

# Adaptation to the Ionizing Radiation–Induced G<sub>2</sub> Checkpoint Occurs in Human Cells and Depends on Checkpoint Kinase 1 and Polo-like Kinase 1 Kinases

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## Abstract

**Checkpoint adaptation was originally defined in yeast as the ability to divide despite the presence of damaged DNA. An important unanswered question is whether checkpoint adaptation also occurs in human cells. Here, we show that following the ionizing radiation–induced G<sub>2</sub> checkpoint, human osteosarcoma cells entered mitosis with  $\gamma$ -H2AX foci, a marker for unrepaired DNA double-strand breaks. Exit from the G<sub>2</sub> checkpoint was accelerated by inhibiting the checkpoint kinase 1 (Chk1) and delayed by overexpressing wild-type Chk1 or depleting the Polo-like kinase 1 (Plk1). Chk1 and Plk1 controlled this process, at least partly, via independent signaling pathways. Our results suggest that human cells are able to exit the checkpoint arrest and divide before the damage has been fully repaired. Such cell division in the presence of damaged DNA may be detrimental for genetic stability and could potentially contribute to cancer development. (Cancer Res 2006; 66(21): 10253-7)**

## Introduction

In response to DNA damage, human cells activate the DNA damage–induced cell cycle checkpoints. These checkpoints help prevent proliferation of potentially genetically unstable cells and support DNA repair. However, a process of checkpoint adaptation was described in *Saccharomyces cerevisiae*, where following a prolonged checkpoint arrest cell division occurred despite the presence of irreparable DNA breaks (1). Cell division in the presence of unrepaired DNA lesions could potentially promote carcinogenesis and was first believed to occur only in single-cell organisms (reviewed in ref. 2). However, an analogous process of checkpoint adaptation was reported recently in *Xenopus* extracts, where entry into mitosis occurred in the presence of incompletely replicated DNA (3). An important unknown issue is whether checkpoint adaptation also exists in human cells (2).

In *S. cerevisiae*, checkpoint adaptation depends on the Polo-like kinase Cdc5, casein kinase II, phosphatases Ptc2 and Ptc3, and the helicase Srs2 (2, 3) and is accompanied by loss of Rad53p kinase activity and checkpoint kinase 1 (Chk1) phosphorylation (4). In *Xenopus* extracts, Polo kinase Plx1-mediated phosphorylation of the checkpoint mediator claspin causes release of claspin from the chromatin and thereby inactivation of the Chk1 kinase (3). In human cells, checkpoint adaptation has not been shown. Normal

recovery after completion of DNA repair in G<sub>2</sub>-arrested human cells requires the Cdc25B phosphatase and Polo-like kinase 1 (Plk1)-mediated degradation of the Wee1 kinase (5), events that increase the activity of cyclin B/cyclin-dependent kinase 1 (Cdk1), the main M phase–promoting kinase.

It has long been known that, in response to ionizing radiation (IR), human cancer cells often undergo one or several rounds of cell division before they die (6). We hypothesized that cell division after a lethal dose may occur in the presence of unrepaired DNA damage; therefore, checkpoint adaptation may exist in human cells. To investigate this issue, we have studied the IR-induced G<sub>2</sub> checkpoint in human U-2-OS osteosarcoma cells. We show that, following a sustained G<sub>2</sub> checkpoint arrest, U-2-OS cells adapt and divide with signs of unrepaired DNA breaks through a process regulated by the Plk1 and Chk1 kinases.

## Materials and Methods

**Cell lines and small interfering RNA treatment.** Human U-2-OS osteosarcoma cells were grown in DMEM with 10% fetal bovine serum. U-2-OS-VP16 cells expressing tetracycline-dependent Chk1-green fluorescent protein (GFP) protein (a gift from T. Schroeder, Danish Cancer Society, Copenhagen, Denmark) were grown with puromycin (5  $\mu$ g/ $\mu$ L), G418 (400  $\mu$ g/ $\mu$ L), and tetracycline (2  $\mu$ g/mL; Calbiochem, San Diego, CA). To induce expression of Chk1-GFP, cells were trypsinized, washed with PBS, and seeded without tetracycline. Chk1 and Plk1 small interfering RNAs (siRNA) were purchased from Dharmacon [Dharmacon SMARTpool M-003255-02 (Chk1) and M-003290-01 (Plk1); four siRNAs combined]. The Oligofectamine reagent (Invitrogen, Carlsbad, CA) was used for transfection.

**Drugs and irradiation.** UCN-01 was a gift from R.J. Schultz (Drug Synthesis and Chemical Branch, National Cancer Institute, Bethesda, MD) and was used at 300 nmol/L. Nocodazole (Sigma, St. Louis, MO) was used at 40 ng/mL. IR was delivered by X-ray generator (Pantak, Berkshire, United Kingdom; HF160; 150 kV, 15 mA, and dose rate of 2.18 Gy/min).

**Antibodies and immunocytochemistry.** Immunoblotting, immunoprecipitation, and *in vitro* kinase assays were described (7). Rabbit antibody to phosphorylated histone H3 (H3-p) and mouse antibody to phosphorylated H2AX ( $\gamma$ -H2AX) were from Upstate Biotechnology (Lake Placid, NY), phosphorylated Chk1 (Ser<sup>317</sup>, Ser<sup>345</sup>, and Ser<sup>296</sup>) and phosphorylated p53 (Ser<sup>15</sup>) antibodies were from Cell Signaling (Danvers, MA). Antibodies to Chk1 (DCS-310 and DCS-316), Mcm7 (DCS-141), Chk2 (DCS-270 and DCS-273), and Cdk7 (MO-1) have been described (7). Mouse antibody to Plk1 was from Zymed (Carlsbad, CA). Rabbit antibody to p53 and mouse antibody to cyclin B were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescence-conjugated secondary antibodies were from Molecular Probes (Carlsbad, CA).

**Flow cytometry.** To assay cell cycle distribution by DNA content, trypsinized cells were fixed in 70% ethanol, stained with 0.1 mg/mL propidium iodide (PI), and analyzed by a FACSCalibur flow cytometer (BD Biosciences, Stockholm, Sweden). Two-variable analysis to assay the number of mitotic cells was described (8).

**Immunofluorescence.** Cells cultured on glass coverslips were treated as indicated in the figure legends, fixed with 4% formaldehyde (12 minutes at

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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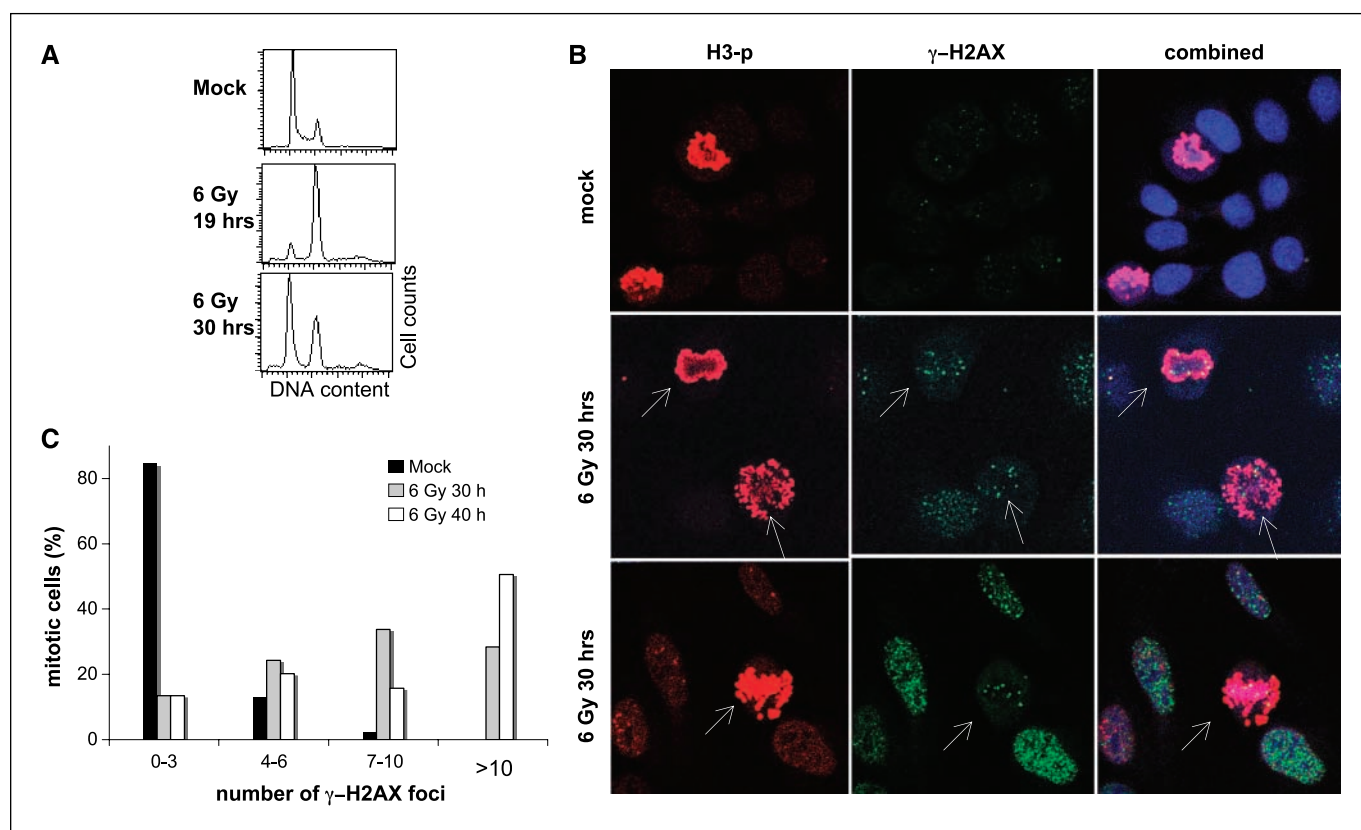
room temperature), permeabilized with PBS/0.25% Triton X-100 (5 minutes at room temperature), and stained with the antibodies indicated in the figure legends followed by DNA staining with Topro3 (1 minute, diluted 1:10,000; Molecular Probes) and mounting in Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Confocal images were obtained by a Zeiss (Carl Zeiss Microimaging Inc., Göttingen, Germany) 510 laser scanning microscope. Wide-field images were obtained by using a Zeiss Axioplan Widefield microscope coupled to a Coolsnap camera and operated by Metamorph software.

## Results and Discussion

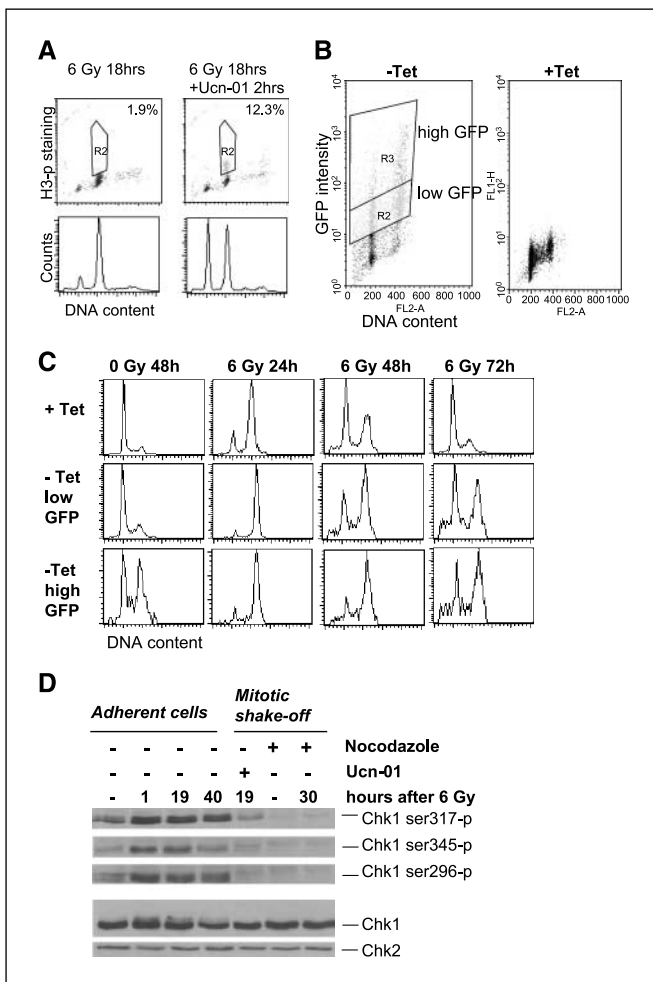
To address whether checkpoint adaptation exists in human cells, we assayed the IR-induced  $G_2$  checkpoint in U-2-OS cells. After exposure to 6 Gy, U-2-OS cells first entered a  $G_2$  checkpoint arrest lasting until ~20 hours. However, in later stages after irradiation, cells began to divide (Fig. 1A). At this dose, the clonogenic survival is only ~4% and defective chromosome segregation occurs in mitosis (8), indicating that many of the dividing cells may contain damaged DNA. To test this prediction, the dividing cells were stained with  $\gamma$ -H2AX, a commonly used marker of DNA double-strand breaks (9, 10). In response to 6 Gy, a rapid induction of  $\gamma$ -H2AX foci occurred within 30 minutes, which decreased over the next 48 hours (Supplementary Fig. S1). Despite this gradual decrease indicating the ongoing DNA repair, mitotic cells examined at 30 and 40 hours after 6 Gy contained significantly more  $\gamma$ -H2AX foci than nonirradiated mitotic cells (Fig. 1B and C). Together,

these results indicate that a process of checkpoint adaptation occurs in the U-2-OS cells.

A key regulator of  $G_2$  checkpoint is Chk1, a protein kinase that controls the activity of the M phase-promoting cyclin B/Cdk1 complex via phosphorylation of the Cdc25 phosphatases (11). To address whether inhibition of Chk1 may be involved in the adaptation process, we first applied chemical inhibitors and Chk1 siRNA to inhibit Chk1 at late times during the  $G_2$  checkpoint arrest. Two distinct Chk1 inhibitors UCN-01 (300 nmol/L) and Cep-3891 (500 nmol/L; ref. 7) caused termination of the  $G_2$  checkpoint when added at 16 hours after irradiation (Fig. 2A; data not shown). Furthermore, transfection with Chk1 siRNA at 12 hours after irradiation accelerated the termination of the checkpoint (Supplementary Fig. S2). We next analyzed U-2-OS cells expressing an inducible GFP-Chk1 fusion protein to test if elevated Chk1 activity would prolong the  $G_2$  checkpoint. The Chk1 kinase activity was increased ~5-fold at 24 hours after induction of GFP-Chk1 expression (Supplementary Fig. S3). Importantly, increase of GFP-Chk1 activity caused a prolonged IR-induced  $G_2$  checkpoint in a dose-dependent fashion (Fig. 2B and C). Cells that expressed higher amount of GFP-Chk1 showed longer  $G_2$  checkpoint arrest, and cells expressing the highest levels of GFP-Chk1 also arrested in  $G_2$  in the absence of IR (Fig. 2C). As many GFP-Chk1-expressing mitotic cells contained between 4 and 15  $\gamma$ -H2AX foci at 50 hours after 6 Gy (data not shown), adaptation was delayed, but not prevented, by Chk1 overexpression in these cells.



**Figure 1.** Following a prolonged IR-induced  $G_2$  checkpoint, U-2-OS cells divide with signs of unrepaired DNA breaks. **A**, U-2-OS cells divide following a sustained IR-induced  $G_2$  checkpoint. DNA histograms are shown at 0, 19, and 30 hours after IR (6 Gy). **B**, the mitotic cells that appear following the IR-induced  $G_2$  checkpoint contain an increased number of  $\gamma$ -H2AX foci. Immunofluorescence of U-2-OS cells stained with antibodies to  $\gamma$ -H2AX and H3-p and the DNA stain Topro3 is shown in nonirradiated cells (mock) and at 30 hours after 6 Gy. **Arrows**, mitotic cells with  $\gamma$ -H2AX foci after IR. Confocal images. **C**, quantification of  $\gamma$ -H2AX foci in mitotic cells after IR. U-2-OS cells were stained as in (B) at 30 and 40 hours after IR (6 Gy) or after mock treatment, and mitotic cells were scored for the number of  $\gamma$ -H2AX foci. About 100 mitotic cells were scored for each treatment. Results are pooled from two independent experiments.



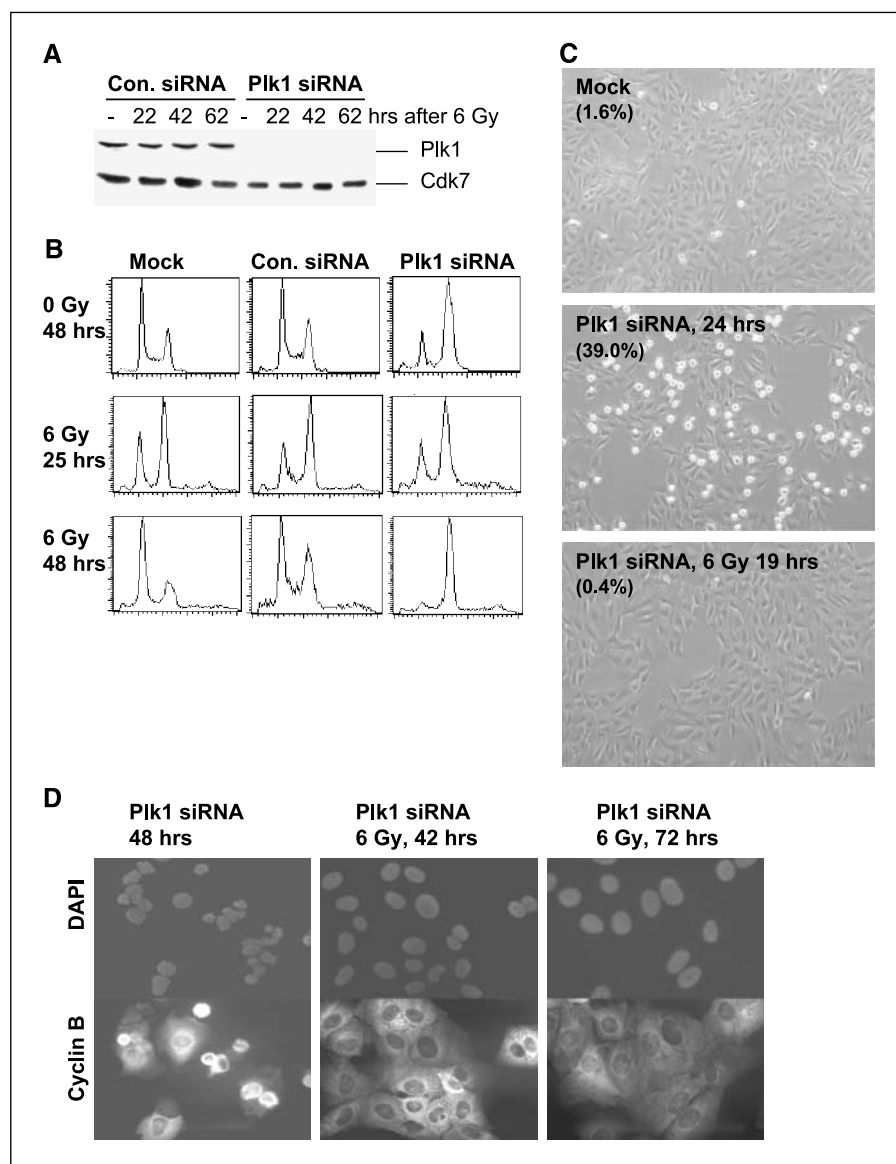
**Figure 2.** Exit from the prolonged IR-induced  $G_2$  checkpoint depends on the Chk1 kinase. **A**, Chk1 inhibition causes termination of the IR-induced  $G_2$  checkpoint. Flow cytometry analysis of U-2-OS cells stained with H3-p antibody and PI is shown at 18 hours after IR (6 Gy) or at 18 hours after IR (6 Gy) with UCN-01 (300 nmol/L) present in the cell culture medium for the last 2 hours. Numbers are the percentages of H3-p-positive cells. **B**, flow cytometry analysis of U-2-OS-VP16 cells expressing tetracycline (*Tet*)-dependent Chk1-GFP protein. Cells were incubated with (+) or without (-) tetracycline and stained with PI. *Left*, cells were gated for low and high expression of Chk1-GFP protein. **C**, overexpression of Chk1 kinase delays the termination of the IR-induced  $G_2$  checkpoint in a Chk1 dose-dependent manner. U-2-OS cells expressing tetracycline-dependent Chk1-GFP protein were seeded with tetracycline-negative or tetracycline-positive medium, irradiated (0 and 6 Gy), and analyzed by flow cytometry at 24, 48, and 72 hours. For the tetracycline-negative cells, DNA histograms are shown for cells expressing low or high amounts of GFP-Chk1 as described in (**B**). **D**, Chk1 phosphorylation is decreased in mitotic cells. Extracts from exponentially growing or mitotic U-2-OS cells were prepared and processed for Western blotting. Extracts from exponentially growing cells were prepared after mock treatment or at 1, 19, or 40 hours after IR (6 Gy; *lanes 1-4*). Extracts from mitotic cells were prepared after mitotic shake-off at 19 hours after IR with UCN-01 (300 nmol/L) present in the cell culture medium for the last 2 hours (*lane 5*), at 10 hours after addition of nocodazole (*lane 6*), or at 30 hours after IR with nocodazole present for the last 10 hours (*lane 7*). Chk2 is included as a loading marker.

In response to DNA damage, the activity of Chk1 kinase is modulated via ATR-mediated phosphorylations of Chk1 on Ser<sup>317</sup> and Ser<sup>345</sup> (11). Chk1 is also phosphorylated on Ser<sup>296</sup>, a likely Chk1 autophosphorylation site (12). We found that the elevated phosphorylation of Ser<sup>317</sup>, Ser<sup>345</sup>, and Ser<sup>296</sup> was maintained at late times of the  $G_2$  checkpoint arrest (Fig. 2D, *lanes 3 and 4*), indicating that the IR-induced Chk1 activity remains high as long as the cells

remain arrested in  $G_2$ . In contrast, these phosphorylations were diminished in cells that had undergone the checkpoint adaptation, as measured by Western blotting of mitotic extracts made by shake-off of the mitotic cells after treatment with 6 Gy and nocodazole (Fig. 2D, *lane 7* compared with *lanes 3 and 4*). Phosphorylation of Chk1 was also diminished when the  $G_2$ -arrested cells were forced to enter mitosis by addition of UCN-01 (Fig. 2D, *lane 5*). Because UCN-01 does not inhibit these phosphorylations in S-phase cells (13), dephosphorylation of Chk1 seems to be a general consequence of mitotic entry. Taken together, these results indicate that the mitotic entry after IR is associated with dephosphorylation and presumably inactivation of Chk1. Dephosphorylation of Chk1 may occur in late  $G_2$  or early M phase during the adaptation process.

The mechanisms of checkpoint adaptation in the *Xenopus* model involve Plk1-mediated inactivation of Chk1 (3). Furthermore, caffeine-mediated release from doxorubicin-induced  $G_2$  checkpoint requires Plk1 kinase activity (5). To address whether Plk1 is involved also in the process of adaptation to the IR-induced  $G_2$  checkpoint, we reduced Plk1 levels by siRNA (Fig. 3A). Cells depleted for Plk1 accumulated with  $G_2$ -M DNA content regardless of IR (Fig. 3B). However, in the absence of IR, Plk1-depleted cells accumulated as round mitotic cells, in contrast to the cells arrested in  $G_2$  after 6 Gy that remained flat (Fig. 3C). These results are consistent with previous reports showing that Plk1 is essential for mitotic exit but does not block mitotic entry in the absence of DNA-damaging agents (5). Because cyclin B translocates from the cytoplasm to the nucleus in early M phase, we also applied immunostaining for cyclin B to distinguish between  $G_2$ - and M-phase cells (Fig. 3D). In the absence of IR, many Plk1-depleted cells showed nuclear cyclin B staining (Fig. 3D, *left*), typical for mitotic cells. After exposure to IR, Plk1-depleted cells maintained cytoplasmic cyclin B for at least 72 hours (Fig. 3D), indicating that Plk1 is required for spontaneous mitotic entry following prolonged IR-induced  $G_2$  arrest. Thus, consistent with the data from yeast and *Xenopus*, Plk1 seems to play a critical role in checkpoint adaptation after IR in human cells.

Having established that both Plk1 and Chk1 control the checkpoint adaptation, we asked whether Chk1 inhibition could cause mitotic entry of Plk1-depleted cells after IR. When UCN-01 was added to Plk1-depleted cells at 48 hours after IR, a proportion of the cells underwent chromatin condensation and nuclear envelope breakdown resulting in nuclear cyclin B staining (Fig. 4A). These data indicate that Chk1 inactivation may be an event downstream of Plk1 in a linear pathway as suggested in *Xenopus*, or alternatively, Chk1 and Plk1 may act via independent signaling to control mitotic entry. To help discriminate between these possibilities, we compared the kinetics of mitotic entry in response to Chk1 inhibition of  $G_2$ -arrested cells transfected with control or Plk1 siRNA (Fig. 4B). Addition of UCN-01 for the last 2 or 4 hours before harvesting cells at 18 hours after 6 Gy caused significantly less mitotic entry of Plk1-depleted cells compared with cells transfected with the control siRNA (Fig. 4B). Because the kinetics of mitotic entry would have been expected to be similar if Chk1 and Plk1 acted solely in a linear pathway, these results indicate that Plk1 and Chk1 act, at least partly, via independent signaling to control checkpoint adaptation in response to IR (Fig. 4C). Plk1 likely acts on other targets than Chk1, such as Wee1 (5) or Emi1 (14). In addition, Plk1 may also regulate Chk1 inactivation during the adaptation process. This could occur by several mechanisms, including activation of phosphatases to dephosphorylate Chk1 (15), inhibition of checkpoint proteins, such



**Figure 3.** Adaptation to the IR-induced G<sub>2</sub> checkpoint depends on the Plk1 kinase. *A*, depletion of Plk1 protein after siRNA transfection. U-2-OS cells transfected with control or Plk1 siRNA were either left nonirradiated and processed for Western blotting at 48 hours after transfection (*lanes 1 and 5*) or treated with IR (6 Gy) at 5 hours after transfection and processed at 22, 42, and 62 hours after IR (*lanes 2-4 and lanes 6-8*). Cdk7 protein is included as a loading marker. *B*, DNA histograms of U-2-OS cells after depletion of Plk1. DNA histograms are shown for nontreated U-2-OS cells (mock) or U-2-OS cells transfected with control or Plk1 siRNA at 48 hours after transfection (*top*) or U-2-OS cells treated with IR (6 Gy) at 5 hours after transfection and harvested at 25 and 48 hours after IR (*bottom*). *C*, phase-contrast images are shown for nontreated U-2-OS cells (mock), U-2-OS cells transfected with Plk1 siRNA at 24 hours after transfection (Plk1 siRNA 24 hours), and U-2-OS cells treated with IR (6 Gy) at 5 hours after transfection and harvested at 19 hours after IR (Plk1 siRNA 6Gy, 19 hours). Numbers are the percentages of H3-p-positive cells as determined by flow cytometry analysis of the same experiment. *D*, immunofluorescence of U-2-OS cells, transfected with Plk1 siRNA, irradiated as in (*C*), and stained with an antibody to cyclin B, and the DNA stain DAPI are shown at 48 hours after transfection (0 Gy) and at 42 and 72 hours after IR.

as claspin, Brca1, Atrp, or Rpa (3, 16), or by direct phosphorylation. The latter scenario seems unlikely, as Chk1 is not a good substrate for Plk1 kinase *in vitro* (data not shown).

A hallmark of IR-treated cells is chromosome aberrations at mitosis, which depends on radiation dose and correlates with cell killing (6). The presence of mitotic cells with such aberrations after IR, particularly acentric fragments, is consistent with checkpoint adaptation, as progression into mitosis occurred in the presence of DNA damage. Thus, checkpoint adaptation is likely not restricted to high radiation doses. Consistent with this notion, ~50% of U-2-OS cells contained more than 4  $\gamma$ -H2AX foci at 24 hours after 2 Gy (data not shown).

In conclusion, our data indicate that following a prolonged DNA damage-induced checkpoint, human cells can divide despite the presence of unrepaired DNA damage (shown in Fig. 4C). To our knowledge, this is the first demonstration of checkpoint adaptation in human cells. Although checkpoint adaptation may facilitate elimination of cells with excessive DNA damage by allowing progression through mitosis, there is a risk that some cells carrying low

amounts of DNA damage might survive. Allowing division of cells with damaged DNA clearly increases the risk for development of genetically unstable cells that could progress toward cancer. We propose that checkpoint adaptation in human cells may potentially contribute to cancer development (Fig. 4C). Consistent with our model, Plk1 is commonly overexpressed in human tumors (17), and mutations of Chk1 were detected in several human cancers (18). Based on our results, both of these events would be predicted to enhance checkpoint adaptation. Further research in this area will reveal more exactly the consequences of checkpoint adaptation in human cells.

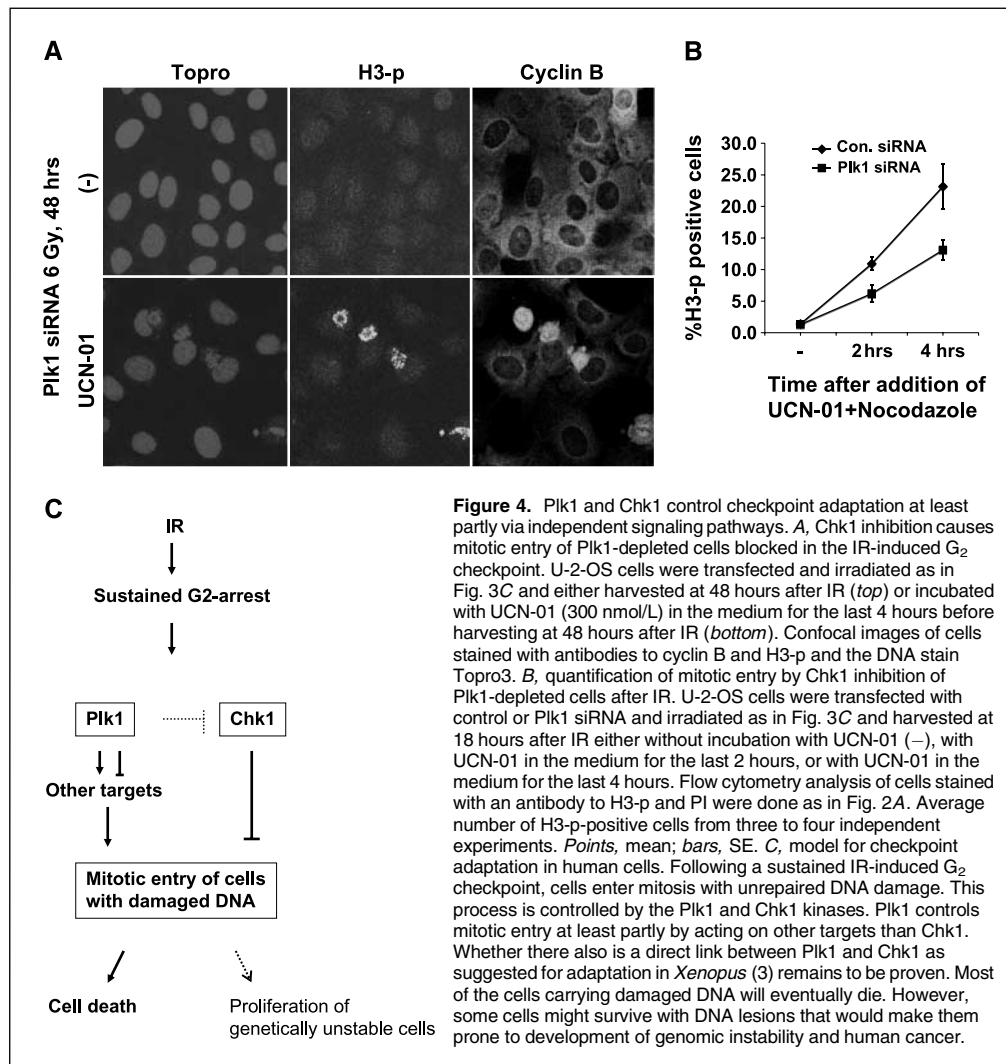
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**Figure 4.** Plk1 and Chk1 control checkpoint adaptation at least partly via independent signaling pathways. **A**, Chk1 inhibition causes mitotic entry of Plk1-depleted cells blocked in the IR-induced G<sub>2</sub> checkpoint. U-2-OS cells were transfected and irradiated as in Fig. 3C and either harvested at 48 hours after IR (*top*) or incubated with UCN-01 (300 nmol/L) in the medium for the last 4 hours before harvesting at 48 hours after IR (*bottom*). Confocal images of cells stained with antibodies to cyclin B and H3-p and the DNA stain Topro3. **B**, quantification of mitotic entry by Chk1 inhibition of Plk1-depleted cells after IR. U-2-OS cells were transfected with control or Plk1 siRNA and irradiated as in Fig. 3C and harvested at 18 hours after IR either without incubation with UCN-01 (–), with UCN-01 in the medium for the last 2 hours, or with UCN-01 in the medium for the last 4 hours. Flow cytometry analysis of cells stained with an antibody to H3-p and PI were done as in Fig. 2A. Average number of H3-p-positive cells from three to four independent experiments. *Points*, mean; *bars*, SE. **C**, model for checkpoint adaptation in human cells. Following a sustained IR-induced G<sub>2</sub> checkpoint, cells enter mitosis with unrepaired DNA damage. This process is controlled by the Plk1 and Chk1 kinases. Plk1 controls mitotic entry at least partly by acting on other targets than Chk1. Whether there also is a direct link between Plk1 and Chk1 as suggested for adaptation in *Xenopus* (3) remains to be proven. Most of the cells carrying damaged DNA will eventually die. However, some cells might survive with DNA lesions that would make them prone to development of genomic instability and human cancer.

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