphorylation sites for PP4R2 have been identified although it is not known how these respond to DNA replication stress in the presence or absence of CHK1.

While our data appear to conflict with some previous work, a major difference in our study is the effect of CHK1 inhibition which greatly enhances the level of stress in cells exposed to replication inhibitors. Inappropriate firing of replication origins during DNA replication stress in the absence of a functional ATR-CHK1 signalling cascade substantially increases the level of ssDNA and RPA binding relative to that found during replication stress in cells containing active CHK1 (18,37) (Figure 7). Our evidence suggests that this in turn amplifies ATR signaling (which is dependent upon RPA coated ssDNA (12)) and consequently

the level of phosphorylated RPA2. Previous work shows that the phosphorylation of Ser33 by ATR is critical for the 'sequential and synergistic' phosphorylation at other sites (29). Consistent with this, DNA-PK activity is also activated in the absence of CHK1 (55) facilitating the phosphorylation of 'downstream' RPA2 phosphorylation sites. Since the loading of the PP4 phosphatase complex onto chromatin is dependent upon CHK1, another important restraint of RPA2 phosphorylation is lost when CHK1 is inhibited (Figure 7). As a result, hyperphosphorylation of RPA2 is amplified in the absence of CHK1 function.

Another characteristic of cells treated with CHK1 and replication inhibitors is their commitment to apoptosis. This is a late event and dependent upon CDC45 (37). CDC45 is an essential helicase cofactor that participates in both the initiation and elongation steps of DNA synthesis (47,49,56). Its depletion suppresses ssDNA formation, recruitment and hyperphosphorylation of RPA, and cell death (37). The data presented here show that widespread hyperphosphorylation of RPA2 promotes the induction of cell death as the substitution of RPA2 with a protein lacking several phosphorylation sites reduced the level of apoptotic cells as measured by subG1 DNA content and caspase 3 activation. Thus we propose that the substantial reduction of cell death by CDC45 depletion reflects an early role in the pathway of events that lead to apoptosis in the absence of CHK1 function while RPA2 hyperphosphorylation is a later, downstream event. This suggests that extensive ssDNA formation under these conditions may be the primary determinant of cell fate (Figure 7).

A number of reports show that cell death following inhibition of the ATR-CHK1 signalling cascade is linked to DSB formation at stalled replication forks (36,38,50). Recently this has been attributed to an exhaustion of RPA pools in cells treated with an ATR inhibitor making ssDNA at stalled forks vulnerable to attack by structure-specific endonucleases, a response called 'replicative catastrophe'(36). This does not appear to be the case for HCT116 following treatment with CHK1 and replication inhibitors as pools of RPA1 and RPA2 do not appear to be exhausted in these cells and there is little DSB formation. Here we tested the possibility that RPA2 hyperphosphorylation protected stalled forks in HCT116 from collapse. However mutant RPA2 lacking hyperphosphorylation was as effective as wild type RPA2 in rescuing cells from DSB formation.

Given our findings that RPA2 hyperphosphorylation is extensive in tumour cells treated with CHK1 and DNA synthesis inhibitors and that this response promotes the cellular commitment to apoptosis, we propose that RPA2 hyperphosphorylation might prove useful as a biomarker to predict the efficacy of therapies including CHK1 inhibitors.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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