UV-induced G2 checkpoint depends on p38 MAPK and minimal activation of ATR-Chk1 pathway

Daniël O. Warmerdam[1,2,*], Eva K. Brinkman[1], Jurgen A. Marteijn[1], René H. Medema[2], Roland Kanaar[1,3] and Veronique A. J. Smits[4,*]

[1]Department of Cell Biology and Genetics, Cancer Genomics Center, Erasmus Medical Center, 3000 CA Rotterdam, The Netherlands
[2]Division of Cell Biology I, Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands
[3]Department of Radiation Oncology, Erasmus Medical Center, 3000 CA Rotterdam, The Netherlands
[4]Unidad de Investigación, Hospital Universitario de Canarias, Instituto de Tecnologías Biomedicas, 38320 Tenerife, Spain
*Authors for correspondence: (d.warmerdam@nki.nl; vsmits@ull.es)

Accepted 18 February 2013
Journal of Cell Science 126, 1923–1930

doi: 10.1242/jcs.118265

Summary

In response to UV light, single-stranded DNA intermediates coated with replication protein A (RPA) are generated, which trigger the ATR-Chk1 checkpoint pathway. Recruitment and/or activation of several checkpoint proteins at the damaged sites is important for the subsequent cell cycle arrest. Surprisingly, upon UV irradiation, Rad9 and RPA only minimally accumulate at DNA lesions in G2 phase, suggesting that only a few single-stranded DNA intermediates are generated. Also, little phosphorylated Chk1 is observed in G2 phase after UV-irradiation, and UV light fails to elicit efficient accumulation of typical DNA damage response proteins at sites of damage in this phase. By contrast, p38 MAPK is phosphorylated in G2 phase cells after UV damage. Interestingly, despite the lack of an obvious activation of the ATR-Chk1 pathway, only the combined inhibition of the ATR- and p38-dependent pathways results in a complete abrogation of the UV-induced G2/M arrest. This suggests that UV light induces less hazardous lesions in G2 phase or that lesions created in this phase are less efficiently processed, resulting in a low activation of the ATR-Chk1 pathway. UV-induced G2 checkpoint activation in this situation therefore relies on signalling via the p38 MAPK and ATR-Chk1 signalling cascades.

Key words: ATR, Chk1, G2 phase, p38, UV

Introduction

After genotoxic stress DNA damage checkpoint and repair pathways are activated that ensure an intact transmission of the DNA (Hoeijmakers, 2001; Kanaar et al., 2008; Shiloh, 2003). Inefficient repair of DNA lesions can lead to genomic instability, a hallmark of cancer (Jeggo and Löbrich, 2006). ATM and ATR kinases are the master regulators of the DNA damage-induced checkpoint response (Abraham, 2001). ATM is predominantly activated in response to double-stranded breaks (DSBs), whereas the ATR pathway is triggered upon replication fork stalling, UV light and after resection of DSBs, although crosstalk between the pathways exists (Callegari and Kelly, 2007; Cimprich and Cortez, 2008; Jazayeri et al., 2006; Shiloh, 2006; Shiotani and Zou, 2009a). The extended single-stranded DNA (ssDNA) intermediates formed after damage, are coated rapidly by RPA (Callegari et al., 2010), after which Rad17-RFC(2–4) and ATRIP-ATR are recruited. Rad17-RFC then loads the Rad9-Rad1-Hus1 complex onto chromatin (Shiotani and Zou, 2009b; Zou and Elledge, 2003; Zou et al., 2003) and TopBP1 binds Rad9 to activate ATR (Kumagai et al., 2006; Lee et al., 2007). Active ATR phosphorylates a number of proteins including effector kinase Chk1 (Ser317/Ser345), resulting in a temporal halt of the cell cycle to gain time for DNA repair (Abraham, 2001). Although ATR signalling mainly functions in response to replication stress, the ATR-Chk1 pathway is also required for the DNA damage-induced G2 phase arrest (Mailand et al., 2000; Takai et al., 2000; Ward and Chen, 2001). p38 and MAPKAP kinase-2 were additionally implicated in the UV-induced G2 checkpoint (Bulavin et al., 2001; Manke et al., 2005).

Recruitment of proteins to sites of DNA damage can be observed as the relocalisation into nuclear foci. Artificial localisation of Mec1 and Ddc1, *S. cerevisiae* ATR and Rad9, to chromatin, triggers a checkpoint response in the absence of DNA damage (Bonilla et al., 2008), suggesting that ATR activation is dependent on the close proximity of these two complexes. We reported that Rad9 localizes to sites of damage in a cell cycle-dependent manner, depending on the type of DNA lesion. Upon ionizing radiation (IR), Rad9 localizes into foci in S and G2 cells, whereas UV light induces foci in G1 and S phase, but not G2 cells (Warmerdam et al., 2009). Here, we analysed the nuclear localisation of different DNA damage response (DDR) proteins upon UV-irradiation in G2 cells to explore the implications for checkpoint activation. Checkpoint proteins minimally accumulate at sites of UV damage, probably because RPA-coated ssDNA intermediates are not formed to the same extent as during G1 and S phase. Conversely, we show that the p38 MAPK is activated in G2 cells upon UV-irradiation. Interestingly, only the combined inhibition of ATR-Chk1 and p38 kinases leads to a complete loss of the UV-induced G2 checkpoint arrest, suggesting that the parallel activation of both pathways is required for UV-induced checkpoint activation.

Results and Discussion

Little Rad9 immobilisation and RPA focus formation after UV damage in G2 phase

We reported that Rad9, ATRIP, Hus1 and Rad1 localise to sites of DNA damage in a cell cycle-dependent manner (Warmerdam

et al., 2009). Localisation of Rad9 to sites of DNA lesions results in a partial, transient immobilisation of the protein (Medhurst et al., 2008; Warmerdam et al., 2009). To further address Rad9 focus formation and immobilisation, stable cells expressing GFP-Rad9 and Cyclin B-mCherry were transfected with CFP-PCNA, to distinguish all cell cycle phases. During S and G2, Cyclin B-mCherry protein levels rise and Cyclin B localises in the cytoplasm and appears on centrosomes shortly before mitosis (Hagting et al., 1999) (Fig. 1A; supplementary material Fig. S1A). PCNA forms foci during DNA replication (Essers et al., 2005). GFP-Rad9 showed a 20–30% immobilisation, determined by fluorescent recovery after photobleaching (FRAP) (Houtsmuller, 2005), after UV damage in G1 and S phase cells, whereas UV-treated G2 cells did not show any immobilised GFP-Rad9 (Fig. 1A). Time-lapse video microscopy of UV-treated cells for 24 hours did not show formation of GFP-Rad9 foci in G2 cells (data not shown). These experiments indicate that Rad9 accumulates to a lesser extent to sites of UV-induced lesions in G2 versus G1/S phase.

Rad9 relocalisation depends on the formation of extended RPA-coated ssDNA intermediates (Wu et al., 2005; Zou et al., 2003). We investigated RPA focus formation upon UV in G2

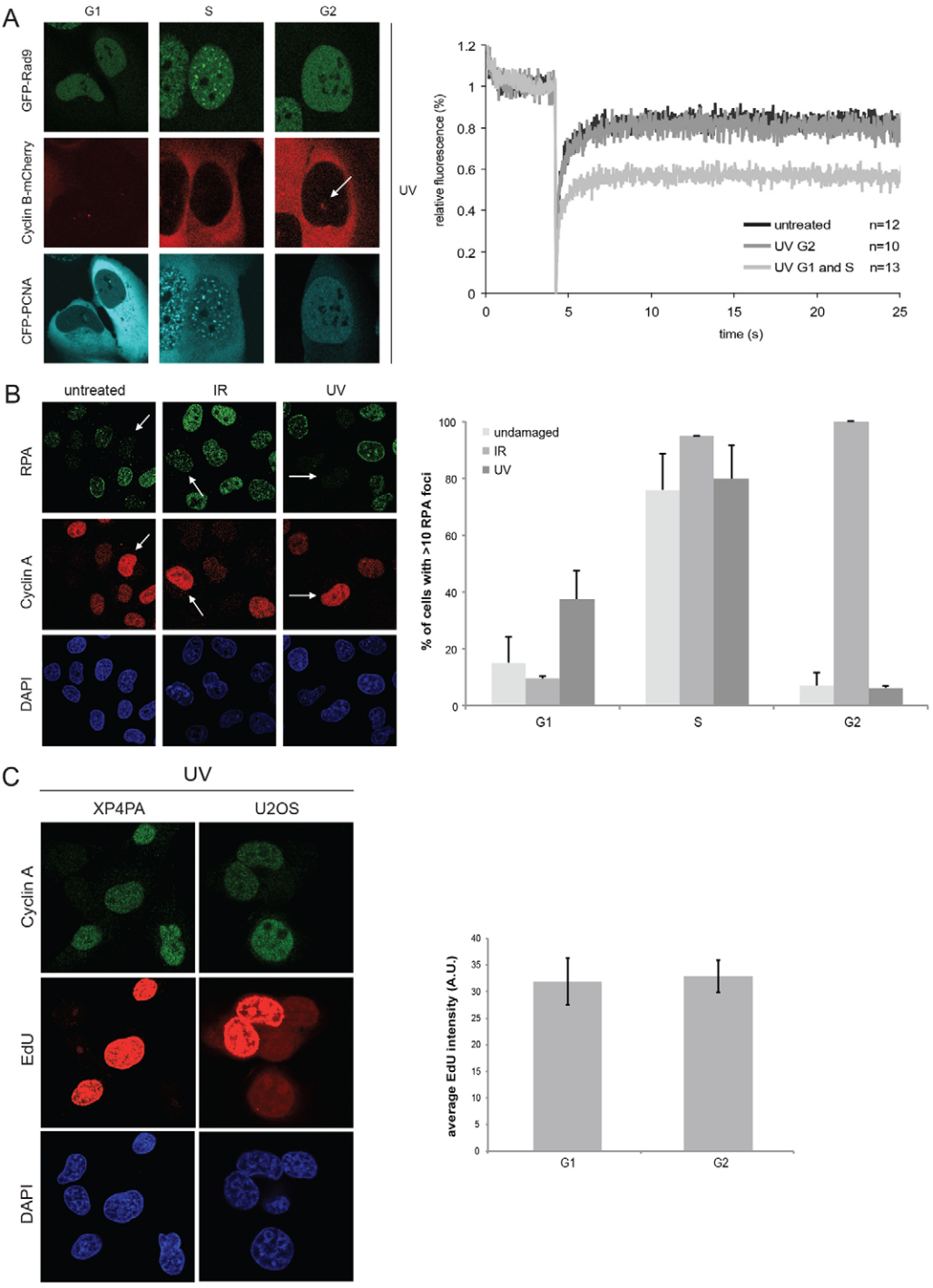

Fig. 1. Cell cycle-dependent Rad9 immobilisation and RPA focus formation. (**A**) U2OS cells expressing GFP-Rad9 and Cyclin B-mCherry were transfected with CFP-PCNA. Left: Images after UV-irradiation. Arrow indicates centromeric Cyclin B-mCherry. Right: FRAP analysis after UV. (**B**) U2OS cells treated with IR or UV irradiation, after which aphidicolin (1 μM) was added for 1 hour. Arrows point to G2 phase cells (left). Quantification of RPA foci throughout the cell cycle (right). (**C**) U2OS and XP4PA cells were incubated with EdU, UV-irradiated and fixed after 1.5 hours. Quantification of average EdU intensity throughout the cell cycle in U2OS was based on cell size (right).

phase cells, that express high levels of Cyclin A (Jazayeri et al., 2006; Warmerdam et al., 2009). Aphidicolin (1 μM) was added after the treatments, which inhibits replication without triggering a checkpoint response (supplementary material Fig. S1B), preventing S phase cells moving to G2. RPA localises into foci during S phase in untreated cells and after IR in S and G2 cells. In contrast, UV light-induced RPA focus formation was observed in G1 and S, but to a much lesser extent in G2 phase (Fig. 1B). As GFP-Rad9, RPA foci also did not form at later time points after UV damage in G2 phase cells (supplementary material Fig. S1C). These results suggest that less RPA-coated ssDNA intermediates are formed in UV-irradiated G2 cells, which is likely the cause of the absence of Rad9 foci under such conditions.

The most common UV-induced lesions are 6–4 photoproducts and cyclobutane pyrimidine dimers that are mainly repaired by nucleotide-excision repair (NER). During NER small ssDNA gaps are formed that likely trigger ATR-mediated checkpoint activation (Marini et al., 2006; Warmerdam et al., 2009). Cell cycle regulation of NER, which could explain low RPA focus formation in G2 cells after UV, was studied by incubating UV-treated cells with nucleotide analogue EdU, which is incorporated in newly synthesized DNA in S phase and during NER (Nakazawa et al., 2010) (Fig. 1C; supplementary material Fig. S1D,E). In untreated cells or in XP4PA cells that have no functional NER, EdU incorporation was only present in replicating cells. In U2OS cells treated with UV light, equal amounts of EdU incorporation were present in G1 and G2 cells, indicating that NER is similarly active during G1 and G2 phases (Fig. 1C; supplementary material Fig. S1E). In addition, we observed accumulation of NER protein XPC to local UV damage in G2 (Marteijn et al., 2009) (supplementary material Fig. S1F). This indicates that although G2 phase cells are proficient in NER activity, this is not sufficient for the formation of extended RPA-coated ssDNA intermediates that trigger ATR signalling.

Marginal ATR-mediated Chk1 phosphorylation upon UV irradiation in G2

The observed low frequency of RPA focus formation and high mobility of Rad9 after UV damage in G2 phase prompted us to study the consequences for ATR-mediated checkpoint signalling. After UV-irradiation of U2OS and HeLa cells, Chk1 was only minimally phosphorylated on Ser317/Ser345 in cells positive for cytoplasmic Cyclin B, whereas treatment with IR resulted in efficient Chk1 phosphorylation (Fig. 2A–C). Chk1 phosphorylation did not increase after higher doses of UV or at longer time points (Fig. 2D,E). The same result was obtained by western blotting. In contrast to asynchronous cells, treating synchronised G2 phase cells with UV resulted in very little Chk1 phosphorylation. p38 MAPK, additionally involved in the UV-induced DDR (Bulavin et al., 2001), is activated in both asynchronous and G2 cells (Fig. 2F).

Transfection with a Chk1-Ser317/Ser345 phosphorylation mutant in U2OS cells and depleting Chk1 or ATR from primary human RPE cells confirmed the specificity of the pS317-Chk1 antibody and demonstrated that the absence of phosphorylated Chk1 in G2 is a general phenomenon (supplementary material Fig. S2A,B).

Chromatin fractionation verified this apparent low activation of the ATR-Chk1 pathway upon UV in G2 phase. UV-treatment induced efficient chromatin binding of RPA and Rad9 in G1 and S phase cells. For ATR minimal recruitment was observed, as described before (Zou et al., 2002). In contrast, the recruitment of all these proteins is marginal in cells irradiated in G2 phase (supplementary material Fig. S2C).

Minimal overall DDR in G2 phase cells after UV damage

We next investigated the localisation and activation of other DNA damage signalling factors in G2 phase. Upon IR, 53BP1 and the phosphorylated forms of ATM and H2AX accumulated into foci in G2 cells (Fig. 3A–C). However, p38 was not phosphorylated upon IR, but after UV in all phases of the cell cycle (Fig. 3E). In contrast, 53BP1, γH2AX and pS1981-ATM did not localise into foci after UV damage during G2 phase, while in cells in G1 and S phase we did observe focus formation (Fig. 3A–D). IR efficiently induced γH2AX focus formation in synchronised G2 cells, whereas this response was only minimal after UV damage (supplementary material Fig. S3A). A tenfold higher UV dose (200 J/m^2) started to induce γH2AX foci in G2 phase, which most likely represents the formation of other additional DNA lesions (supplementary material Fig. S3B).

Accumulation of DDR proteins to sites of local UV damage is virtually absent in G2 phase cells

In regions of localised UV damage, a similar amount of UV-induced CPD lesions was observed during all cell cycle phases (Fig. 3F,G). γH2AX accumulated at such local UV lesions (CPD-positive), in cells without cytoplasmic Cyclin B, but not in G2 phase cells (Fig. 3F). The absence of γH2AX signal at the local UV damage site was independent of dose or time (Fig. 3F,G). Combined, these results indicate that also in the presence of a large number of close proximity UV-induced lesions the accumulation of DDR proteins is not very efficient in G2 phase, although the reason is not clear. Since XPC is recruited and the NER machinery is active in G2 phase, the DNA lesions seem accessible (Fig. 1C; supplementary material Fig. S1D,E). The different level of checkpoint activation might be due to the same dose of UV light causing more damage during S phase than in G2 as a result of ongoing DNA replication. Indeed, UV-treating asynchronous cells in which replication was blocked by a low dose of aphidicolin without triggering a checkpoint [low EdU incorporation and the absence of γH2AX signal (supplementary material Fig. S3C)], resulted in very low γH2AX focus formation as compared to control cells in which replication was not perturbed (supplementary material Fig. S3C). These results suggest that the difference in checkpoint response between S and G2 phases might be caused by a different damage load, for example stalled or collapsed replication forks during S phase.

UV-induced G2 phase arrest is regulated by ATR-Chk1 and p38 pathways

Despite the lack of an obvious PIKK-mediated checkpoint upon UV in G2 cells in our experimental setup, various reports strongly suggest that the ATR-Chk1 pathway is important in regulating damage-induced G2 arrest. However, mostly IR or DNA damaging drugs are used and the number of studies that investigate the UV-induced G2 arrest is limited. Inhibition of Chk1 in HeLa cells abrogates the G2 arrest after UV and cells from Chk1$^{-/-}$ mouse embryos have a similar checkpoint defect (Phong et al., 2010; Takai et al., 2000). Moreover, Seckel cells, with mutated ATR, fail to arrest at the G2/M transition upon UV (Alderton et al., 2004; Stiff et al., 2008). ATR and Chk1 are

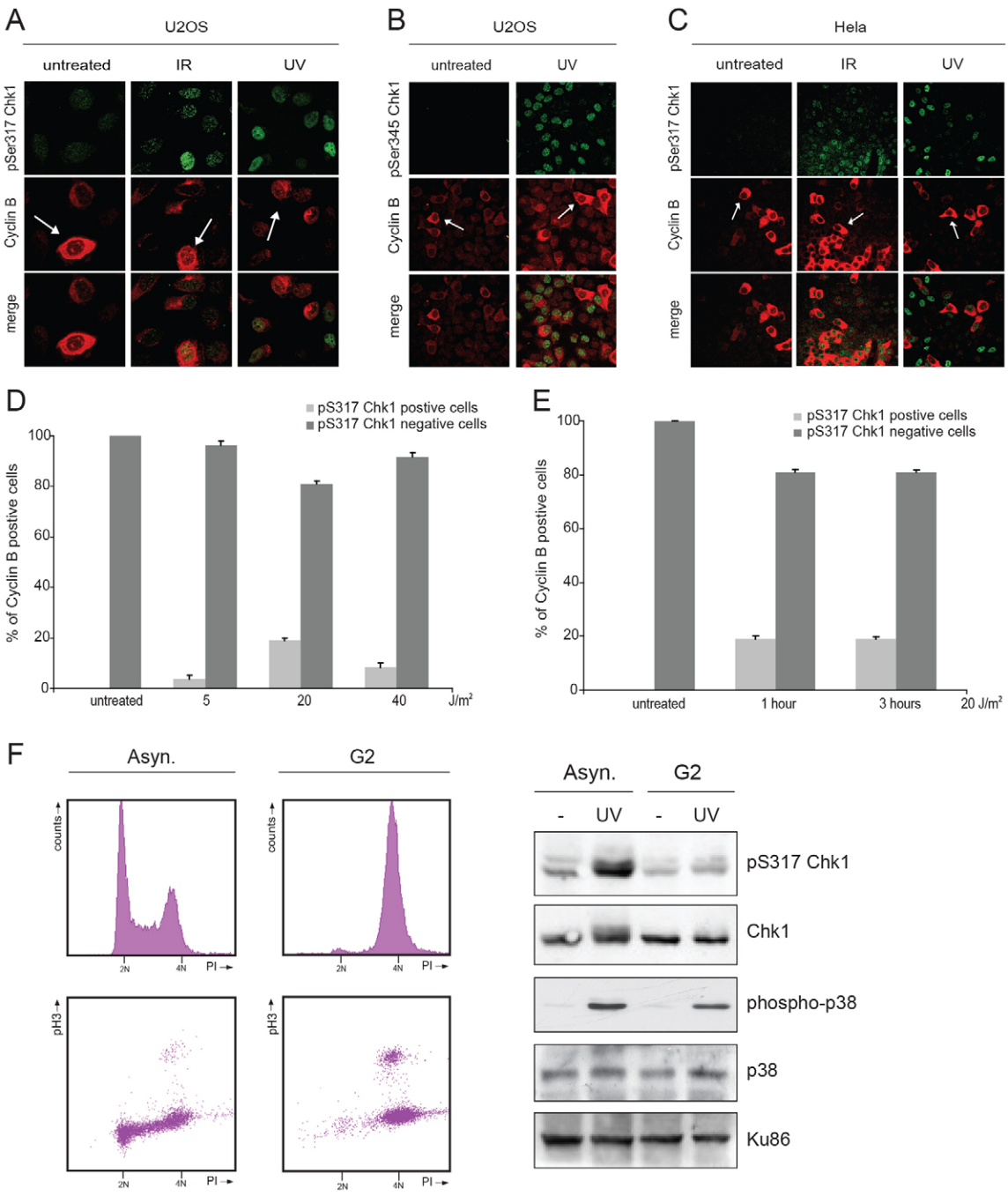

Fig. 2. Minimal UV-induced ATR-mediated checkpoint activation in G2 cells. (**A**) U2OS cells exposed to IR or UV irradiation and analysed by immunofluorescence. Arrows indicate G2 cells. (**B**) As for A, but after UV. (**C**) As for A, but HeLa cells. (**D**) U2OS cells were treated with UV light (5, 20, 40 J/m^2) for 1 hour. Percentage of Cyclin B-positive cells, negative or positive for pSer317-Chk1. Total number of cells in each cell cycle phase was set to 100%. (**E**) As for D, but 1–3 hours after UV (20 J/m^2). (**F**) U2OS cells synchronised in G2. Left: Cell cycle profile and pHH3 by FACS. Right: Asynchronous or G2 cells were UV-treated and analysed by western blotting.

required for UV-induced degradation of Cdc25A, thereby regulating the G2 arrest (Mailand et al., 2000), whereas p38 and downstream target MAPKAP2 contribute to the G2 arrest after UV by regulating Cdc25B/C (Bulavin et al., 2001; Manke et al., 2005). We investigated the contribution of these two pathways in the UV-induced arrest using a range of kinase inhibitors: caffeine for all PIKK kinases and specific inhibitors for ATM, ATR, Chk2, Chk1 and p38. Validation for the ATR and p38 inhibitors was shown in supplementary material Fig. S4A,B. U2OS cells were pre-incubated with inhibitor(s), treated with UV light and thereafter incubated with BrdU and nocodazole. G2/M cells were selected by gating for BrdU-negative cells with a 4N DNA content, thereby excluding possible S phase cells that moved into G2/M phase (supplementary material Fig. S4C). Pre-treatment with any of the single inhibitors did not significantly abrogate the G2 arrest. Interestingly, only the combined

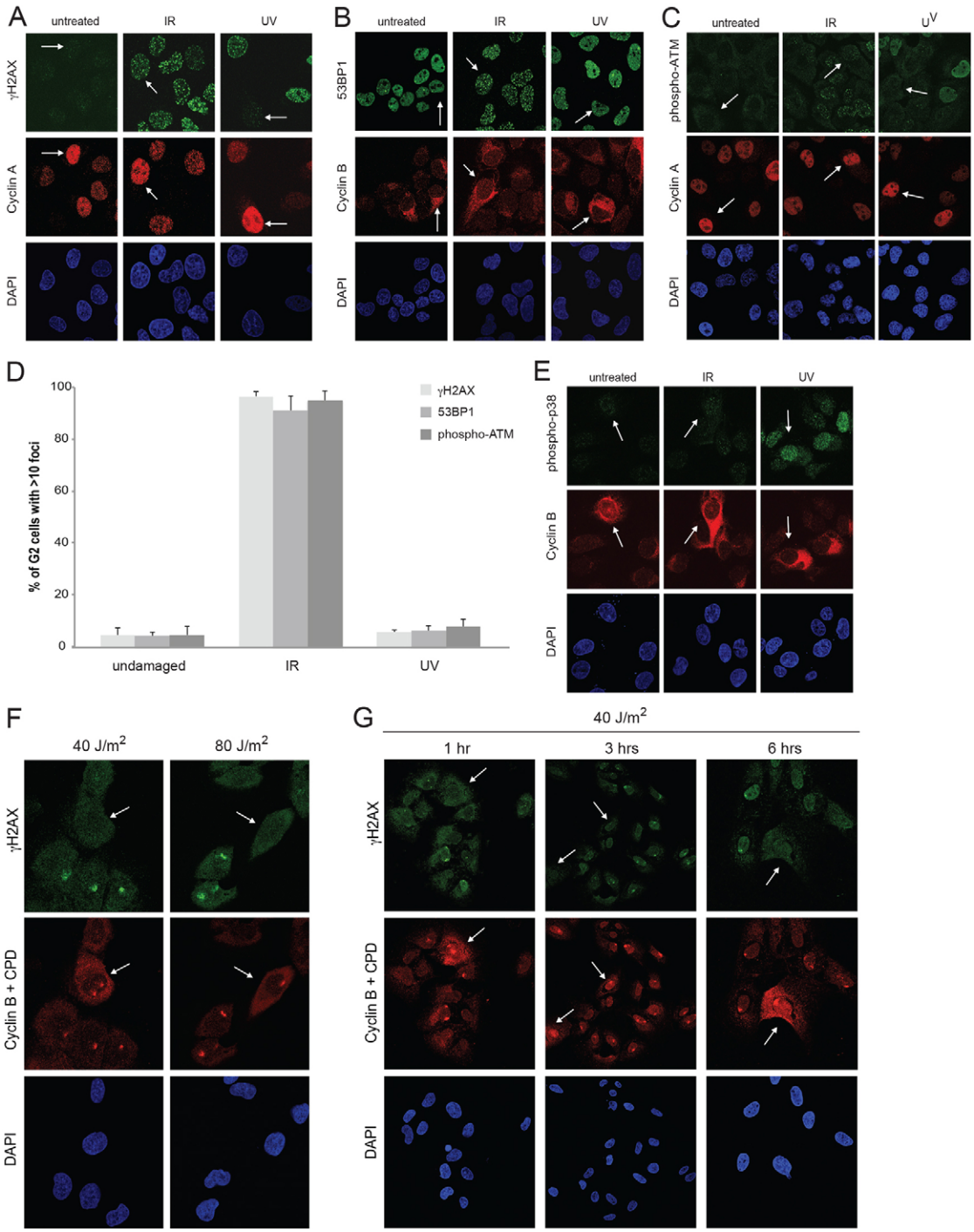

Fig. 3. UV-induced DDR of the ATR pathway is marginal in G2 cells. (**A–C**,**E**) U2OS cells treated with IR or UV irradiation, fixed and stained with the indicated antibodies. Arrows indicate G2 cells. (**D**) Quantification of G2 cells with foci from A–C. (**F**) U2OS cells locally UV-irradiated (40/80 J/m^2) and fixed after 1 hour. (**G**) U2OS cells locally irradiated (40 J/m^2) and fixed after 1, 3 or 6 hours. In all experiments, aphidicolin (1 μM) was added to prevent S phase cells moving to G2.

treatment with caffeine and p38 inhibitor led to a complete loss of the arrest. Similar results were obtained with p38 and specific ATR/Chk1 inhibitors, whereas a combination of p38 and ATM/Chk2 inhibitors did not abrogate the UV-induced G2 checkpoint (Fig. 4A). Analysing mitotic entry by time-lapse microscopy showed a similar result (Fig. 4B). In undamaged conditions mitotic entry is somewhat affected by incubation with p38 and ATR inhibitors, which might be due to the control of Cdc25A by ATR-Chk1 in the absence of exogenous DNA damage (Sørensen et al., 2004; Zhao et al., 2002). As expected, inflicting UV damage resulted in a delay in mitotic entry of ~7 hours. Inhibiting p38 resulted in a slight advantage in mitotic entry but a checkpoint override was only observed when p38 and ATR were inhibited simultaneously (Fig. 4B). Together these results demonstrate that the ATR-Chk1 and p38 signalling modules function as independent but cooperative pathways in the UV-induced arrest

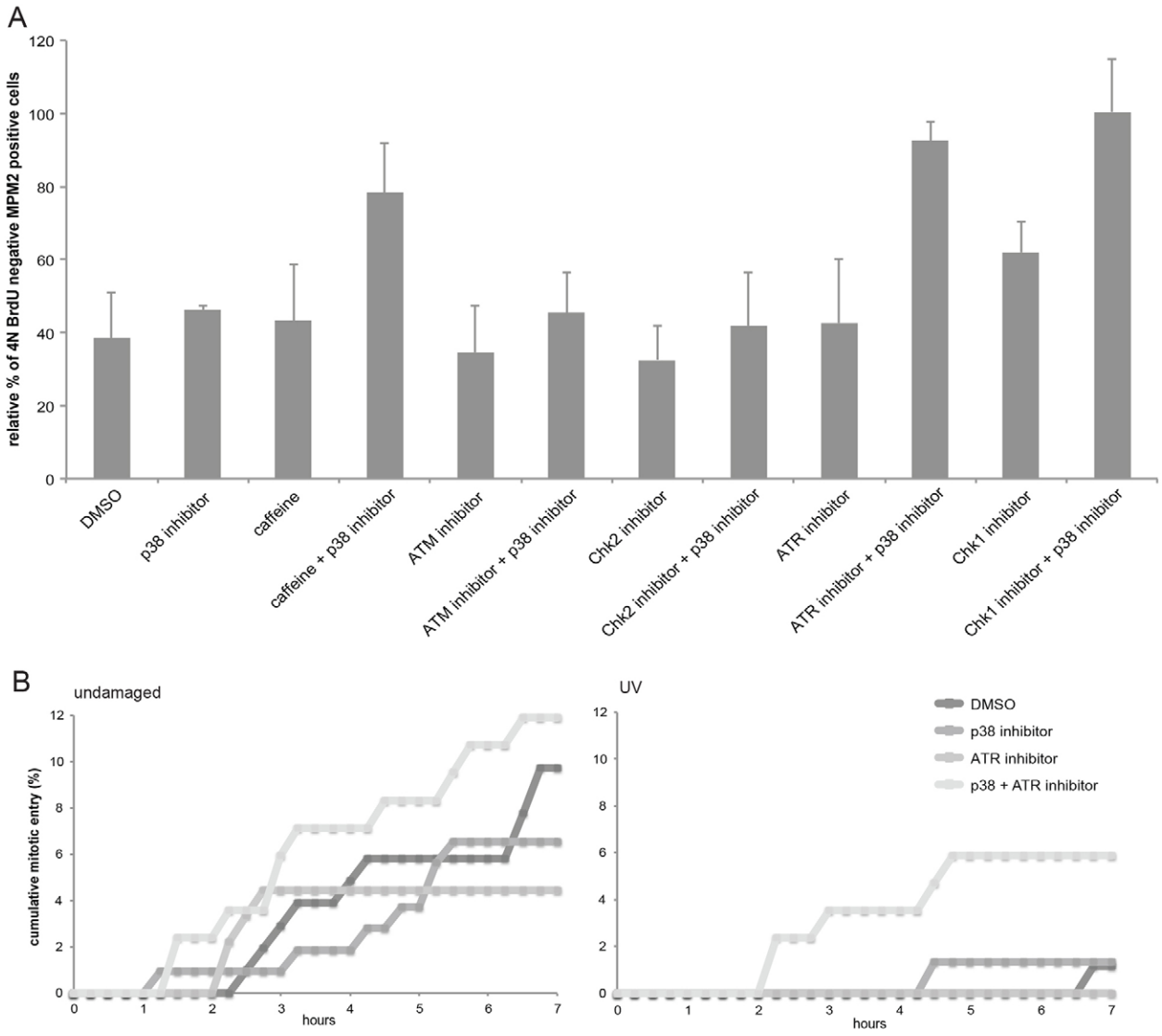

Fig. 4. UV-induced cell cycle arrest in G2 depends on ATR-Chk1 and p38. (**A**) U2OS cells pre-incubated with inhibitors before UV irradiation. Then, cells were incubated with nocodazole, BrdU and inhibitor for 8 hours. Cells were analysed for MPM2 and BrdU by FACS. Shown is the relative percentage of BrdU-negative mitotic cells compared to undamaged controls. (**B**) As for A, but cells expressing GFP-Rad9 and Cyclin B-mCherry were followed by time-lapse microscopy after UV irradiation (5 J/m^2, without nocodazole). Represented is the cumulative mitotic entry. Shown is a representative experiment from three.

in G2 cells. Differences with other studies, that show a G2 checkpoint defect in the absence of a functional ATR-Chk1 pathway, are probably due to different experimental conditions, such as cell type or the use of mutant/knock out versus pharmacological inhibitors. It should be stressed that the ATR-Chk1 and MK2-p38 pathways collaborating in response to genotoxic stress is in accordance with reports in the literature (Bulavin et al., 2002; Reinhardt et al., 2007; Reinhardt et al., 2010). However, although we observe UV-induced phosphorylation of p38 in G2 phase cells, we fail to observe significant activation of the ATR-Chk1 pathway. An explanation is that the ATR-Chk1 pathway is activated at a very low level, possibly due to limited generation of ssDNA, not enough to trigger extensive phosphorylated Chk1 or the localisation of DDR-associated proteins into nuclear foci. This proposed low level of ATR-Chk1 activation might explain why cells come to rely on p38-MK2 as an additional pathway for the execution of the UV-induced G2 arrest. In addition, as ATR-Chk1 are thought to regulate Cdc25A levels, whereas Cdc25B/C levels are largely regulated by p38-MK2, the two pathways might fulfill complementing roles in the G2 checkpoint arrest upon exposure to UV light.

Materials and Methods

Cell culture

U2OS, HeLa, XP4PA primary fibroblasts and RPE (retinal pigment epithelium) cells were grown using standard procedures. For stable expression of Cyclin B-mCherry, U2OS-GFP-Rad9 cells (Medhurst et al., 2008) were transfected with Cyclin B1-mCherry (Gavet and Pines, 2010) and pBabepuro, and positive clones were picked after puromycin selection.

Cell synchronisation

Cells were synchronised using thymidine (2.5 mM) block and release. Alternatively, cells were incubated with thymidine, washed and incubated with

nocodazole (250 ng/ml). After 16 hours, detached cells were removed and remaining cells, G2 phase cells – as confirmed by flow cytometry, were used.

Antibodies and inhibitors

Antibodies: RPA (Ab2, Oncogene), FLAG (Sigma), CPD (TDM-2, MBL International), pS10-Histone H3 (Genescript), MPM2, pS139-Histone H2AX and pS1987-ATM (Upstate Biotechnology), 53BP1 and pS317-Chk1 (Bethyl), pThr180/Tyr182-p38, pS317-Chk1 and pS345-Chk1 (Cell Signaling), BrdU (Abcam), Rad9 (Novus Biologicals), Orc2 (BD Pharmingen), ATR, Cyclin A, Cyclin B1, Ku86, p38 and Chk1 from Santa Cruz Biotechnology. Anti-XPC was generated by Wim Vermeulen (Erasmus MC, The Netherlands).

Inhibitors: Caffeine (5 mM, Sigma), p38 inhibitor SB203580 (10 μM, Sigma), Chk1 inhibitor SB218078 (2.5 μM, Calbiochem), ATR inhibitor ATR-45 (10 μM, Medicinal Chemistry Shared Resource, Ohio State University), ATM inhibitor KU55933 (10 μM, Sigma) and Chk2 inhibitor Chk2-II (10 μM, Sigma).

Cell fractionation

Biochemical fractionation of cells was performed as previously described (Méndez and Stillman, 2000; Smits et al., 2006).

Unscheduled DNA synthesis (UDS) assay

The UDS assay was performed as described (Nakazawa et al., 2010) using Click-iT Alexa Fluor 488 (Invitrogen).

Immunofluorescence

Immunostaining was performed as previously described (Warmerdam et al., 2009). >50 cells were analysed for each point and error bars represent the SEM of three independent experiments, except supplementary material Fig. S3A that was performed twice. Cells with >10 foci were scored as positive.

Images were made using a Cell Observer fluorescent microscope (Zeiss) or a Confocal Laser Scanning Microscope LSM 510 (Zeiss). The latter was also used for strip-FRAP experiments and time-lapse imaging.

Generation of DNA damage and photobleaching techniques

Unless indicated, UV-irradiation was performed at 20 J/m^2 and cells were processed 1 hour post treatment. IR (10 Gy) was induced using a ^{137}Cs source for 2 hours. Local UV-irradiation experiments were performed as previously described (Moné et al., 2001). FRAP was performed as described (Warmerdam et al., 2009).

Time-lapse imaging

Cells were plated in 8-well chambered glass bottom slides (LabTek) and imaged in a heated chamber using a 20× air objective on a Deltavision RT imaging system. Images were taken every 15 minutes and analysed using ImageJ software. Cumulative mitotic entry was determined by monitoring the number of Cyclin B-positive cells that entered mitosis in time. >100 cells were analysed in each condition.

G2 checkpoint assay

After mock- or UV-irradiation, cells were incubated for 8 hours in the presence of BrdU (10 μM) and nocodazole, to label S-phase cells and to trap cells in mitosis, respectively. Cells were stained and analysed by a FACSCaliber and CellQuest Pro software (Becton Dickinson), for BrdU, MPM2 and PI. To exclude S phase cells, MPM2-positivity was scored in BrdU-negative cells with a 4N DNA content.

Acknowledgements

The authors are grateful to Jeroen Essers, Wim Vermeulen, Rob Wolthuis and Jiri Bartek for sharing reagents, Cecile Beerens for technical assistance and Wim Vermeulen, Andrés Clemente-Blanco and Raimundo Freire for helpful discussions.

Author contributions

D.O.W. designed experiments, performed most of them and participated in writing the manuscript. E.K.B. assisted in the RPA and pChk1 immunofluorescence experiments. J.A.M. performed the UDS assays. R.H.M. and R.K. participated in data interpretation and manuscript writing. V.A.J.S. designed experiments, performed chromatin fractionation/western blot experiments on synchronised cells, supervised the study and wrote the manuscript.

Funding

This work was supported by grants from the Dutch Cancer Society [grant number EMCR 2005-3412], NWO/ZonMW TOP-GO [TOPGO.L.10.037] and the Spanish Ministry of Economy and Competitiveness [SAF2010-22126, CONSOLIDER-Ingenio 2010 CDS2007-0015 and Ramón y Cajal Program]. The research leading to these results has received funding from the European Community's Seventh Framework Programme [FP7/2007-2013, grant agreement number HEALTH-F2-2010-259893] and the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.118265/-/DC1

References

Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev.* **15**, 2177-2196.

Alderton, G. K., Joenje, H., Varon, R., Børglum, A. D., Jeggo, P. A. and O'Driscoll, M. (2004). Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway. *Hum. Mol. Genet.* **13**, 3127-3138.

Bonilla, C. Y., Melo, J. A. and Toczyski, D. P. (2008). Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage. *Mol. Cell* **30**, 267-276.

Bulavin, D. V., Higashimoto, Y., Popoff, I. J., Gaarde, W. A., Basrur, V., Potapova, O., Appella, E. and Fornace, A. J., Jr (2001). Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* **411**, 102-107.

Bulavin, D. V., Amundson, S. A. and Fornace, A. J. (2002). p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphony. *Curr. Opin. Genet. Dev.* **12**, 92-97.

Callegari, A. J. and Kelly, T. J. (2007). Shedding light on the DNA damage checkpoint. *Cell Cycle* **6**, 660-666.

Callegari, A. J., Clark, E., Pneuman, A. and Kelly, T. J. (2010). Postreplication gaps at UV lesions are signals for checkpoint activation. *Proc. Natl. Acad. Sci. USA* **107**, 8219-8224.

Cimprich, K. A. and Cortez, D. (2008). ATR: an essential regulator of genome integrity. *Nat. Rev. Mol. Cell Biol.* **9**, 616-627.

Essers, J., Theil, A. F., Baldeyron, C., van Cappellen, W. A., Houtsmuller, A. B., Kanaar, R. and Vermeulen, W. (2005). Nuclear dynamics of PCNA in DNA replication and repair. *Mol. Cell. Biol.* **25**, 9350-9359.

Gavet, O. and Pines, J. (2010). Activation of cyclin B1-Cdk1 synchronizes events in the nucleus and the cytoplasm at mitosis. *J. Cell Biol.* **189**, 247-259.

Hagting, A., Jackman, M., Simpson, K. and Pines, J. (1999). Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. *Curr. Biol.* **9**, 680-689.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366-374.

Houtsmuller, A. B. (2005). Fluorescence recovery after photobleaching: application to nuclear proteins. *Adv. Biochem. Eng. Biotechnol.* **95**, 177-199.

Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J. and Jackson, S. P. (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat. Cell Biol.* **8**, 37-45.

Jeggo, P. A. and Löbrich, M. (2006). Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability. *DNA Repair (Amst.)* **5**, 1192-1198.

Kanaar, R., Wyman, C. and Rothstein, R. (2008). Quality control of DNA break metabolism: in the 'end', it's a good thing. *EMBO J.* **27**, 581-588.

Kumagai, A., Lee, J., Yoo, H. Y. and Dunphy, W. G. (2006). TopBP1 activates the ATR-ATRIP complex. *Cell* **124**, 943-955.

Lee, J., Kumagai, A. and Dunphy, W. G. (2007). The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. *J. Biol. Chem.* **282**, 28036-28044.

Mailand, N., Falck, J., Lukas, C., Syljuåsen, R. G., Welcker, M., Bartek, J. and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. *Science* **288**, 1425-1429.

Manke, I. A., Nguyen, A., Lim, D., Stewart, M. Q., Elia, A. E. and Yaffe, M. B. (2005). MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. *Mol. Cell* **17**, 37-48.

Marini, F., Nardo, T., Giannattasio, M., Minuzzo, M., Stefanini, M., Plevani, P. and Muzi Falconi, M. (2006). DNA nucleotide excision repair-dependent signaling to checkpoint activation. *Proc. Natl. Acad. Sci. USA* **103**, 17325-17330.

Marteijn, J. A., Bekker-Jensen, S., Mailand, N., Lans, H., Schwertman, P., Gourdin, A. M., Dantuma, N. P., Lukas, J. and Vermeulen, W. (2009). Nucleotide excision repair-induced H2A ubiquitination is dependent on MDC1 and RNF8 and reveals a universal DNA damage response. *J. Cell Biol.* **186**, 835-847.

Medhurst, A. L., Warmerdam, D. O., Akerman, I., Verwayen, E. H., Kanaar, R., Smits, V. A. and Lakin, N. D. (2008). ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage. *J. Cell Sci.* **121**, 3933-3940.

Méndez, J. and Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Mol. Cell. Biol.* **20**, 8602-8612.

Moné, M. J., Volker, M., Nikaido, O., Mullenders, L. H., van Zeeland, A. A., Verschure, P. J., Manders, E. M. and van Driel, R. (2001). Local UV-induced DNA damage in cell nuclei results in local transcription inhibition. *EMBO Rep.* **2**, 1013-1017.

Nakazawa, Y., Yamashita, S., Lehmann, A. R. and Ogi, T. (2010). A semi-automated non-radioactive system for measuring recovery of RNA synthesis and unscheduled DNA synthesis using ethynyluracil derivatives. *DNA Repair (Amst.)* **9**, 506-516.

Phong, M. S., Van Horn, R. D., Li, S., Tucker-Kellogg, G., Surana, U. and Ye, X. S. (2010). p38 mitogen-activated protein kinase promotes cell survival in response to DNA damage but is not required for the G(2) DNA damage checkpoint in human cancer cells. *Mol. Cell. Biol.* **30**, 3816-3826.

Reinhardt, H. C., Aslanian, A. S., Lees, J. A. and Yaffe, M. B. (2007). p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. *Cancer Cell* **11**, 175-189.

Reinhardt, H. C., Hasskamp, P., Schmedding, I., Morandell, S., van Vugt, M. A., Wang, X., Linding, R., Ong, S. E., Weaver, D., Carr, S. A. et al. (2010). DNA damage activates a spatially distinct late cytoplasmic cell-cycle checkpoint network controlled by MK2-mediated RNA stabilization. *Mol. Cell* **40**, 34-49.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat. Rev. Cancer* **3**, 155-168.

Shiloh, Y. (2006). The ATM-mediated DNA-damage response: taking shape. *Trends Biochem. Sci.* **31**, 402-410.

Shiotani, B. and Zou, L. (2009a). ATR signaling at a glance. *J. Cell Sci.* **122**, 301-304.

Shiotani, B. and Zou, L. (2009b). Single-stranded DNA orchestrates an ATM-to-ATR switch at DNA breaks. *Mol. Cell* **33**, 547-558.

Smits, V. A., Reaper, P. M. and Jackson, S. P. (2006). Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. *Curr. Biol.* **16**, 150-159.

Sørensen, C. S., Syljuåsen, R. G., Lukas, J. and Bartek, J. (2004). ATR, Claspin and the Rad9-Rad1-Hus1 complex regulate Chk1 and Cdc25A in the absence of DNA damage. *Cell Cycle* **3**, 941-945.

Stiff, T., Cerosaletti, K., Concannon, P., O'Driscoll, M. and Jeggo, P. A. (2008). Replication independent ATR signalling leads to G2/M arrest requiring Nbs1, 53BP1 and MDC1. *Hum. Mol. Genet.* **17**, 3247-3253.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y. A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., Nakanishi, M. and Nakayama, K. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. *Genes Dev.* **14**, 1439-1447.

Ward, I. M. and Chen, J. (2001). Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J. Biol. Chem.* **276**, 47759-47762.

Warmerdam, D. O., Freire, R., Kanaar, R. and Smits, V. A. (2009). Cell cycle-dependent processing of DNA lesions controls localization of Rad9 to sites of genotoxic stress. *Cell Cycle* **8**, 1765-1774.

Wu, X., Shell, S. M. and Zou, Y. (2005). Interaction and colocalization of Rad9/Rad1/Hus1 checkpoint complex with replication protein A in human cells. *Oncogene* **24**, 4728-4735.

Zhao, H., Watkins, J. L. and Piwnica-Worms, H. (2002). Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. *Proc. Natl. Acad. Sci. USA* **99**, 14795-14800.

Zou, L. and Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**, 1542-1548.

Zou, L., Cortez, D. and Elledge, S. J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. *Genes Dev.* **16**, 198-208.

Zou, L., Liu, D. and Elledge, S. J. (2003). Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc. Natl. Acad. Sci. USA* **100**, 13827-13832.

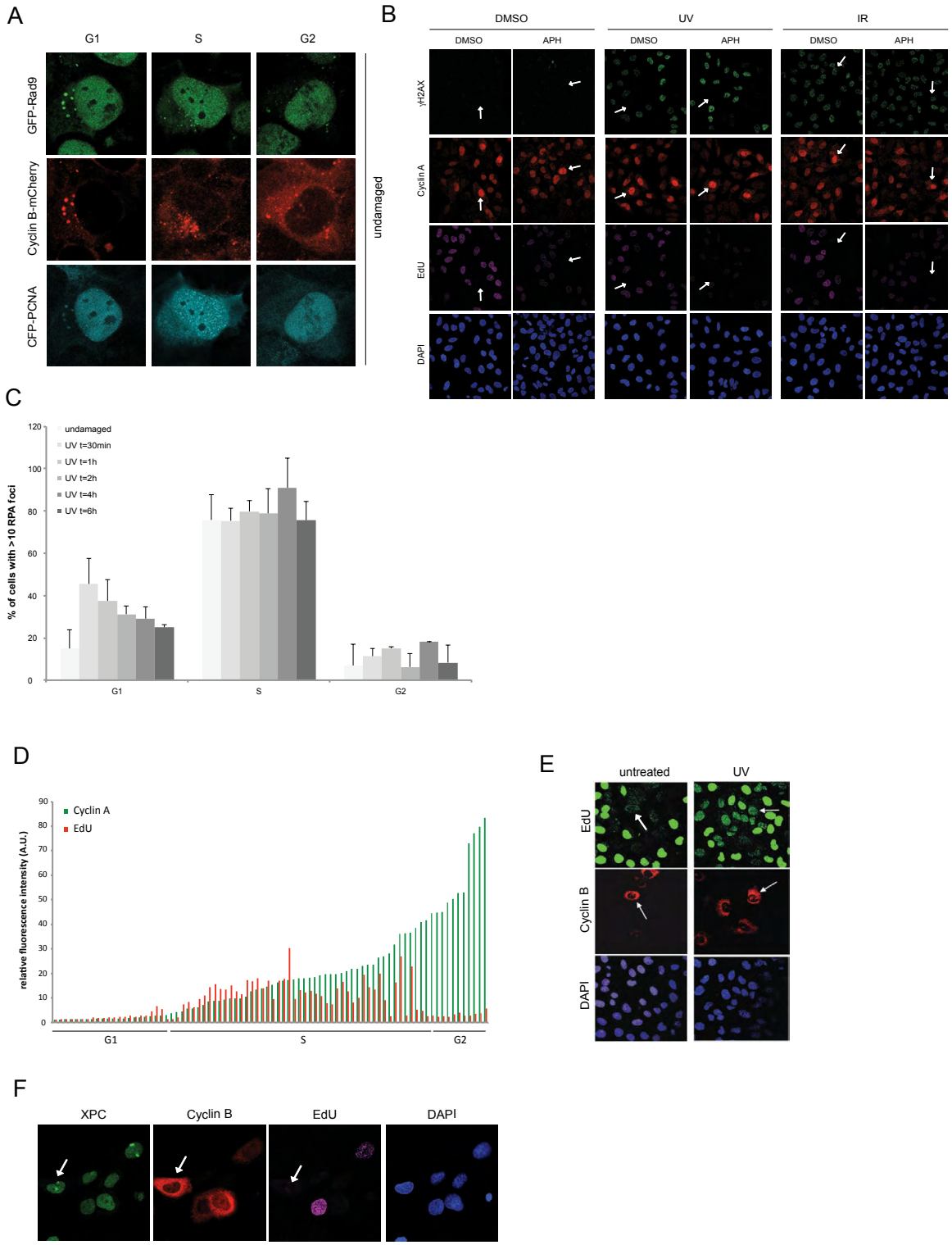

Fig. S1. (**A**) U2OS cells stably expressing GFP-Rad9 and Cyclin B-mCherry (kindly provided by Rob Wolthuis, Netherlands Cancer Institute, The Netherlands) were transiently transfected with CFP-PCNA (kindly provided by Jeroen Essers, ErasmusMC, The Netherlands). Undamaged cells accompanying Fig. 1A. (**B**) 1μM of aphidicolin is added directly after damage induction and prevents S phase cells (EdU positive) from entering G2 (EdU negative). Cells were fixed 4 hours after treatment. Immunofluorescence was performed with the indicated antibodies. (**C**) U2OS cells were treated with 20 J/m^2 UV and EdU was added directly after irradiation. In addition, 1μM of aphidicolin was added to prevent S phase cells moving to G2 during the time course of the experiment. Cells were fixed at the indicated time points and immunofluorescence was performed with antibodies against RPA, Cyclin A and EdU. Cell cycle phase was determined based on Cyclin A and EdU levels. Represented is the percentage of cells with >10 RPA foci in the different phases of the cell cycle. (**D**) Single cell quantification of U2OS cells stained for EdU and Cyclin A by immunofluorescence. Cells were sorted on their relative Cyclin A intensity (green bars) and their corresponding EdU signal intensity is given (red bars). (**E**) U2OS cells were treated with UV light, after which EdU was added for 1 hour. Cells were fixed and stained for EdU and Cyclin B. (**F**) U2OS cells were locally UV-irradiated (80 J/m^2), EdU was added and the cells were fixed after 1 hour and analysed by immunofluorescence with the indicated antibodies.

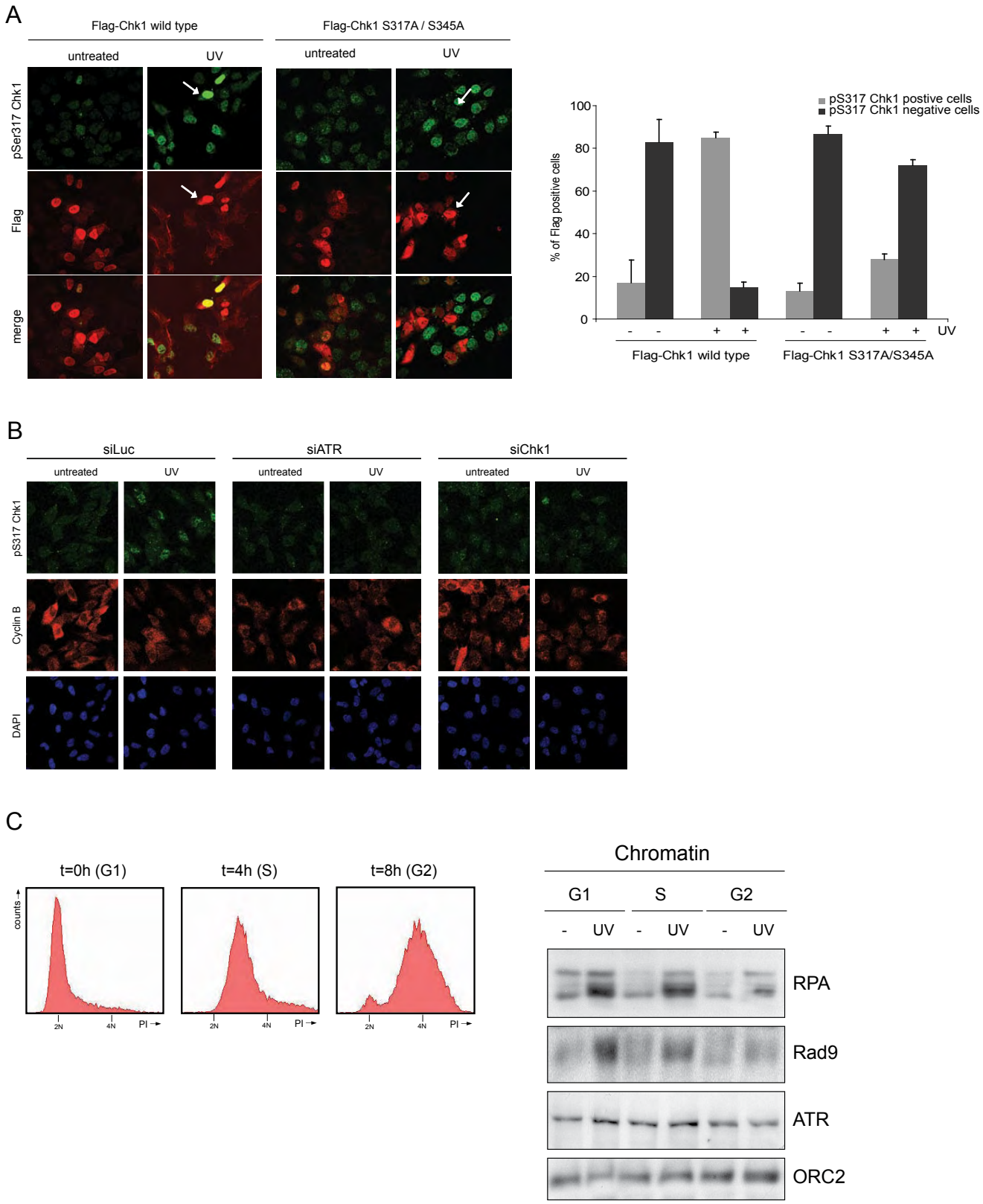

Fig. S2. (**A**) U2OS cells were transfected with wild type or S317A/S345A Flag-labelled Chk1 (kindly provided by Jiri Bartek, Institute of Cancer Biology and Centre for Genotoxic Stress Research, Danish Cancer Society, Denmark). 48 hours after transfection cells were UV-treated, and analysed by immunofluorescence with the indicated antibodies. Right panel: the percentage of Flag-positive cells that were either negative or positive for phosphorylated Ser317 Chk1. (**B**) RPE cells were transfected with siRNA oligos against luciferase (Luc, CGUACGCGGAAUACUUCGAdTdT), ATR (CCUCCGUGAUGUUGCUUGAdTdT) or Chk1 (UCGUGAGCGUUUGUUGAACdTdT). 48 hours later, cells were UV-damaged and immunofluorescence was performed with the indicated antibodies. (**C**) U2OS cells were synchronised using thymidine block and release. At the indicated time points (t=0h, G1; t=4h, S; t=8h, G2), cells were left untreated or treated with UV light (40 J/m^2). One hour later, cells were harvested for cellular fractionation or FACS analysis. Right panel: Western blot analysis of the chromatin fractions using the indicated antibodies.

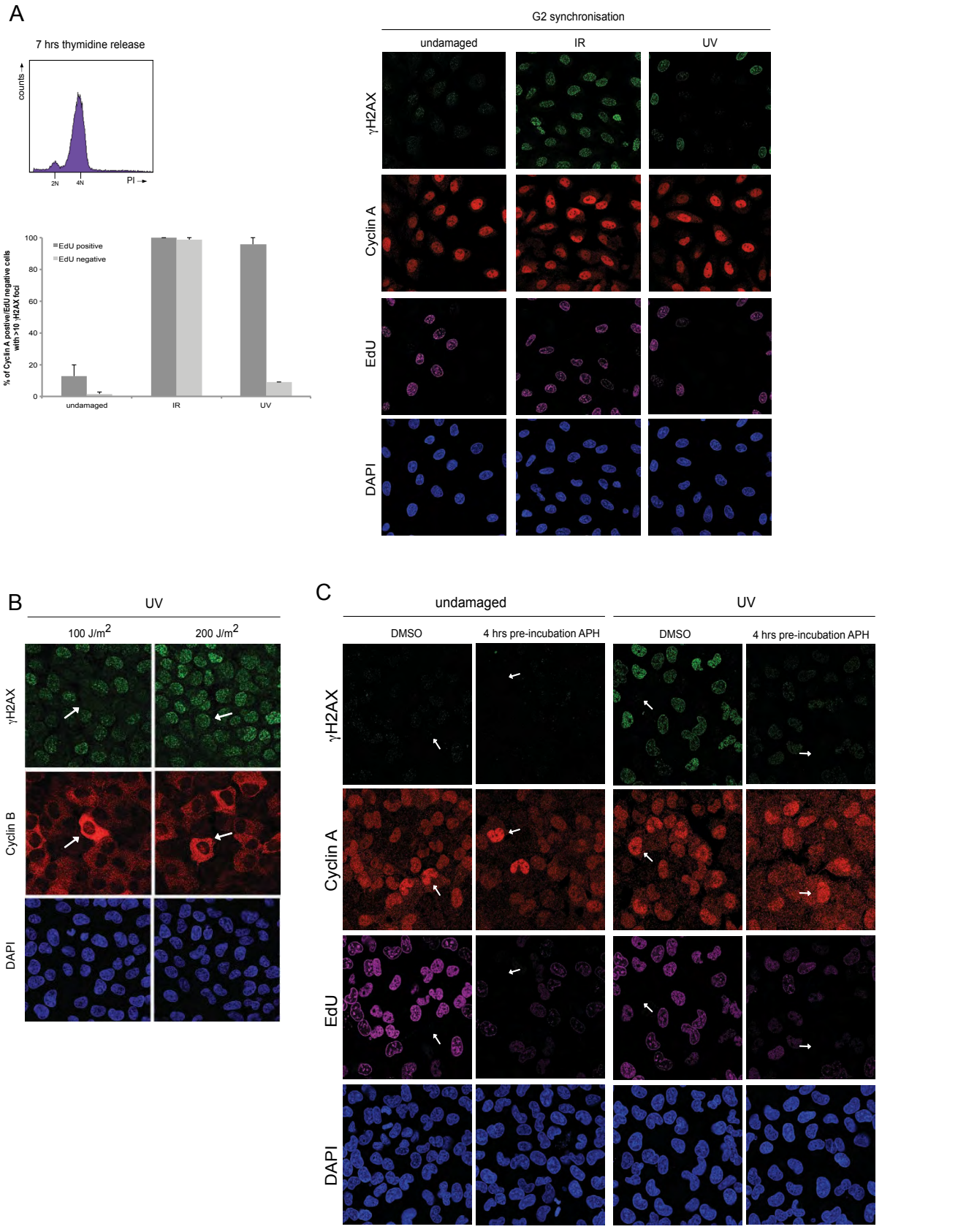

Fig. S3. (**A**) U2OS cells were synchronised using thymidine for 24 hours. Cells were released for 7 hours (G2 phase) and either treated with IR or UV, and EdU was added to the medium. After 1 hour, cells were fixed and immunofluorescence was performed with the indicated antibodies. Cyclin A-positive cells with (S phase cells) and without EdU (G2 phase cells) were quantified for γH2AX focus formation. The right panel shows representative images. (**B**) U2OS cells were treated with 100 or 200 J/m^2 of UV light. Cells were fixed after 1 hour and stained for immunofluorescence with the indicated antibodies. (**C**) U2OS cells were incubated with 1μM aphidicolin for 4 hours after which cells were UV-irradiated with 20 J/m^2, EdU was added and fixed 1 hour later. Immunofluorescence was performed with the indicated antibodies.

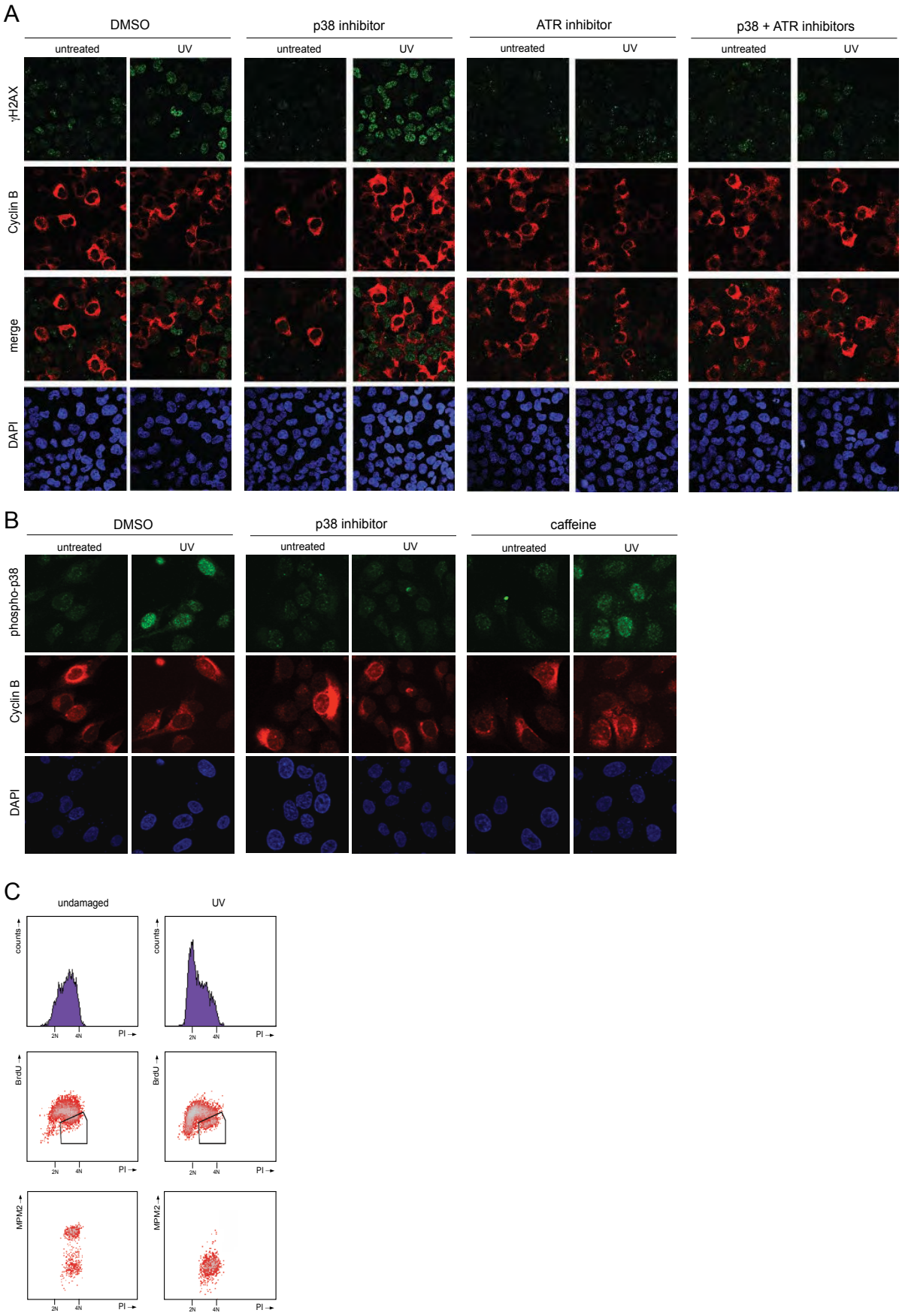

Fig. S4. (**A**) U2OS cells were pre-incubated for 30 minutes with DMSO, p38 inhibitor (SB203580), ATR inhibitor (ATR-45) or a combination. Thereafter cells were UV-treated and incubated for 1 hour in the presence of the inhibitor. Immunofluorescence was performed with the indicated antibodies. (**B**) As in A, but here cells were pre-incubated with DMSO, p38 inhibitor (SB203580) or caffeine (10mM). (**C**) Example of analysis performed for Fig. 4A. BrdU-negative cells with 4N DNA content were gated and the mitotic cells were determined by MPM2-positivity in undamaged and UV-treated samples.