Cell cycle arrest at the initiation step of human chromosomal DNA replication causes DNA damage

Dávid Szüts and Torsten Krude*

Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK
*Author for correspondence (e-mail: tk1@mole.bio.cam.ac.uk)

Accepted 21 June 2004
Journal of Cell Science 117, 4897-4908 Published by The Company of Biologists 2004
doi:10.1242/jcs.01374

Summary

Cell cycle arrest in response to environmental effects can lead to DNA breaks. We investigated whether inhibition of DNA replication during the initiation step can lead to DNA damage and characterised a cell-cycle-arrest point at the replication initiation step before the establishment of active replication forks. This arrest can be elicited by the iron chelators mimosine, ciclopirox olamine or 2,2′-bipyridyl, and can be reversed by the removal of the drugs or the addition of excess iron. Iron depletion induces DNA double-strand breaks in treated cells, and activates a DNA damage response that results in focal phosphorylation of histone H2AX, focal accumulation of replication protein A (RPA) and ATR (ATM and Rad3-related kinase), and activation of CHK1 kinase. Abrogation of the checkpoint response does not abolish the cell cycle arrest before the establishment of active DNA replication forks. DNA breaks appear concomitantly with the arrival of cells at the arrest point and persist upon release from the cell cycle block. We conclude that DNA double-strand breaks are the consequence, and not the cause, of cell cycle arrest during the initiation step of DNA replication by iron chelation.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/117/21/4897/DC1

Key words: DNA replication initiation, Iron chelator, Mimosine, DNA damage

Introduction

The maintenance of genome stability is essential for normal cell proliferation. Genome stability is threatened by genotoxic agents that damage DNA, resulting in the direct or indirect induction of DNA breaks. DNA damage can activate checkpoint pathways that lead to cell cycle arrest in the G1, S or G2 phases of the cell cycle (Bartek and Lukas, 2001a; Bartek and Lukas, 2001b; Nyberg et al., 2002; O'Connell et al., 2000). DNA breaks can occur after an interference with the DNA replication machinery (Michel et al., 1997; Saintigny et al., 2001). Inhibition of the elongation stage of DNA replication activates the replication checkpoint which is closely related to the S phase DNA damage checkpoint, and leads to cell cycle arrest (Nyberg et al., 2002; Osborn et al., 2002). The mechanisms proposed to generate DNA breaks during the elongation phase of DNA replication rely on localised events arising from the inhibition of replication fork progression (Hyrien, 2000; Rothstein et al., 2000). At present, it is unclear what kind of DNA structure is detected at the replication checkpoint; inhibition of replication elongation has been shown to result in the accumulation of single stranded stretches of DNA in yeast (Sogo et al., 2002), and can also lead to the formation of double-strand breaks (DSBs) in bacteria (Michel et al., 1997), yeast (Sogo et al., 2002) and higher eukaryotes (Saintigny et al., 2001). Damage-sensing might be achieved when single stranded regions at stalled replication forks, which are associated with replication protein A (RPA), are detected by the checkpoint kinase ATR when its partner-protein ATRIP binds to RPA (Costanzo et al., 2003; Zou and Elledge, 2003). In response, ATR kinase phosphorylates the histone variant H2AX, possibly aided by the DNA-dependent protein kinase (DNA-PK) and ATM (ataxia-telangiectasia mutated) kinases which are activated by DSBs. The appearance of a focal pattern of the phosphorylated form (γ-H2AX) in the nuclei is generally used as a marker for DSBs (Rogakou et al., 1998; Rogakou and Sekeri-Pataryas, 1999). Indeed, ATR has been shown to phosphorylate H2AX in response to replicational stress (Ward and Chen, 2001), and ATR activity is required to minimise DNA breakage, because a replication block by aphidicolin causes up to 20-times more DNA breaks in the absence of ATR (Casper et al., 2002).

The key regulatory step of DNA replication is initiation. We therefore set out, to investigate whether interference with replication initiation also leads to DNA damage and poses a threat to genome stability. To examine whether inhibition of replication initiation in human cells, at a stage preceding the establishment of active replication forks, leads to DNA damage, we chemically induced cell cycle arrest at or just before the G1-to-S-phase transition. In human cells, this kind of cell cycle arrest is achieved by using the plant amino acid, mimosine (Krude, 1999; Lalande, 1990). At a concentration of 500 μM, mimosine arrests asynchronously proliferating human cells with a G1-phase DNA-content before replication forks are established (Krude, 1999; Krude, 2000). The exact point of arrest is not characterised at the molecular level, however, this arrest is reversible (Watson et al., 1991). When mimosine is removed, DNA replication soon commences in early, but not in late-replicating chromatin domains (Keller et al., 2002).

More specifically, close examination of an early-firing replication origin in human cells has shown that nascent DNA is enriched at the origin when compared with sequences less than 3.5 kb away, indicating an origin-specific initiation of DNA replication (Keller et al., 2002). The speed and origin-specificity of DNA replication upon mimosine removal suggests that the arrest is very close to the events of replication initiation. This arrest is distinctly different from the mid-S phase cell cycle block, caused by activation of the replication checkpoint (reviewed in Osborn et al., 2002).

Mimosine is a well-known iron chelator, and its effects on the cell cycle have been attributed to iron depletion (Clement et al., 2002; Kulp and Vulliet, 1996). In agreement with this, several other iron chelating compounds have been shown to block cell cycle progression near the G1-S phase boundary (Clement et al., 2002; Gao and Richardson, 2001) (reviewed in Le and Richardson, 2002). Therefore, we have used iron chelators to study the susceptibility of chromosomal DNA to damage during a block prior to the establishment of active DNA replication forks.

In this study, we demonstrate that mimosine and the other iron chelators ciclopirox olamine and bipyridyl (Clement et al., 2002; Linden et al., 2003) similarly arrest human cells at the G1-S phase boundary. The cell cycle arrest can be reversed by either removing the chemicals or restoring the iron level, both of which leads to the establishment of active DNA replication forks. In addition to cell cycle arrest, iron depletion also causes DNA breaks that are accompanied by the phosphorylation of H2AX, the focal accumulation of RPA and ATR, and the activation of the CHK1 kinase. We show that the damage caused by mimosine is cell cycle dependent and is concomitant with cell cycle arrest. Release from the cell cycle block allows entry into and progression through S phase before the damage is repaired, indicating that DNA damage is not the cause of the arrest. Rather, we suggest that, DNA damage is a consequence of the cell cycle arrest at the G1-S phase boundary before the active DNA replication forks are establishment.

Materials and Methods

Cell culture and synchronisation

Human HFF primary fibroblasts, EJ30 and HeLa cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 10 U/ml penicillin, 0.1 mg/ml streptomycin and 2.5 μg/ml amphotericin B (Fungizone) (all from Gibco BRL). Cells were arrested at the G1-S phase boundary by treatment with 500 μM mimosine, 100 μM ciclopirox olamine or 200 μM 2,2′-bipyridyl (all from Sigma) as described in the Results section. Lower concentrations of each iron chelator resulted in an enrichment of S phase cells, as observed earlier in the case of mimosine (Krude, 1999). Cell cycle arrest owing to iron chelators was released by either replacing the medium or by adding 1 mM $FeSO_4$ from a freshly made stock. Bromodeoxyuridine (BrdU) was added to the medium at a concentration of 50 μM where indicated. Mitotic arrest was obtained by treatment with 40 nM nocodazole (Calbiochem). The replication machinery was either blocked by addition of 5 μg/ml aphidicolin (Sigma) or slowed down by the addition of 2.5 mM thymidine (Sigma). DSBs were induced by treating the cells with 40 μg/ml etoposide (Sigma) or 1-10 μg/ml Bleocin™ (Calbiochem). The S-phase checkpoint was inhibited with 25 μM wortmannin or 5 mM caffeine (Sigma). Quiescent EJ30 cells were arrested by culturing confluent plates in 0.5% serum for 7 days (Krude, 1999). All cell-synchronisations were verified by flow cytometry of isolated nuclei (Krude et al., 1997) on a Becton-Dickinson FACScan apparatus using the CellQuest software. The cell cycle distribution was quantitated using the same software.

Immunocytochemistry, western blot analyses and DNA-replication reactions

For immunofluorescence staining, EJ30 cells were grown on coverslips, fixed in 4% paraformaldehyde, and blocked in PBS with 1% BSA, 0.1% Triton X-100 and 0.02% SDS. Primary and secondary antibodies were diluted in the same blocking buffer. For BrdU staining, cells were treated with 4M HCl for 30 minutes after fixation. The following primary antibodies were used: anti-γ-H2AX antibodies (S139 mouse monoclonal JBW304 and rabbit no. 07-164, both Upstate), anti-RPA antibody [rabbit polyclonal pAb-RPA1 (Szüts et al., 2003) and mouse monoclonal 34A (Kenny et al., 1990)], anti-ATR antibody (sc-1887, Santa Cruz), anti-phospho-CHK1 antibody (S317, Cell Signaling), anti-phospho-CHK2 antibody (T68, Cell Signaling) and anti-BrdU antibody (MAS 250b, Harlan). Fluorescence-labelled secondary antibodies were obtained from Amersham Biosciences and from Molecular Probes. Propidium iodide was used at a concentration of 50 μg/ml for DNA counterstaining. Stained cells were analysed using a laser-confocal microscope (Leica). Western blot analyses was performed using standard techniques, with the phospho-specific antibodies specified above.

Nucleic and cytosolic extracts were prepared, and in vitro DNA replication assays were performed exactly as described (Szüts et al., 2003), using 100 μg HeLa cell cytosolic extract where stated. DNA replication was detected by confocal microscopy as described (Szüts et al., 2003).

Detection of DNA breaks by electrophoresis

10 million (alkaline gels) or 2 million (pulsed field gels) isolated HeLa cell nuclei were used per sample. Nuclei were embedded in a block of 0.5% low-melting-point agarose in PBS, and incubated with 2 mg/ml proteinase K, 0.5 M EDTA and 1% Sarcosyl (N-lauroylsarcosine, Sigma) at 55°C for 20 hours. For alkaline gel electrophoresis, the agarose blocks were equilibrated in 50 mM NaOH and 1 mM EDTA, and inserted into the wells of a 0.7% agarose gel prepared with the same buffer. Following electrophoresis at 4 V/cm for 4 hours, the gels were neutralised in 1 M NaCl with 0.5 M Tris-HCl pH 7.2, stained with ethidium bromide and photographed. For pulsed field gel electrophoresis, the agarose blocks were equilibrated in 1×Tris-HCl–EDTA (TE) buffer pH 8, melted, and poured into the wells of an 1.2% agarose gel. Following electrophoresis at 195 V with 40 second pulse-times for 24 hours in a pulsed field gel electrophoresis (PFGE) apparatus by Pharmacia, the gel was stained with SYBR® Gold (Molecular Probes).

Results

Mimosine and other iron chelators cause cell cycle arrest at the G1-S phase boundary

We first compared the effect of mimosine to that of other iron chelators implicated in cell cycle arrest. Asynchronously growing human cells were treated with mimosine, ciclopirox olamine and bipyridyl for 24 hours at a range of concentrations. We found that a treatment with 500 μM mimosine was equivalent to a treatment with 100 μM ciclopirox olamine or 200 μM bipyridyl, resulting in the enrichment of cells with a G1 phase DNA content and the near complete depletion of the S phase population (Fig. 1A). This cell cycle distribution appears to be similar to that obtained by the inhibition of DNA polymerases using aphidicolin (Fig. 1A).

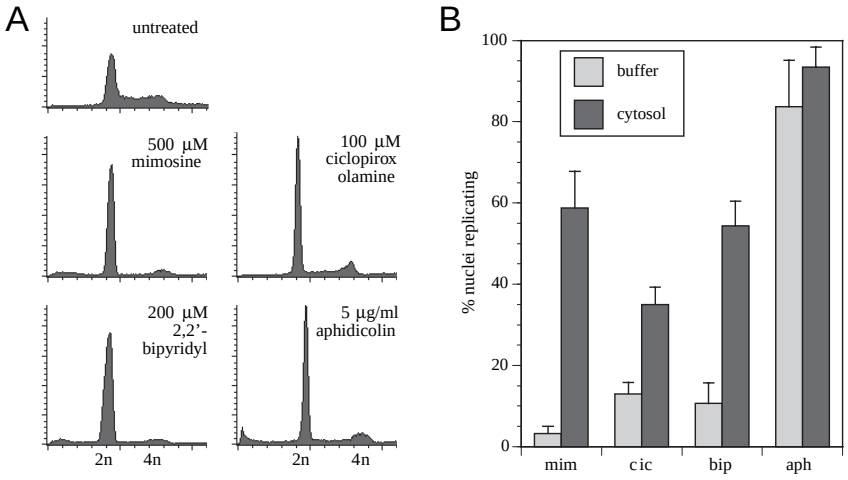

Fig. 1. Cell cycle arrest at the G1-S phase boundary caused by iron chelators. (A) Flow cytometry analysis of propidium iodide stained nuclei, isolated from HeLa cells that were treated for 24 hours with the iron chelators mimosine, ciclopirox olamine or 2,2′-bipyridyl, and, as control, with the replication-elongation inhibitor aphidicolin, at the indicated concentrations. The positions of unreplicated (2n) and fully replicated DNA content (4n) are indicated. (B) In vitro replication reactions of nuclei, isolated from cells treated with the iron chelators and aphidicolin as in A. Nuclei were incubated in an elongation buffer ('buffer', light grey bars) or in the same buffer supplemented with 100 μg HeLa cell cytosolic extract (cytosol, dark grey bars). Percentages of nuclei that incorporated labelled nucleotides were determined. The mean of 3-4 experiments and the standard deviations are shown.

To investigate whether this arrest occurs before or after the establishment of DNA replication forks, nuclei were isolated from the treated cells and assayed in vitro for their replication potential. Nuclei from S-phase cells are capable of continuing DNA synthesis in vitro in the presence of an 'elongation buffer' containing nucleoside and deoxynucleoside triphosphates (Krude et al., 1997). The in vitro DNA-chain-elongation is performed by replication forks that were established in vivo during S phase. Indeed, about 85% of nuclei isolated from aphidicolin-treated cells (which are arrested in S phase) continued elongation in vitro (Fig. 1B). By contrast, only 5-15% of nuclei isolated from cells treated with either one of the iron chelators were capable of continuing DNA replication, indicating that 85-95% of these cells were not in S phase at the time of preparation (Fig. 1B).

DNA replication initiation can be elicited in vitro in nuclei isolated from late G1-phase cells by adding cell extracts that contain essential initiation factors (Krude, 2000; Krude et al., 1997; Szüts et al., 2003). The addition of cytosolic extract from proliferating cells triggers the initiation of DNA replication in about 50% of nuclei isolated from mimosine-treated cells (Fig. 1B) (Krude, 2000). We also observed that 20-30% of nuclei from ciclopirox-treated cells, and 40-50% of nuclei from bipyridyl-treated cells, initiated DNA synthesis when cytosolic extract was added (Fig. 1B). This indicates that all three iron chelators arrest human cells at the same point in the cell cycle, at or near the G1/S-phase boundary and before active DNA replication forks are established, with a significant proportion of the cells blocked in a replication initiation competent state.

The reversible cell cycle arrest is caused by iron depletion

Cell cycle arrest by mimosine is reversible, as cells progress through S phase after mimosine is removed (Krude, 1999; Lalande, 1990). To investigate, whether the removal of mimosine is functionally equivalent to restoring the iron level, cells were kept in 500 μM mimosine for 24 hours. Then either mimosine was removed by replacing the medium, or an excess of iron (1 mM $FeSO_4$) was added to the mimosine-containing medium. In both cases, analysis of DNA content by flow-cytometry showed a significant widening of the G1 peak, 4 hours after release. Also, a distinct population of cells were moving through S phase at later time points (Fig. 2A). Quantification of the flow cytometry results indicates that more than half of the original G1-phase population had moved into S phase 4 hours after mimosine had been removed (Fig. 2B). Approximately 16 hours after release, the proportion of cells in G2 or M-phase reaches its peak, and by 24 hours many of the cells have completed mitosis and return to G1 phase (Fig. 2A,B). No significant cell death was observed during the period up to 3 days after the removal of mimosine (data not shown). A similar release into S phase was observed, when the medium was replaced or iron was added to ciclopirox olamine or bipyridyl-treated cells (data not shown). These data suggest that the cell cycle arrest caused by the examined iron-chelating compounds is due to the depletion of free iron.

To determine the time of S-phase entry upon release from iron chelation more accurately, nuclei were isolated from cells at various time points after the release and were incubated in elongation buffer to visualise the presence of active DNA replication forks. One hour after the release, cells had not entered S phase. However, 2 hours after mimosine removal and 4 hours after iron addition, 30-40% of the cells had progressed into S phase (Fig. 2C), suggesting that the removal of the iron chelators is more efficient in reversing the cell cycle block than the addition of excess iron. These data are in good agreement with the results obtained by flow cytometry (Fig. 2A). Taken together, they confirm that iron-chelation arrests cell cycle progression shortly before active DNA replication forks at the G1/S phase transition are established.

Treatment by iron chelators causes DNA damage

Any interference with DNA replication fork progression is expected to cause single-strand nicks and free ends, which can also be processed into DSBs (Hyrien, 2000; Rothstein et al., 2000). We decided to investigate whether iron depletion, which results in a block before the establishment of DNA replication forks, also causes DNA damage. Proliferating human EJ30 cells were treated for 24 hours with iron chelators and, as controls, with a range of compounds commonly used for inducing either S-phase arrest or DNA damage. Initially, DNA damage was monitored through the phosphorylation of the

histone variant H2AX. In untreated cells, only a very low level of focal γ-H2AX staining was sporadically detected (Fig. 3A and data not shown). Treatment with excess thymidine yielded a weak focal γ-H2AX staining, whereas aphidicolin, an inhibitor of DNA polymerases, produced a strong focal pattern of H2AX phosphorylation (Fig. 3A). These data confirm that interference with DNA replication fork progression results in DNA damage. Importantly, all three tested iron chelators also caused strong and clear focal γ-H2AX staining (Fig. 3A). As positive control, we also analysed two agents that directly cause DNA DSBs. Etoposide, an inhibitor of topoisomerase II, and Bleocin, a component of the DNA damaging antibiotic bleomycin, both produced strong, and in case of Bleocin, dose-dependent focal γ-H2AX staining (Fig. 3A). Taken together, these data suggest that iron chelators cause extensive and localised DNA damage, most probably in the form of DSBs.

It is possible that phosphorylation of H2AX not only marks DNA strand breaks, but rather some other disturbances at the DNA level. Therefore, we obtained independent confirmation of DNA damage by separating the DNA content of treated cell nuclei on alkaline agarose gels. Genomic DNA from untreated cells showed no migration into the alkaline gel under the experimental conditions (Fig. 3B, lanes 1 and 7). However, the tested iron chelators produced a smear of heterogeneous DNA fragments that enter the gel, down to the 1 kb size-range, indicating the presence of DNA breaks (Fig. 3B, lanes 4-6, see also Fig. 6D). Etoposide and Bleocin produced a similar, but more pronounced smear (Fig. 3B, lanes 8-10). The elongation blockers thymidine and aphidicolin also induced DNA breaks, although the smear visible after thymidine treatment is rather weak (Fig. 3B, lanes 2-3).

The conditions in an alkaline agarose gel do not allow a distinction between single and double-strand breaks. To investigate, whether iron depletion causes the formation of DSBs, we analysed representative samples by neutral pulsed field gel electrophoresis (PFGE). Genomic DNA from untreated proliferating cells (Fig. 3C, lane 1) or from cells treated with thymidine (lane 2) showed no migration into the gel under these conditions, and therefore did not contain a significant amount of DSBs. However, both the replication inhibitor aphidicolin (lane 3) and the iron chelator mimosine (lane 4) produced a significant amount of DSBs, indicated by a smear of DNA fragments across a broad size-range (Fig. 3C). These results confirm that iron depletion causes genomic double-strand DNA breaks under conditions that induce cell cycle arrest before the establishment of active DNA replication forks. Therefore, we asked next whether these breaks result in the activation of DNA-damage response pathways.

Activation of DNA damage checkpoint and repair pathways

Previously we observed that treatment with mimosine results in the appearance of a focal pattern of RPA in the nuclei (Szüts

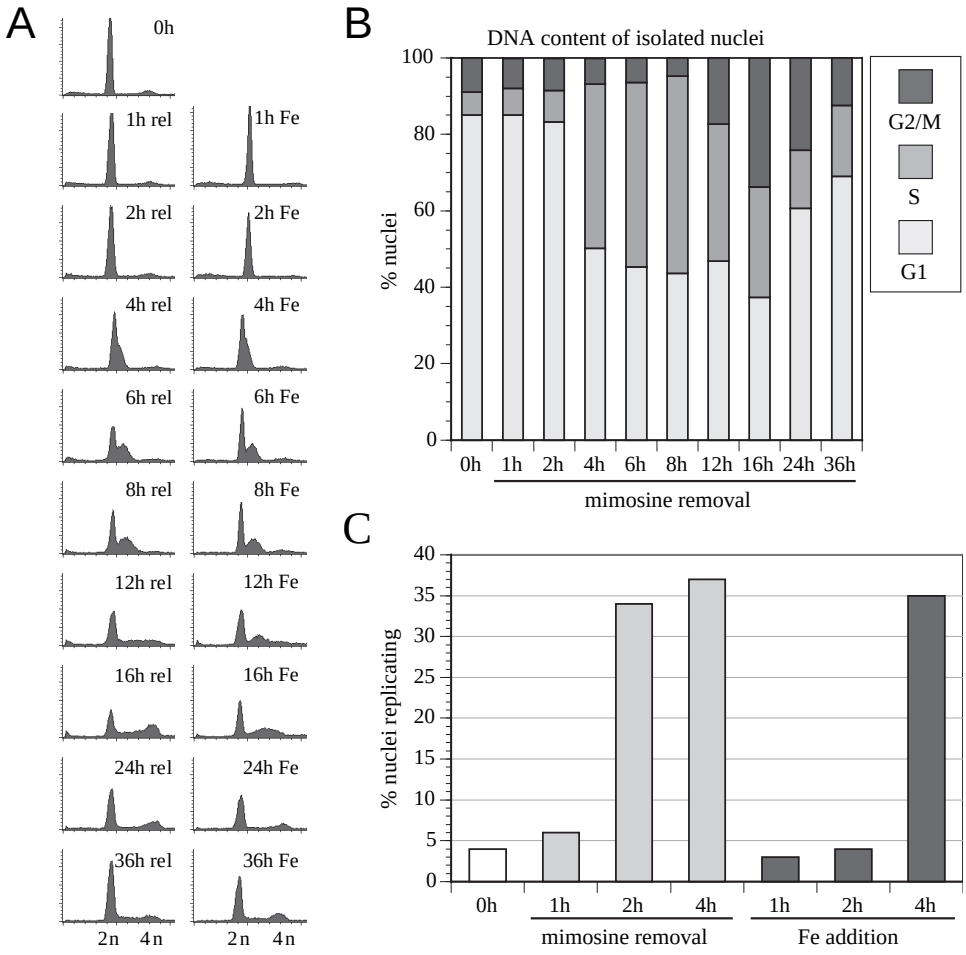

Fig. 2. The cell cycle arrest caused by iron chelation is reversible. (A) HeLa cells were kept in 500 μM mimosine for 24 hours. Then, either mimosine was removed by replacing the medium (mimosine release, rel) or 1 mM $FeSO_4$ (Fe) was added. Nuclei were prepared and their DNA content was analysed by flow cytometry at the indicated time points. The '0 h' control indicates a 24-hour treatment with mimosine only. (B) Proportion of cells in the G1, S or G2-M phases of the cell cycle at the indicated time points after release from mimosine-induced cell cycle arrest, as determined from the flow cytometry histograms shown in A. (C) In vitro replication assay of nuclei from the 0, 1, 2 and 4-hour samples from A. Nuclei were incubated in elongation buffer and the percentage of nuclei that incorporated labelled nucleotides is shown for each time point.

et al., 2003). The formation of RPA foci at sites of both single and double-strand DNA breaks is a known phenomenon (Burns et al., 1996; Golub et al., 1998; Lao et al., 2000). Therefore, we investigated the localisation of chromatin-bound RPA in relation to γ-H2AX after treating proliferating cells with mimosine. As seen before (Szüts et al., 2003), RPA assumes a focal pattern in nuclei of mimosine-treated cells, in contrast to a low level of staining in the majority of untreated cells (Fig. 4A). Upon close observation, most of these RPA foci colocalise with γ-H2AX, because most γ-H2AX-staining patches have a bright RPA focus in the centre. The signalling protein kinase ATR, which has been shown to move to sites of DNA repair (Tibbetts et al., 2000), perfectly colocalised with RPA in mimosine-treated cell nuclei (Fig. 4B). This result, together with the observation that ATR and RPA relocalisation into foci depends on ATR activity (Barr et al., 2003), indicates that an ATR-dependent DNA damage pathway is activated in response to iron depletion by mimosine.

Amongst the downstream targets of ATR is the checkpoint kinase CHK1 (Zhao and Piwnica-Worms, 2001). ATR activity should therefore result in the phosphorylation of CHK1, which can be assayed using a phospho-specific antibody. Upon mimosine treatment, γ-H2AX-positive cells showed uniform nuclear staining with the phospho-CHK1 antibody (Fig. 4C). In addition, we observed the phosphorylation of both CHK1 and CHK2 after mimosine treatment in whole-cell extracts (see below, Fig. 7), which provides further evidence for the activation of checkpoint pathways acting through ATR and possibly ATM. These results indicate that DNA damage signalling pathways are activated during the cell cycle arrest induced by iron chelation.

Cell cycle arrest by iron chelators is independent of checkpoint activation

The activation of DNA damage response pathways operating through the CHK1 and CHK2 kinases raises the possibility that the cell cycle arrest observed under conditions of iron depletion is the result of a DNA damage checkpoint. To test this hypothesis, we first performed a set of experiments using wortmannin, an inhibitor of ATM, ATR and DNA-PK (Sarkaria et al., 1998). We found that the inclusion of 25 μM wortmannin during a 24-hour treatment of EJ30 cells with mimosine or aphidicolin, completely prevents H2AX phosphorylation (Fig. 5A). Wortmannin treatment also prevented the accumulation of RPA into strong intranuclear foci in the presence of mimosine, but not in the presence of aphidicolin (Fig. 5B). These results strongly suggest that wortmannin abolishes the DNA damage response in mimosine-arrested cells, whereas the remaining focal RPA in cells treated with aphidicolin and wortmannin is because of the continued presence of stalled replication forks.

Next, we investigated whether this checkpoint inhibition abolishes cell cycle arrest by iron depletion. We included caffeine in this analysis as an alternative to wortmannin, because caffeine is a more potent inhibitor of ATR, while it has only little effect on DNA-PK (Sarkaria et al., 1999). When

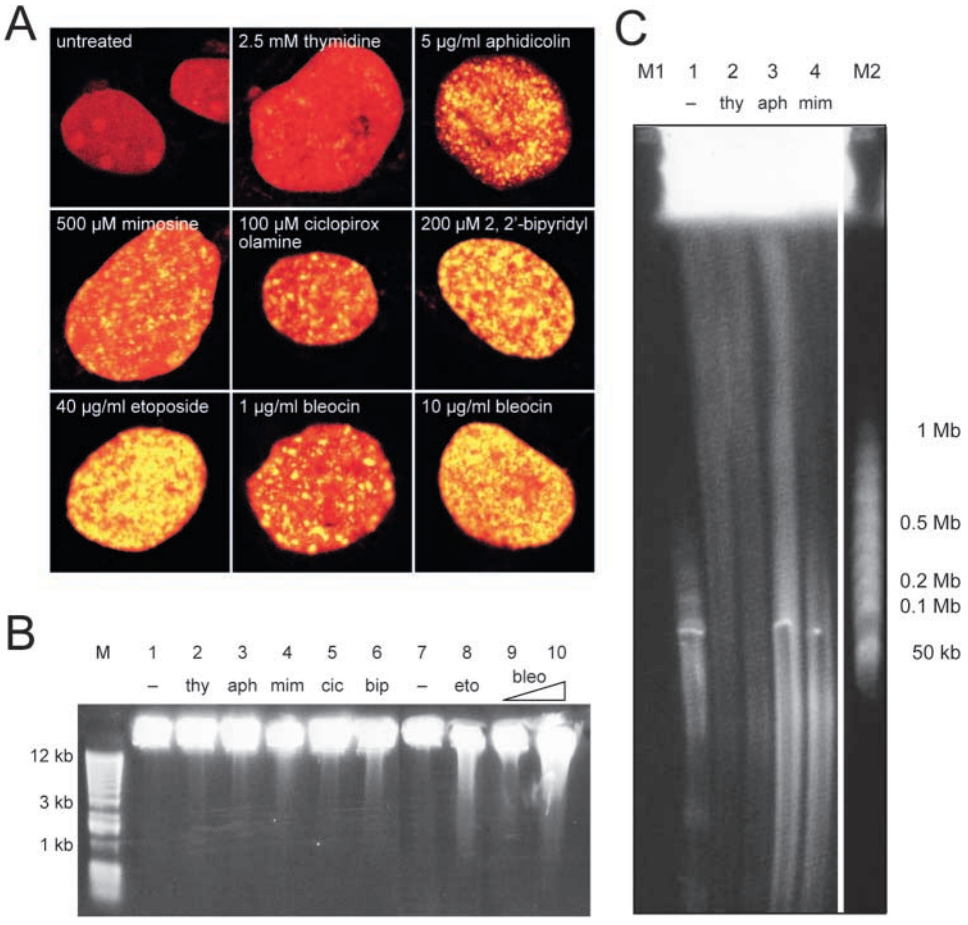

Fig. 3. Treatment with iron chelators causes DNA breaks. (A) EJ30 cells were treated with the indicated compounds for 24 hours, fixed, treated with RNase A and stained with propidium iodide for DNA (red) and with antibodies for γ-H2AX (green). Merged images are shown. (B) HeLa cells were subjected to treatments exactly as in A and the generated DNA breaks were analysed by alkaline-agarose-gel electrophoresis. Lanes 1 and 7, untreated control; 2, thymidine; 3, aphidicolin; 4, mimosine; 5, ciclopirox olamine; 6, bipyridyl; 8, etoposide; 9, 1 μg/ml Bleocin; 10, 10 μg/ml Bleocin. The marker (M) is a 1 kb ladder (Roche). (C) Samples 1-4 from B were subjected to neutral pulsed-field-gel electrophoresis to assay for the presence of DSBs. The markers cover the 0.1-200 kb (M1) or 50-1000 kb (M2) size-range.

wortmannin or caffeine were added for 4 hours to cells arrested with mimosine, no release from the G1-S block and entry into S phase was observed by flow cytometry (Fig. 5C). Simultaneous 24-hour treatment with mimosine and either of the inhibitors produced a similar cell cycle profile, but also caused significantly high incidences of cell death (data not shown). We analysed the in vitro replication potential of nuclei isolated from cells treated for 24 hours with mimosine and wortmannin together (Fig. 5D), or with caffeine (Fig. 5E). Importantly, the elimination of checkpoint signalling by either wortmannin or caffeine did not increase the proportion of mimosine-treated cell nuclei capable of run-on DNA replication in elongation buffer (Fig. 5D,E). Furthermore, neither inhibitor abolished the ability of these nuclei to initiate DNA replication in the presence of cytosolic extract. We conclude that cell cycle arrest by iron depletion is independent of checkpoint signalling through ATR or ATM.

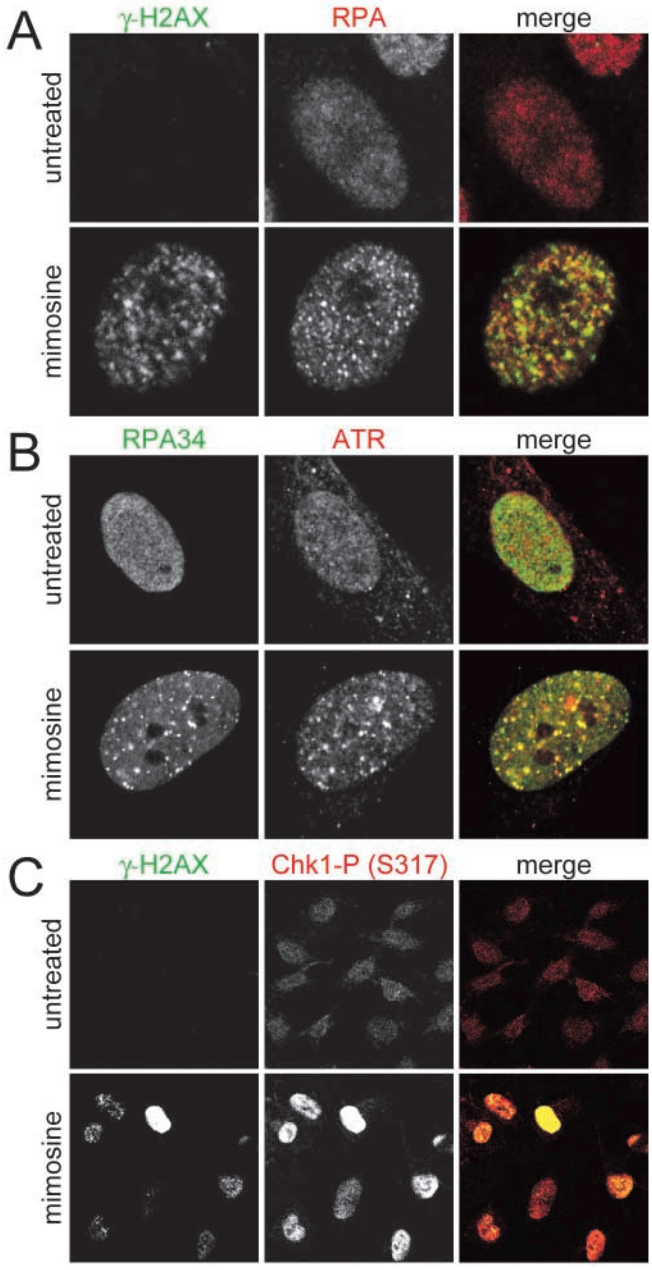

Fig. 4. Activation of DNA damage response pathways. EJ30 cells were grown on coverslips and were either used as control (untreated) or treated with 500 μM mimosine for 24 hours. After fixation, the cells were stained with antibodies against (A) γ-H2AX (green) and RPA (red), (B) RPA34 (green) and ATR (red) and (C) γ-H2AX (green) and phosphorylated CHK1-S317-P (red). Representative nuclei are shown in A and B. (C) To illustrate the correlation between the intensity of γ-H2AX staining and the level of CHK1-P, a low magnification field of nuclei is shown. Merged RGB images are shown in the right hand panels.

Mimosine-induced DNA damage detected by H2AX phosphorylation is cell cycle dependent and is concomitant with the arrest

As shown above, cell cycle arrest by iron chelation is not dependent on DNA-damage signalling. This raises the possibility that DNA damage is a consequence of the cell cycle arrest rather than its cause. We began to address this issue by asking if damage induction is restricted to a particular phase of the cell cycle. First, we examined whether DNA damage can be elicited in non-cycling, quiescent cells. Quiescent human EJ30 cells were treated with 500 μM mimosine for 24 hours. We found that less than 5% of the cells displayed a focal γ-H2AX pattern both before and after the mimosine treatment (Fig. 6A), indicating that iron depletion does not cause DSBs in non-proliferating cells. By contrast, mimosine treatment of asynchronously proliferating EJ30 cells resulted in the appearance of γ-H2AX in 75-95% of the cells (Fig. 6B).

Next we investigated, whether the induction of DNA damage is restricted to a particular phase of the proliferative cell cycle. To examine G1 phase and the G1-S transition, EJ30 cells were released from a mitotic arrest induced by nocodazole, and treated with mimosine or aphidicolin for 4-hour intervals, starting 4, 8 or 12 hours after the removal of nocodazole. Cell cycle progression of untreated control cells was monitored by flow cytometry and BrdU incorporation. In these experiments, S-phase cells were first seen in significant numbers 12 hours after the release from the nocodazole-induced arrest (Fig. 6D). In parallel, DNA damage after the 4-hour treatments was visualised by γ-H2AX immunofluorescence (Fig. 6C). Intriguingly, almost no cells showed γ-H2AX staining following the 4-8-hour post-mitotic treatment, whereas a significant number of cells displayed focal γ-H2AX staining after the 8-12-hour treatment with either mimosine or aphidicolin (Fig. 6C). The number of cells with γ-H2AX foci further increased after the 12-16 hour treatment, in good agreement with the number of control cells that enter S phase during this period (Fig. 6C,D). These results show that DNA damage induced by mimosine becomes detectable approximately the same time untreated control cells enter S phase. Aphidicolin only causes DNA damage in S phase, when it inhibits DNA polymerases. The close similarity in the number of cells with damaged DNA after mimosine or aphidicolin treatment, therefore supports the conclusion that damage induced by mimosine occurs at a time when cells have entered S phase.

As a control, we performed, in parallel, 4-hour-long etoposide treatments of cells synchronised in G1 and S phase. We found that etoposide damages DNA and induces H2AX

phosphorylation in all cells, regardless of their cell cycle position (not shown). Taken together, we conclude that DNA damage is induced by mimosine in a cell-cycle-specific manner, concomitant with the cells' arrival at the arrest point at the G1-S phase boundary.

Cells released from a cell cycle arrest by iron depletion replicate DNA in the presence of DNA damage

To address further whether DNA damage is the cause or consequence of the cell cycle arrest induced by iron chelators, we released EJ30 cells from a mimosine-induced cell cycle block by either the removal mimosine or by the addition of iron (as in Fig. 2), and then followed the fate of the DNA damage. If DNA damage causes cell cycle arrest, one expects a complete repair of the damage before the cells exit from the block. However, we found that the strong focal γ-H2AX staining observed in most cells after mimosine treatment (Fig. 7A), remained almost constant for up to 16 hours after the removal of mimosine. Even after 24-48 hours, approximately half of the cells still contained γ-H2AX (Fig. 7B,D). Adding an excess amount of iron to the mimosine-containing medium had a similar effect on γ-H2AX foci in the released cells (Fig. 7C,D). Interestingly, the proportion of cells with γ-H2AX initially remained constant, whereas the overall amount of γ-H2AX in whole-cell extracts significantly decreased within 4-8 hours after mimosine removal (Fig. 7E). We also observed the rapid dephosphorylation of both CHK1 and CHK2 after the removal of mimosine, followed by fluctuating levels of phosphorylation at later time points (Fig. 7E).

By using alkaline gel electrophoresis, we found that the extent of DNA damage in nuclei from released cells had not significantly changed 4 hours after release by either method, and even 24 hours after release there was still a much higher level of DNA breakage than in the untreated control sample (Fig. 7F). Bearing in mind that already 4 hours after the release most of the cells had left the block and made significant progression into S phase (Fig. 2A), these results show that DNA damage alone does not sustain the cell cycle arrest caused by iron chelators. Therefore, DNA damage is not the cause of the cell cycle arrest, but appears to be its consequence.

Finally, we investigated the spatial relationship between DNA damage and DNA replication within the same nuclei. Mimosine-containing medium was removed from cells after 24 hours and replaced with fresh medium containing BrdU to label the sites of DNA replication after release. We observed strong BrdU incorporation within 1 hour, indicating that DNA replication had begun. As expected, approximately half of the cell nuclei incorporated BrdU, and these cells all showed focal γ-H2AX in a similar pattern (Fig. 7G, top panels). An in vitro equivalent of this experiment was also performed, because entirely semi-conservative origin-specific chromosomal DNA-replication can be initiated in nuclei isolated from mimosine-treated cells by the addition of cytosolic extract (Keller et al., 2002; Krude, 2000). After 5 minutes, DNA replication was observed in 30-40% of the nuclei in vitro, in all cases in the presence of focal γ-H2AX (Fig. 7G, bottom panels), similarly to the in vivo release from mimosine. The γ-H2AX staining seen in these nuclei probably indicates phosphorylation that originated from the arrested

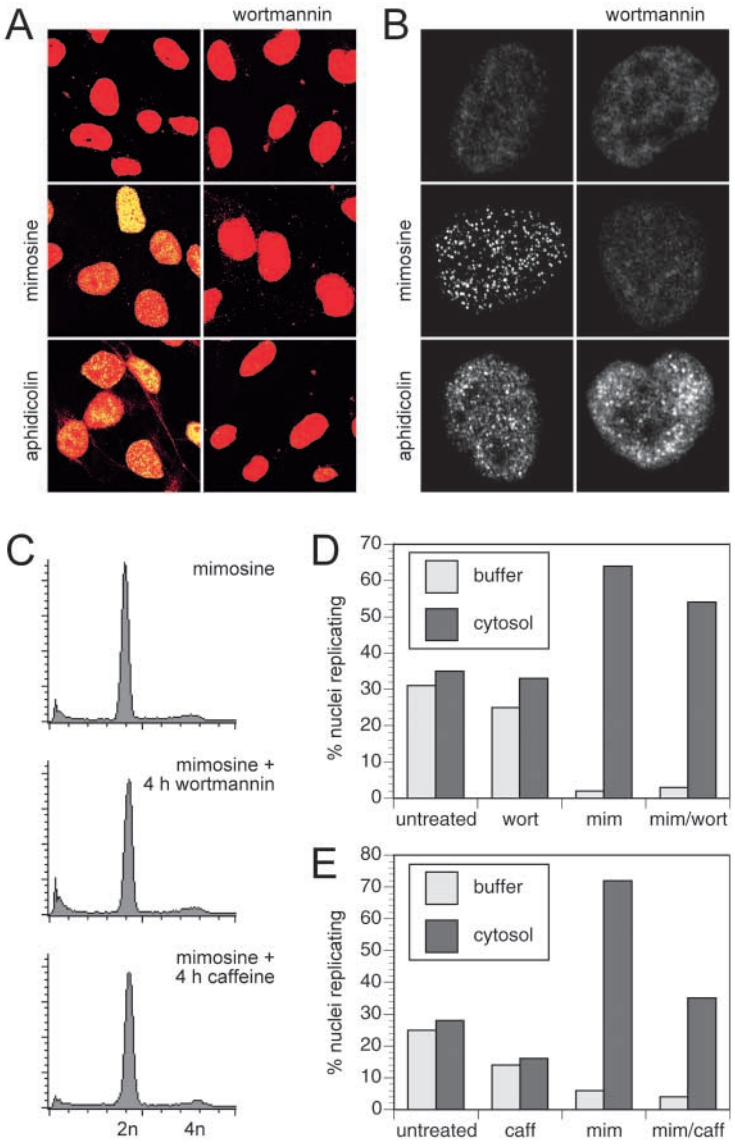

Fig. 5. The iron depletion-induced cell cycle arrest is independent of checkpoint activation. (A) EJ30 cells were subjected to 24-hour treatments with mimosine or aphidicolin in the absence or presence of 25 μM wortmannin, and stained for DNA (red) and γ-H2AX (green). (B) Representative cell nuclei from these experiments, stained for RPA. (C) Flow cytometry profiles of HeLa cells treated with mimosine for 28 hours (top); with mimosine only for 24 hours, followed by mimosine plus wortmannin for 4 hours (middle); or with mimosine only for 24 hours, followed by mimosine plus caffeine for 4 hours (bottom). (D) Nuclei from cells subjected to 24-hour treatments with mimosine (mim) or wortmannin (wort) or both (mim/wort) as in A were analysed by in vitro replication reactions. Nuclei were incubated in an elongation buffer (buffer) or in the same buffer supplemented with 100 μg HeLa cell cytosolic extract (cytosol). Percentages of nuclei incorporating labelled nucleotides are shown. (E) Nuclei from cells subjected to 24-hour treatments with caffeine (caff), mimosine (mim) or both (mim/caff) were analysed by in vitro replication reactions as in D.

state in vivo, as the signal rapidly disappears in vitro with longer incubations, and it is therefore unlikely that significant H2AX phosphorylation would take place in the in vitro system. Most γ-H2AX foci colocalise with sites of DNA replication in vitro. In addition, the overall patterns of γ-H2AX and DNA replication look similar in vivo and in vitro, suggesting that iron chelators cause DNA damage in early replicating chromatin domains.

Discussion

Inhibition of DNA replication fork progression is known to cause DNA breaks and elicit cellular responses leading to cell cycle arrest. In the present work we show that blocking the human cell cycle shortly before the initiation of DNA replication also induces DNA breaks. These breaks are the consequence of a cell cycle arrest by compounds that share one key property, the ability to chelate iron and other transition metal ions. Upon removal of the chemicals or restoration of iron levels, cells can enter and make significant progress through S phase with DNA that has not been repaired. Therefore, whilst cell cycle arrest by iron chelators can lead to DNA damage at the initiation stage, this damage itself cannot cause or maintain the arrest at the G1-S phase boundary.

Human cell populations treated with 500 μM mimosine have a G1-phase DNA content (Krude, 1999; Lalande, 1990). In this study, we found that analogous 24-hour treatment of human cells with 100 μM ciclopirox olamine or 200 μM 2,2′-bipyridyl has the same effect on cell cycle distribution. For the following reasons, the arrest takes place before the cells enter the S phase (at the G1-S phase boundary): First, it has been shown in the case of mimosine that these cells do not incorporate BrdU when it is added together with the drug, before the arrival of the cells at the point of arrest (Keller et al., 2002; Watson et al., 1991). Second, the results of in vitro replication run-on experiments provide evidence that the arrest happens before S-phase entry. Nuclei of S-phase cells are capable of continuing the elongation of DNA-replication in vitro at pre-existing sites, using replication forks and their accessory factors established in vivo (Krude et al., 1997). On the other hand, although nuclei of late G1-phase cells cannot replicate DNA under the same conditions, they can initiate DNA replication in vitro in the presence of extracts from proliferating cells (Krude, 2000; Krude et al., 1997). This property was also observed for nuclei from cells that were arrested with the iron chelators ciclopirox olamine or bipyridyl (this study). Therefore, unlike an arrest caused by aphidicolin or hydroxyurea, this arrest takes place before S-phase entry. However, the point of arrest appears to be close to S phase, because cells released from the block soon start replication. In vivo, BrdU incorporation has been seen within 1 hour (this study) or 15 minutes (Watson et al., 1991) of release from mimosine; DNA synthesis in vitro is detectable in as little as 5 minutes. DNA synthesis observed in vivo and in vitro cannot solely be due to DNA repair for the following reasons: In vivo, cells synthesise large amounts of DNA (detectable by flow cytometry) within hours (Fig. 2A) and eventually complete S phase. In vitro, isolated nuclei synthesise less DNA, but the observed DNA synthesis has been shown to be entirely semiconservative (Krude, 2000). Therefore, upon iron chelation, human cells are arrested in a replication–initiation-

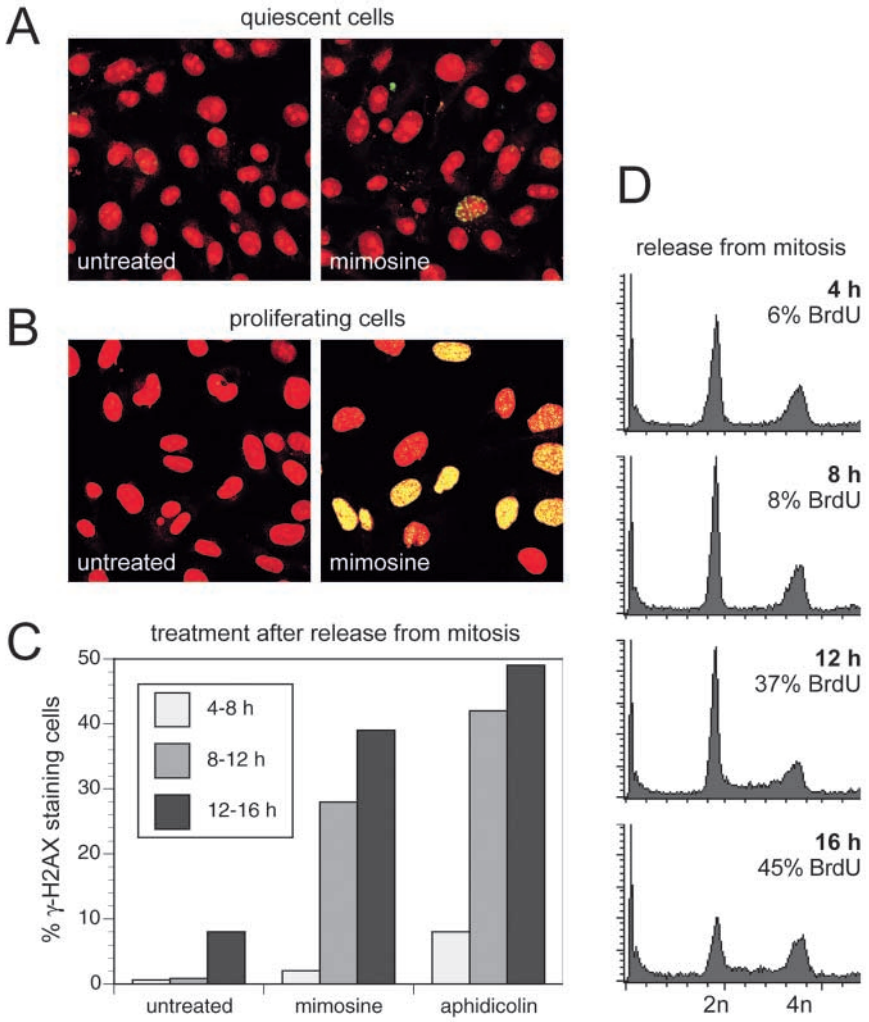

Fig. 6. Mimosine-induced DNA damage is cell cycle dependent. (A) Quiescent EJ30 cells were incubated in 500 μM mimosine for 24 hours, fixed, treated with RNase A, and stained for DNA (red) and for γ-H2AX (green). (B) Proliferating EJ30 cells were incubated for 24 hours and stained as in A. (C) EJ30 cells were arrested in mitosis by a 24-hour nocodazole treatment, released by replacing the medium, and then subjected to a treatment of 500 μM mimosine or 5 μg/ml aphidicolin in intervals of 4-8, 8-12 and 12-16 hours post release. The percentage of cells showing focal γ-H2AX staining were determined by immunofluorescence microscopy. (D) Flow-cytometry analysis of the DNA content of untreated control cells at the indicated time points after release from nocodazole as used in C. The percentages of cells that incorporated BrdU in the 15 minutes before preparation are shown in each panel.

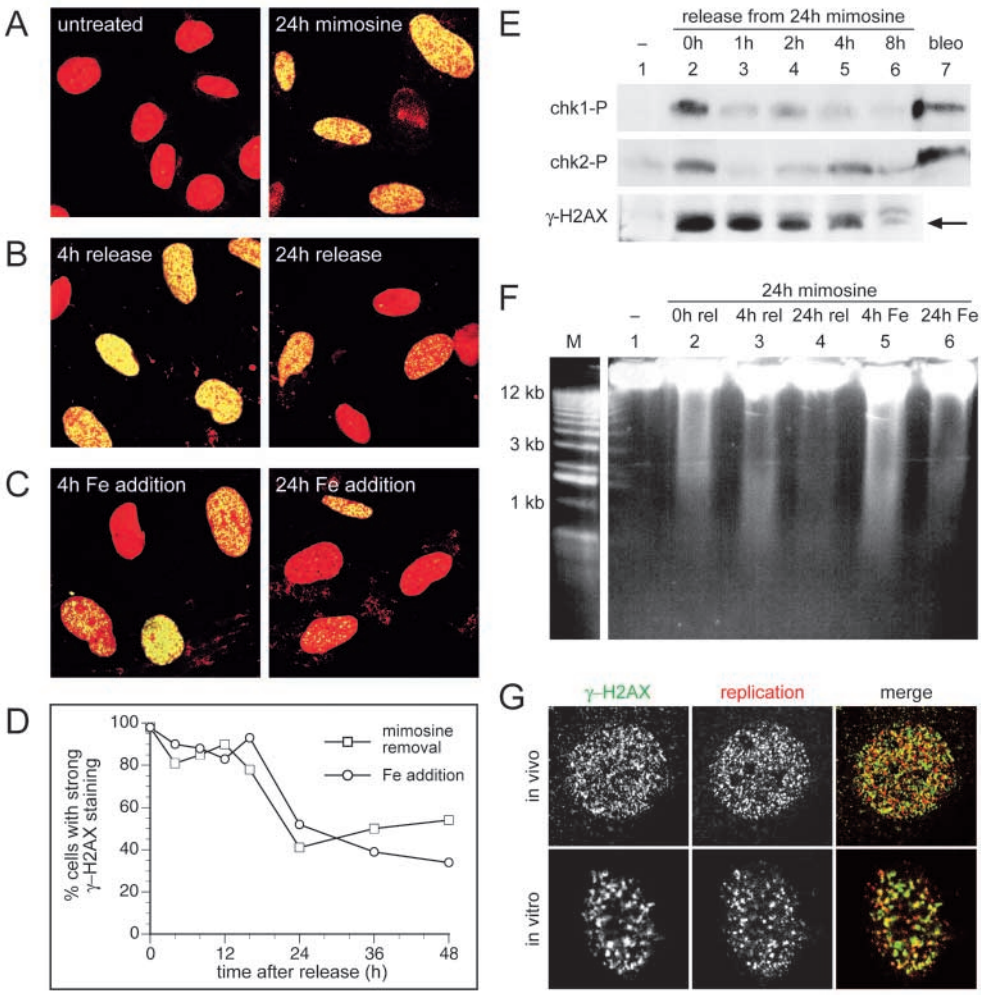

Fig. 7. DNA replication proceeds in the presence of DNA breaks upon release from mimosine. (A) Control EJ30 cells and cells treated with 500 μM mimosine for 24 hours were stained for γ-H2AX (green) and DNA (red). (B) Cells, stained as in A, 4 or 24 hours after the removal of mimosine. (C) Cells, stained as in A, 4 or 24 hours after the addition of 1 mM $FeSO_4$, which was added 24 hours after the addition of 500 μM mimosine. (D) Graph, showing the percentage of cells with strong focal γ-H2AX staining, as seen in B-C, at the indicated time points after cells had been released from arrest by either mimosine removal (squares) or iron addition (circles). (E) Western blot, showing the phosphorylation of the CHK1, CHK2 and H2AX proteins in whole cell extracts, prepared at the indicated time points after release from a mimosine arrest; the arrow indicates the specific γ-H2AX band. (F) HeLa cells, subjected to identical treatments as in A-C. The presence of DNA breaks was analysed by alkaline gel electrophoresis as in Fig. 3B. (G) EJ30 cells were treated with 500 μM mimosine for 24 hours and assayed for their competence to initiate DNA replication in the presence of DNA damage in vivo (top panels) or in vitro (bottom panels). In vivo, cells were released into fresh medium containing BrdU for 1 hour, fixed and stained for γ-H2AX (using a rabbit polyclonal antibody, green) and BrdU (red). For the in vitro test, nuclei of mimosine-treated cells were incubated for 5 minutes in a replication assay mix containing cytosolic extract and digoxigenin-dUTP (Roche), fixed and stained for γ-H2AX (green) and digoxigenin (red). Representative nuclei of the class that stains for both γ-H2AX and replication markers are shown. Merged RGB images are shown in the right hand panels.

competent state in late G1 phase, near the G1-S phase boundary.

What might cause the formation of DNA breaks in cells arrested at the G1–S-phase boundary? Our cell-synchronisation experiments show that mimosine, used as a representative for iron chelators, does not cause H2AX phosphorylation in early G1 phase, and that the formation of DNA damage coincides with the time when cells are entering S phase. Moreover, the cells can progress into S phase with unrepaired DNA as soon as the iron chelators are removed or iron levels are replenished, indicating that the arrest is not due to damaged DNA, nor is the maintenance of the arrest dependent upon persistent DNA breaks. Therefore, the DNA breaks have to be a consequence of the cell cycle arrest. One of the iron chelators, mimosine, was shown before (in an alkaline comet assay) to generate DNA breaks (Mikhailov et al., 2000). These authors concluded that the induced damage is independent of the cell cycle phase, and can even be generated in quiescent cells. In our experiments, mimosine clearly did not induce H2AX phosphorylation in either quiescent or G1 phase cells. Therefore, our cell-synchronisation experiments allow us to conclude that any potential cell-cycle-independent effect of mimosine that possibly induces apoptosis (Mikhailov et al., 2000), is distinctly different from the generation of DNA breaks as a consequence of a G1/S phase cell cycle arrest, caused by iron depletion.

A known mechanism of indirectly generating DNA breaks and H2AX phosphorylation is by inhibiting the progression of the replication fork (Michel et al., 1997), as done by aphidicolin or hydroxyurea. In this case, breaks are thought to result from fork-reversion by annealing the nascent strands, and the resolution of the resulting Holliday junction. However, cells treated by mimosine do not replicate any detectable amount of DNA before the G1-S arrest (Keller et al., 2002), making it unlikely that the DNA damage arises from collapsed replication forks. An additional argument against this possibility is provided by observations on focus formation by RPA. In the nuclei of cells arrested by mimosine, RPA appears in a focal pattern (Szüts et al., 2003). Importantly, this amount of RPA is not sufficient to initiate replication (Szüts et al., 2003). In the presence of wortmannin, an inhibitor of DNA damage signalling, mimosine does not induce the formation of RPA foci. Therefore, it is more plausible that RPA is recruited

to nuclear sites in mimosine-treated cells predominantly in response to the formation of DNA breaks. By contrast, RPA foci, that are visible after the inhibition of DNA replication by aphidicolin are not abolished by wortmannin, showing that focus formation of RPA at sites of replication is independent of checkpoint signalling.

DNA breaks are only generated by iron depletion when cells arrive at the G1-S phase boundary. Many of these breaks seem to colocalise with sites of replication immediately after the release of arrest. Therefore, it is possible that the breaks are a consequence of early stages in the series of events leading to the initiation of replication. For example, if origin unwinding occurred in the presence of iron chelators, it could explain the localised recruitment of ATR and the activation of ATR and CHK1, because the prolonged presence of single-stranded regions can activate an ATR-dependent checkpoint mechanism (Costanzo et al., 2003). The DNA DSBs visualised by PFGE, on the contrary, cannot be explained by this mechanism. It is possible that they are the consequence of long, continuously unwound regions of DNA that might be more susceptible to nucleolytic attacks. Alternatively, the base-pairing of potential inverted repeats in the unwound regions might give rise to the phenomenon of cruciform extrusion, and the resolution of the resulting Holliday junction would produce a DSB. Clearly, more experiments are required to explain the formation of these breaks at the molecular level.

An interesting aspect of the studied cell cycle arrest is its reversibility. Unexpectedly, cells can progress through S phase in the presence of damaged DNA and phosphorylated H2AX. The fact that these cells take at least 16 hours to complete the S phase, and that both CHK1 and CHK2 seem to get re-activated during this period in a fluctuating manner suggests that DNA breaks are indeed still present and replication is slowed by the need to repair the breaks. Eventually, those cells that completed S phase also manage to go through mitosis, losing the remaining γ-H2AX at the same time. By this time and in this proportion of cells, DNA repair is probably complete. By contrast, the DNA damage that is still observed 24 hours after the release of the cell cycle arrest caused by mimosine, is most probably present in those cells that did not manage to be efficiently released from the cell cycle block.

We considered the possibility that the observed behaviour of the tumour cell lines used throughout this study is influenced by potential abnormalities of their checkpoint mechanisms. We therefore repeated most of the key experiments with human primary fibroblasts and found that they produce a very similar response to iron chelators, including arrest at the G1-S boundary, induction of DNA damage and release from the arrest in the presence of damaged DNA (see Fig. S1 in supplementary material).

While the iron chelators used in this study are useful tools to investigate the initiation of replication, the results presented in this article might also shed some light on their mode of action. How can iron depletion lead to cell cycle arrest at the G1-S boundary? A reduction of certain cell-cycle-protein levels upon iron chelation, including the ones of cyclin D, cyclin A, cyclin B and CDK2 has been reported (Alcantara et al., 2001; Gao and Richardson, 2001) as has the hypo-phosphorylation of Rb (Gao and Richardson, 2001; Kulp and Vulliet, 1996; Terada et al., 1991). Iron chelators have also been reported to increase the level of the cdk inhibitory proteins p21 or p27 (Alpan and Pardee, 1996; Chang et al., 1999; Wang et al., 2004). However, in HeLa cells we did not see a significant change in the level on any of these proteins (D.S. and T.K., unpublished results). Nevertheless, it is possible that the cell cycle arrest at the G1-S boundary operates through a direct or indirect inhibition of kinases, required for cell cycle progression through this point. A decrease in the activity of either CDK2 or CDC7 would prevent the firing of early-replication origins (Jares and Blow, 2000; Walter, 2000).

An intriguing explanation for the mechanism of G1-S arrest by iron chelation would be the activation of the hypoxia-response pathway. Hypoxia-induced genes are regulated by the HIF-1 transcription factor (Wang and Semenza, 1993), which under normoxic conditions is targeted for degradation by the iron-dependent HIF-1α prolyl hydroxylase enzymes (Ivan et al., 2001; Jaakkola et al., 2001). Iron depletion has been shown to inhibit the HIF-1α prolyl hydroxylases, thereby triggering the hypoxic response. Indeed, both mimosine (Warnecke et al., 2003) and ciclopirox olamine (Linden et al., 2003) have been shown to activate the HIF-1 pathway. The activation of the hypoxic response, in turn, leads to the inhibition of DNA replication initiation in SV40 viral replication (Riedinger et al., 1999) and in human HeLa cellular replication (Probst et al., 1999). Although the method of inhibition is not clear, the hypoxia-arrested nuclei contain the components of the pre-replication complex (pre-RC) but not PCNA (van Betteraey-Nikoleit et al., 2003). This scenario would be consistent with properties of mimosine-arrested HeLa cells, which are very near S-phase entry and have pre-RC proteins present, but which have much lower nuclear bound RPA levels than S-phase HeLa cells (Szüts et al., 2003). It will be interesting to see whether HIF-1 directly regulates the expression of any of the proteins involved in the initiation of replication.

Cell cycle arrest by iron depletion seems to operate through a unique checkpoint mechanism, because the known p53, ATM or ATR-dependent G1 or S-phase arrests all result in a different arrest state. The G1-phase DNA content of the arrested cells, and their inability to support chromosomal DNA replication in the absence of initiation factors in vitro, makes this arrest distinct from those triggered by the S-phase replication or damage checkpoints. A p53-dependent, late G1-phase arrest can also be ruled out. Although activation of p53 in response to iron chelation has been shown (Abeysinghe et al., 2001), it is unlikely to be important in HeLa cells, where the half-life of p53 is much reduced (Talis et al., 1998). Moreover, mimosine also arrests p53-null-mutant SaOS cells at the G1-S phase boundary (D.S. and T.K., unpublished observation). Most importantly, however, cell cycle arrest caused by iron depletion does not depend on prior DNA damage, but rather causes DNA breaks as a secondary effect. Therefore, while the iron chelating drugs used in this work are very useful to study the initiation of chromosomal DNA replication, their DNA damaging effect must also be taken into account in cell-synchronisation experiments.

We thank Rolf Knippers, Manuel Stucki, Tony Mills and Arman Nabatiyan for critically reading this manuscript and for discussions. This work was supported by Cancer Research UK (Project Grant C1471/A2612) and the Isaac Newton Trust.

References

Abeysinghe, R. D., Greene, B. T., Haynes, R., Willingham, M. C., Turner, J., Planalp, R. P., Brechbiel, M. W., Torti, F. M. and Torti, S. V. (2001). p53-independent apoptosis mediated by tachpyridine, an anti-cancer iron chelator. *Carcinogenesis* **22**, 1607-1614.

Alcantara, O., Kalidas, M., Baltathakis, I. and Boldt, D. H. (2001). Expression of multiple genes regulating cell cycle and apoptosis in differentiating hematopoietic cells is dependent on iron. *Exp. Hematol.* **29**, 1060-1069.

Alpan, R. S. and Pardee, A. B. (1996). p21WAF1/CIP1/SDI1 is elevated through a p53-independent pathway by mimosine. *Cell Growth Differ.* **7**, 893-901.

Barr, S. M., Leung, C. G., Chang, E. E. and Cimprich, K. A. (2003). ATR kinase activity regulates the intranuclear translocation of ATR and RPA following ionizing radiation. *Curr. Biol.* **13**, 1047-1051.

Bartek, J. and Lukas, J. (2001a). Mammalian G1- and S-phase checkpoints in response to DNA damage. *Curr. Opin. Cell Biol.* **13**, 738-747.

Bartek, J. and Lukas, J. (2001b). Pathways governing G1/S transition and their response to DNA damage. *FEBS Lett.* **490**, 117-122.

Burns, J. L., Guzder, S. N., Sung, P., Prakash, S. and Prakash, L. (1996). An affinity of human replication protein A for ultraviolet-damaged DNA. *J. Biol. Chem.* **271**, 11607-11610.

Casper, A. M., Nghiem, P., Arlt, M. F. and Glover, T. W. (2002). ATR regulates fragile site stability. *Cell* **111**, 779-789.

Chang, H. C., Lee, T. H., Chuang, L. Y., Yen, M. H. and Hung, W. C. (1999). Inhibitory effect of mimosine on proliferation of human lung cancer cells is mediated by multiple mechanisms. *Cancer Lett.* **145**, 1-8.

Clement, P. M., Hanauske-Abel, H. M., Wolff, E. C., Kleinman, H. K. and Park, M. H. (2002). The antifungal drug ciclopirox inhibits deoxyhypusine and proline hydroxylation, endothelial cell growth and angiogenesis in vitro. *Int. J. Cancer.* **100**, 491-498.

Costanzo, V., Shechter, D., Lupardus, P. J., Cimprich, K. A., Gottesman, M. and Gautier, J. (2003). An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. *Mol. Cell* **11**, 203-213.

Gao, J. and Richardson, D. R. (2001). The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents, IV, The mechanisms involved in inhibiting cell-cycle progression. *Blood* **98**, 842-850.

Golub, E. I., Gupta, R. C., Haaf, T., Wold, M. S. and Radding, C. M. (1998). Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA. *Nucleic Acids Res.* **26**, 5388-5393.

Hyrien, O. (2000). Mechanisms and consequences of replication fork arrest. *Biochimie* **82**, 5-17.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G., Jr (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation, implications for O2 sensing. *Science* **292**, 464-468.

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* **292**, 468-472.

Jares, P. and Blow, J. J. (2000). Xenopus cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. *Genes Dev.* **14**, 1528-1540.

Keller, C., Hyrien, O., Knippers, R. and Krude, T. (2002). Site-specific and temporally controlled initiation of DNA replication in a human cell-free system. *Nucleic Acids Res.* **30**, 2114-2123.

Kenny, M. K., Schlegel, U., Furneaux, H. and Hurwitz, J. (1990). The role of human single-stranded DNA binding protein and its individual subunits in simian virus 40 DNA replication. *J. Biol. Chem.* **265**, 7693-7700.

Krude, T. (1999). Mimosine arrests proliferating human cells before onset of DNA replication in a dose-dependent manner. *Exp. Cell Res.* **247**, 148-159.

Krude, T. (2000). Initiation of human DNA replication in vitro using nuclei from cells arrested at an initiation-competent state. *J. Biol. Chem.* **275**, 13699-13707.

Krude, T., Jackman, M., Pines, J. and Laskey, R. A. (1997). Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell* **88**, 109-119.

Kulp, K. S. and Vulliet, P. R. (1996). Mimosine blocks cell cycle progression by chelating iron in asynchronous human breast cancer cells. *Toxicol. Appl. Pharmacol.* **139**, 356-364.

Lalande, M. (1990). A reversible arrest point in the late G1 phase of the mammalian cell cycle. *Exp. Cell Res.* **186**, 332-339.

Lao, Y., Gomes, X. V., Ren, Y., Taylor, J. S. and Wold, M. S. (2000). Replication protein A interactions with DNA. III. Molecular basis of recognition of damaged DNA. *Biochemistry* **39**, 850-859.

Le, N. T. and Richardson, D. R. (2002). The role of iron in cell cycle progression and the proliferation of neoplastic cells. *Biochim. Biophys. Acta* **1603**, 31-46.

Linden, T., Katschinski, D. M., Eckhardt, K., Scheid, A., Pagel, H. and Wenger, R. H. (2003). The antimycotic ciclopirox olamine induces HIF-1alpha stability, VEGF expression, and angiogenesis. *FASEB J.* **17**, 761-763.

Michel, B., Ehrlich, S. D. and Uzest, M. (1997). DNA double-strand breaks caused by replication arrest. *EMBO J.* **16**, 430-438.

Mikhailov, I., Russev, G. and Anachkova, B. (2000). Treatment of mammalian cells with mimosine generates DNA breaks. *Mutat. Res.* **459**, 299-306.

Nyberg, K. A., Michelson, R. J., Putnam, C. W. and Weinert, T. A. (2002). Toward maintaining the genome, DNA damage and replication checkpoints. *Annu. Rev. Genet.* **36**, 617-656.

O'Connell, M. J., Walworth, N. C. and Carr, A. M. (2000). The G2-phase DNA-damage checkpoint. *Trends Cell Biol.* **10**, 296-303.

Osborn, A. J., Elledge, S. J. and Zou, L. (2002). Checking on the fork, the DNA-replication stress-response pathway. *Trends Cell Biol.* **12**, 509-516.

Probst, G., Riedinger, H. J., Martin, P., Engelcke, M. and Probst, H. (1999). Fast control of DNA replication in response to hypoxia and to inhibited protein synthesis in CCRF-CEM and HeLa cells. *Biol. Chem.* **380**, 1371-1382.

Riedinger, H. J., van Betteraey, M. and Probst, H. (1999). Hypoxia blocks in vivo initiation of simian virus 40 replication at a stage preceding origin unwinding. *J. Virol.* **73**, 2243-2252.

Rogakou, E. P. and Sekeri-Pataryas, K. E. (1999). Histone variants of H2A and H3 families are regulated during in vitro aging in the same manner as during differentiation. *Exp. Gerontol.* **34**, 741-754.

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S. and Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* **273**, 5858-5868.

Rothstein, R., Michel, B. and Gangloff, S. (2000). Replication fork pausing and recombination or "gimme a break". *Genes Dev.* **14**, 1-10.

Saintigny, Y., Delacote, F., Vares, G., Petitot, F., Lambert, S., Averbeck, D. and Lopez, B. S. (2001). Characterization of homologous recombination induced by replication inhibition in mammalian cells. *EMBO J.* **20**, 3861-3870.

Sarkaria, J. N., Tibbetts, R. S., Busby, E. C., Kennedy, A. P., Hill, D. E. and Abraham, R. T. (1998). Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. *Cancer Res.* **58**, 4375-4382.

Sarkaria, J. N., Busby, E. C., Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M. and Abraham, R. T. (1999). Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res.* **59**, 4375-4382.

Sogo, J. M., Lopes, M. and Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science* **297**, 599-602.

Szüts, D., Kitching, L., Christov, C., Budd, A., Peak-Chew, S. and Krude, T. (2003). RPA is an initiation factor for human chromosomal DNA replication. *Nucleic Acids Res.* **31**, 1725-1734.

Talis, A. L., Huibregtse, J. M. and Howley, P. M. (1998). The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. *J. Biol. Chem.* **273**, 6439-6445.

Terada, N., Lucas, J. J. and Gelfand, E. W. (1991). Differential regulation of the tumor suppressor molecules, retinoblastoma susceptibility gene product (Rb) and p53, during cell cycle progression of normal human T cells. *J. Immunol.* **147**, 698-704.

Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J. and Abraham, R. T. (2000). Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. *Genes Dev.* **14**, 2989-3002.

van Betteraey-Nikoleit, M., Eisele, K. H., Stabenow, D. and Probst, H. (2003). Analyzing changes of chromatin-bound replication proteins occurring in response to and after release from a hypoxic block of replicon initiation in T24 cells. *Eur. J. Biochem.* **270**, 3880-3890.

Walter, J. C. (2000). Evidence for sequential action of cdc7 and cdk2 protein kinases during initiation of DNA replication in Xenopus egg extracts. *J. Biol. Chem.* **275**, 39773-39778.

Wang, G. L. and Semenza, G. L. (1993). General involvement of hypoxia-

inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl. Acad. Sci. USA* **90**, 4304-4308.

Wang, G., Miskimins, R. and Miskimins, W. K. (2004). Regulation of p27(Kip1) by intracellular iron levels. *Biometals* **17**, 15-24.

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.

Warnecke, C., Griethe, W., Weidemann, A., Jurgensen, J. S., Willam, C., Bachmann, S., Ivashchenko, Y., Wagner, I., Frei, U., Wiesener, M. et al. (2003). Activation of the hypoxia-inducible factor-pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors. *FASEB J.* **17**, 1186-1188.

Watson, P. A., Hanauske-Abel, H. H., Flint, A. and Lalande, M. (1991). Mimosine reversibly arrests cell cycle progression at the G1-S phase border. *Cytometry* **12**, 242-246.

Zhao, H. and Piwnica-Worms, H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. *Mol. Cell. Biol.* **21**, 4129-4139.

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