

Report

Radiosensitization by Chir-124, a selective CHK1 inhibitor

Effects of p53 and cell cycle checkpoints

Yungan Tao,^{1,3} Céline Leteur,^{1,3} Ceyao Yang,^{1,2} Ping Zhang,¹ Maria Castedo,^{3,4} Alain Pierré,⁵ Roy M. Golsteyn,⁶ Jean Bourhis,^{1,3} Guido Kroemer^{3,4,*} and Eric Deutsch^{1,3,*}

¹UPRES EA27-10 Laboratoire Radiosensibilité des Tumeurs et Tissus Sains; ²University Paris-Sud XI Kremlin-Bicêtre; ³Institut Gustave-Roussy; ⁴U848 INSERM; Villejuif; ⁵Research Centre; Servier Laboratory; Croissy-sur-Seine, France; ⁶University of Lethbridge; Alberta, Canada

Key words: CHK1, radiation, G₂-M checkpoint, p53

Checkpoint kinase-1 (CHK1) is a key regulator of the DNA damage-elicited G₂-M checkpoints. The aim of the present study was to investigate the effects of a selective CHK1 inhibitor, Chir124, on cell survival and cell cycle progression following ionizing radiation (IR). Treatment with Chir-124 resulted in reduced clonogenic survival and abrogated the IR-induced G₂-M arrest in a panel of isogenic HCT116 cell lines after IR. This radiosensitizing effect was relatively similar between p53^{-/-} and p53-sufficient wild type (WT) HCT116 cells. However, the number of mitotic cells (as measured by assessing the phosphorylation of mitotic proteins) increased dramatically in p53^{-/-} HCT116 cells after concomitant Chir-124 exposure, compared to IR alone, while no such effect was observed in p53-sufficient WT HCT116 cells. In p53^{-/-} cells, Chir-124 treatment induced a marked accumulation of polyploid cells that were characterized by micronucleation or multinucleation. p21^{-/-} HCT116 cells displayed a similar pattern of response as p53^{-/-} cells. Chir-124 was able to radiosensitize HCT116 cells that lack checkpoint kinase-2 (CHK2) or that were deficient for the spindle checkpoint protein Mad2. Finally, Chir-124 could radiosensitize tetraploid cell lines, which were relatively resistant against DNA damaging agents. Altogether these results suggest that Chir-124-mediated radiosensitization is profoundly influenced by the p53 and cell cycle checkpoint system.

Introduction

Cells commonly respond to DNA-damaging agents by activating cell cycle checkpoints.¹ These checkpoints provide a controlled temporary arrest at a specific stage of the cell cycle

to allow the cell to correct possible defects. Two prominent checkpoints control the G₁-S and G₂-M transitions.² The G₂-M checkpoint prevents the initiation of mitosis in cells that have suffered DNA damage during G₂, or that have progressed into G₂ in spite of some unrepaired damage that has been inflicted during previous S or G₁ phases.³

The critical target of the G₂ checkpoint is the mitosis-promoting activity of the cyclin B/CDK1 kinase.⁴ Initiation of mitosis is marked by an abrupt activation of CDK1 kinase activity. CDK1 activation requires binding to its positive regulatory subunit, cyclin B. Cyclin B/CDK1 complexes are held inactive by the phosphorylation of two residues located within the ATP binding loop (Thr14 and Tyr15 of CDK1) by the Wee1,⁵ and Myt1 kinases.⁶ Checkpoint kinase 1 (CHK1) is a major checkpoint kinase in mammals.⁷ Upon DNA damage, CHK1 is activated by ATM/ATR-mediated phosphorylation and then phosphorylates Cdc25 phosphatases, thereby preventing the activation of CDK1 kinase. Once activated, CHK1 delays the onset of mitosis by modulating the activity of these pathways at multiple levels.⁸ CHK1 phosphorylates both Cdc25A and Cdc25C, which facilitates their binding to 14-3-3 binding, and hence inhibits their activating interaction with the cyclin B/CDK1 complex.^{9,10} In addition, CHK1 phosphorylates the Wee1 kinase, resulting in the inhibition of CDK1.¹¹ These modifications interrupt the positive feedback loop leading to CDK1 activation, and thus delay the entry into mitosis.⁸

During the interphase of the cell cycle, a subset of CHK1 proteins localizes to centrosomes where CHK1 prevents premature activation of cyclin B/CDK1 through local inhibition of Cdc25B.¹² CHK1 phosphorylates Cdc25B at multiple sites during unperturbed cell cycle progression.¹³ Although active cyclin B/CDK1 complexes appear first at the centrosome, subsequent activation of the more abundant nuclear pool of cyclin B/CDK1 by Cdc25A and Cdc25C is essential for mitosis, and again CHK1 appears to play an important regulatory role.¹⁴ A recent report has identified a functional requirement for CHK1 in the regulation of the spindle assembly checkpoint (SAC). CHK1-deficient cells manifest a decreased Aurora-B activity and impaired kinetochore

*Correspondence to: Guido Kroemer; INSERM; U848; Institut Gustave Roussy, PR1; 39, rue Camille Desmoulins; Villejuif F-94805 France; Tel.: 33.1.42.11.60.46; Email: kroemer@igr.fr/ Eric Deutsch; UPRES EA27-10 Laboratoire Radiosensibilité des Tumeurs et Tissus Sains; Institut Gustave-Roussy; 39, rue Camille-Desmoulins; Villejuif 94805 France; Tel.: 33.1.42.11.52.96; Email: deutsch@igr.fr

Submitted: 02/12/09; Accepted: 02/16/09

Previously published online as a *Cell Cycle* E-publication:
<http://www.landesbioscience.com/journals/cc/article/8203>

localization of BubR1, both of which are restored by reintroduction of wild type but not kinase-dead CHK1.¹⁵

The p53 tumor suppressor mediates DNA damage-induced G₁ arrest, in part through the activation of p21, a CDK inhibitor.¹⁶ While normal cells mainly arrest in G₁ in response to genotoxic stress, p53-deficient tumor cells are defective in the G₁ checkpoint and therefore rely more heavily on the S or G₂ checkpoints to repair their damaged DNA than normal cells.¹ It has been proposed that specific abrogation of the S and G₂ checkpoints would still permit normal cells to arrest in the G₁ phase and to repair any adventitious DNA damage. In contrast, tumor cells that lack the G₁ checkpoint would undergo mitotic catastrophe and eventually die when the S and G₂ checkpoints are perturbed.¹⁷ Thus, CHK1 has been proposed as an attractive target to potentiate the effects of chemotherapeutics in p53-deficient tumors.¹⁸

CHK1 siRNA enhances DNA damage-induced apoptosis in p53-deficient cancer cell lines and augments growth inhibition by different DNA-damaging agents.¹⁹ One Chk1 inhibitor, CEP 3891 has previously been shown to increase cytotoxicity after irradiation *in vitro*²⁰ by increasing the number of cells that enter mitotic catastrophe, a marker for CHK1 inhibition. A role for CHK1 in radiation resistance of cancer stem cells has recently been proposed.²¹ To validate CHK1 as a target for cancer therapy, we decided to investigate whether CHK1 inhibitors can specifically sensitize p53-deficient tumors to radiotherapy yet avoid an increase in the toxicity of irradiation for normal, p53-proficient cells. To this aim, we took advantage of Chir-124, a potent and selective small molecule CHK1 inhibitor²² and evaluated its radiosensitizing effect on a panel of isogenic HCT116 colon cancer cell lines that are proficient or deficient for p53, p21, CHK2 or the essential SAC protein MAD2.

Results

The p53 status influences cell cycle changes after inhibition of CHK1 by Chir-124 and IR. We first evaluated the effects of the CHK1 inhibitor Chir-124 on the advancement of the cell cycle in p53^{-/-} or wild type (WT) HCT116 cell lines. Few cell cycle changes were observed by 24 h after treatment at various doses of Chir-124 alone: 100 nM, 250 nM (Fig. 1A) or 500 nM (data not shown). More cells accumulated in the G₂-M phase in the p53^{-/-} cell line (without a functional G₁ checkpoint) than in the WT HCT116 cells, in accord with published data.²³ However, fewer cells in G₂-M were found 24 h after IR (10 Gy) in the presence of Chir-124, indicating that Chir-124 attenuated the IR induced G₂-M block in p53^{-/-} or WT HCT116 cells. When the two HCT116 cell lines were compared, we observed a similar increase of sub-G₁ (bona fide apoptotic) cells after Chir-124 and IR combinatorial treatment as with IR or Chir-124 alone (Fig. 1C). Moreover, we noted a marked accumulation of polyploid cells following the combined action of Chir-124 and IR in p53^{-/-} HCT116 cells. In contrast, no significant accumulation of >4N cells was observed in the WT cell line. Next, we treated the p53^{-/-} HCT116 cell line with 250 nM Chir-124 for 1 h followed by a single dose IR (10 Gy) and kinetic cell cycle analyses (Fig. 1B). A clear augmentation in sub-G₁ cells was observed starting from 8 h after IR, while

polyploid cells increased at 24 h post-IR. These data suggest an enhancement of radiation-induced cell killing and polyploidization after treatment with Chir-124 in p53^{-/-} HCT116 cells.

CHK1 activation after irradiation *in vitro* and *in vivo*. Total CHK1 expression and its activating phosphorylation on serine 345 (CHK1-Pho) was monitored at different times, either in WT HCT116 and p53^{-/-} HCT116 that were irradiated *in vitro* (Fig. 2A) or in p53^{-/-} HCT116 tumour xenografts that were irradiated *in vivo* (Fig. 2B). We found that irradiation *in vitro* (10 Gy) and *in vivo* (10 Gy), the phosphorylation of CHK1 increased near-to-immediately, as rapidly as 15 min or 30 min post-IR and remained detectable for at least 24 h. In contrast, the levels of total CHK1 protein remained relatively stable throughout the post-IR phase. We did not observe any significant differences in the patterns of CHK1 phosphorylation between p53^{-/-} and WT cells.

p53-independent effects of Chir-124 on clonogenic survival after IR. Clonogenic survival assays, which constitute the gold standard for the evaluation of *in vitro* radiation response,²⁴ were used to test the survival of cells treated with Chir-124 plus IR. We found that treatment of HCT116 cells with Chir-124 (cells were exposed to Chir-124 for 24 h and then transferred into fresh medium) reduced clonogenic survival following IR in p53^{-/-} HCT116 cells (SF2 = 37.4% for control versus 27.7% for Chir-124, respectively, with a dose enhancement ratio DER 1.35) as well as in WT HCT116 cell lines (SF2 = 33.8% versus 25.6% for Chir-124, respectively, with DER 1.32) (Fig. 3A). Hence the clonogenic survival was similarly compromised in p53-negative and p53-expressing cells and the radiosensitizing effect of Chir-124 was little influenced by the p53 status. We also found that the deletion of p21 moderately affected the radiosensitivity of HCT116 cells and slightly modulated the radiosensitizing function of Chir-124 (Fig. 3A).

Differential effects of Chir-124 in p53^{-/-} and WT HCT116 cells on the G₂ checkpoint. The immunofluorescence detection of the MPM2 epitope, which is a marker of mitosis,²⁵ was used to quantify the mitotic index by cytofluorometry. Exposure of p53^{-/-}, and WT HCT116 cells to 10 Gy IR resulted in G₂ arrest and hence reduced the frequency of MPM2-positive cells (Fig. 3C). This effect was found in WT, p53^{-/-} and p21^{-/-} HCT 116 cells (Fig. 3C). p21^{-/-} HCT 116 cells generally showed a similar pattern in the modification of their cell cycle after irradiation ± Chir-124 as did p53^{-/-} cells (Fig. 3B). When added to p53^{-/-} cells, Chir-124 treatment avoided the IR-induced G₂ arrest and actually increased the frequency of cells expressing MPM2, indicating that it abolishes the G₂ arrest and triggers entry into (premature) mitosis (Fig. 3C). In contrast, we observed a clear diminution of the mitotic index after irradiation in the p53-proficient wild type cell line, be it in the presence or absence of Chir-124. This suggests that only the simultaneous inhibition of CHK1 and p53 can abolish the irradiation-induced G₂ arrest.

We monitored the time-dependent change in the mitotic index of p53^{-/-} and WT HCT116 cells after treatment with Chir-124 ± IR (Fig. 4A). In the absence of Chir-124, mitotic cells reduced progressively from 4 h to 8 h post-IR in p53^{-/-} and WT cells, and then increased from 24 h post-IR in p53^{-/-} cell line but not in WT

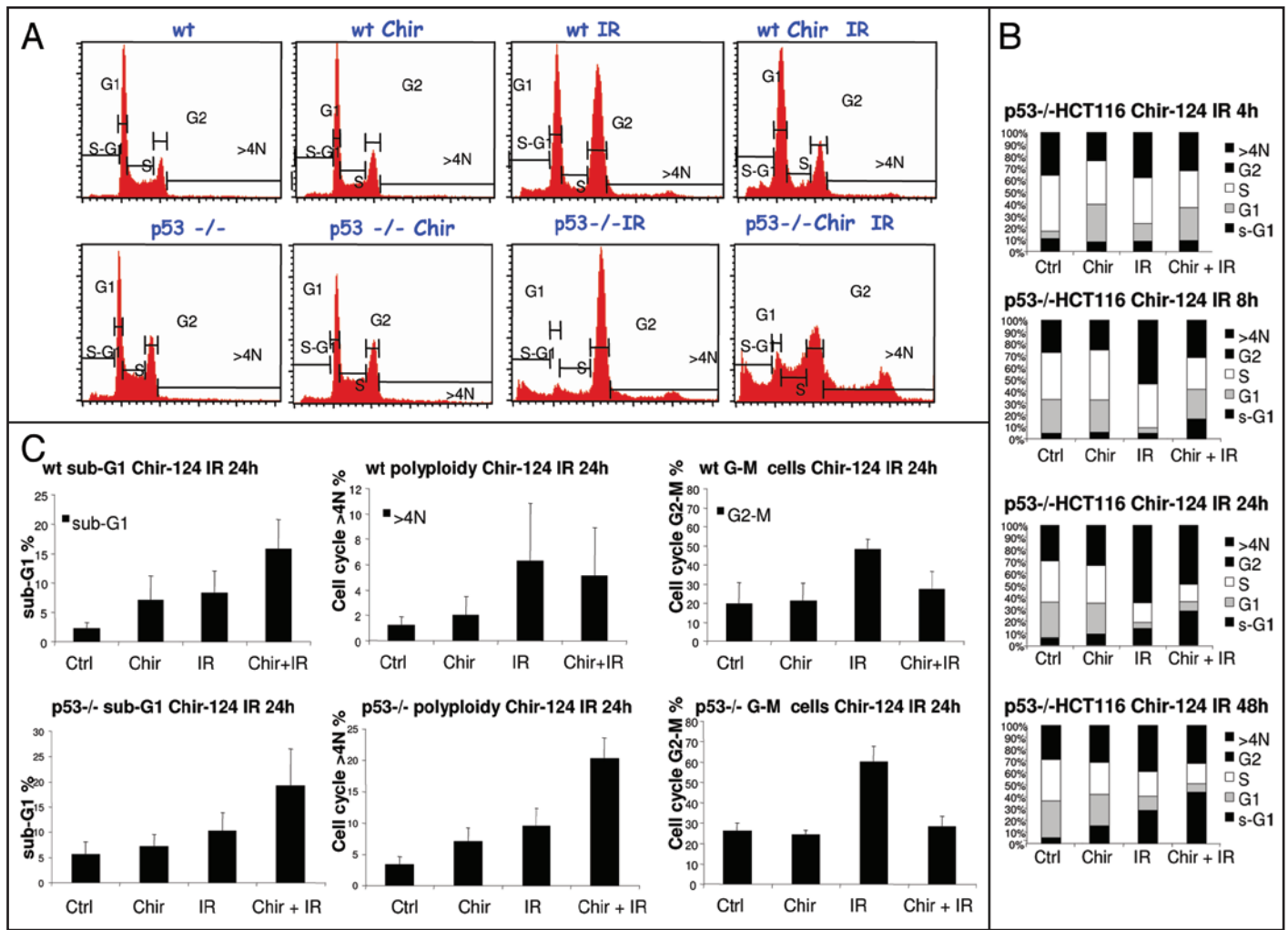


Figure 1. Cell cycle analysis in $p53^{-/-}$ and wild type (WT) HCT116 cells after treatment with 250 nM Chir-124 and 10 Gy irradiation. (A) Cytofluorometric analysis of different cell cycle sub-populations in the following conditions: 24 h after IR alone (10 Gy), 25 h after Chir-124 alone (Chir) or combination of both (Chir 10 Gy: 1 h Chir-124 followed by IR 10 Gy with a further 24 h exposure) or no treatment at all (control). Representative FACS pictograms of three independent experiments are shown. (B) Distribution of the cell cycle in $p53^{-/-}$ HCT116 cells at different time point (4 h, 8 h, 24 h, 48 h) after treatment with IR \pm Chir-124. (C) Quantification of the data obtained in (A). Error bar represents SEM of three independent experiments.

cell line. Nonetheless, in the presence of Chir-124, mitotic $p53^{-/-}$ cells increased dramatically from 4 h to 24 h, yet were not (or only slightly) affected in WT HCT116 cells (Fig. 4A).

Next, we assessed whether cells entered mitotic catastrophe in the $p53^{-/-}$ and WT HCT116 cell lines after combined IR and CHK1 inhibition. Mitotic catastrophe was identified by fluorescence microscopy as micro- or multinucleation, after staining of cells with the chromatin dye Hoechst 33324. We found that after IR, micronuclei or multinucleated cells increased dramatically in all groups. A higher percentage of cells with micronuclei or multinucleation was detected when the cells were pretreated by Chir-124, and this effect was more pronounced in $p53^{-/-}$ HCT116 cells than in WT control cells (Fig. 4D).

Chir-124 does not affect DNA damage foci in WT and $p53^{-/-}$ HCT116. We evaluated DNA damage by staining permeabilized cells with an antibody specific for a phospho-neoepitope that arises in histone H2AX as a result of its phosphorylation by

ATM. Immunofluorescence staining of such γ H2AX foci followed by cytometric analysis revealed a time-dependent DNA damage response. The intensity of γ H2AX increased 20 min after IR and then decreased progressively to nearly normal levels at 24 h. The kinetic variation of γ H2AX was not affected by pretreatment with Chir-124, be it in WT HCT116 or in $p53^{-/-}$ cells (Fig. 4C). This suggests that DNA damage repair after IR is probably not influenced by Chir-124 (Fig. 4B and C).

Role of CHK2 and 14-3-3 in the radiosensitivity induced by Chir-124. Chir-124 does not inhibit CHK2 at the concentration used in our experiments.²² We explored the role of CHK2, as well as that of 14-3-3 on radiation responses after CHK1 inhibition (Fig. 5). Chir-124 markedly enhanced the frequency of apoptotic (sub-G₁) found in irradiated CHK2^{-/-} HCT116 cultures (Fig. 5A) and reduced the clonogenic survival in CHK2^{-/-} HCT116 cells (Fig. 5C and Table 1). 14-3-3 σ normally sequesters cyclin B/CDK1 complexes in the cytoplasm during the G₂ arrest, meaning that its

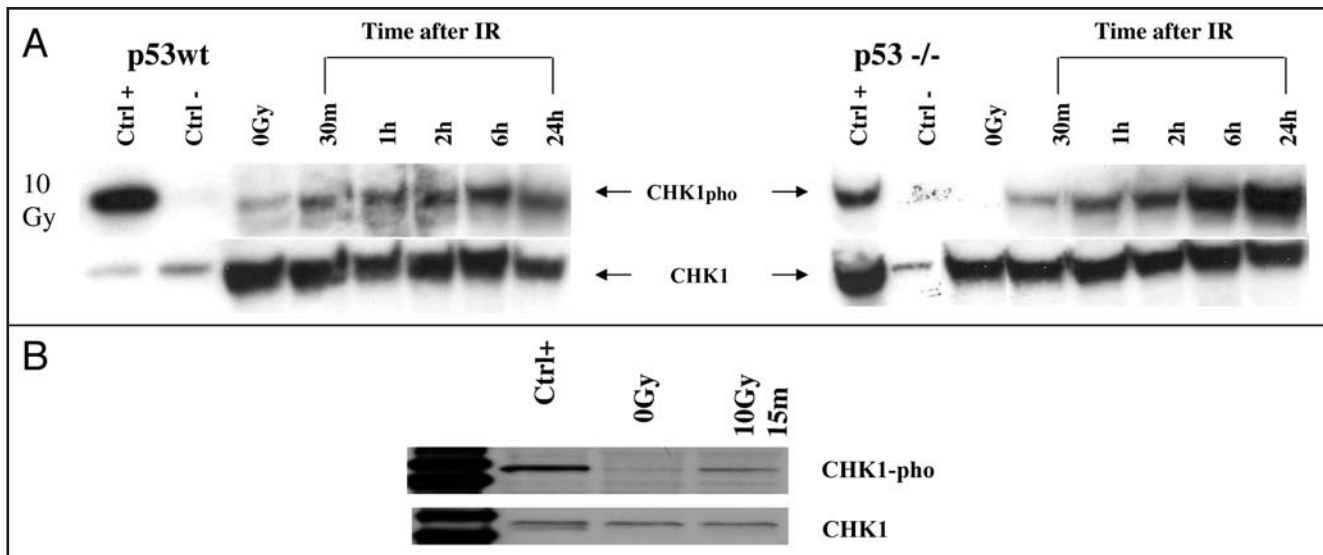


Figure 2. (A) Immunoblot detection of phospho-CHK1 (on serine 345) after irradiation in WT and p53^{-/-} cells. (A) Cells were treated with 10 Gy irradiation, cultured *in vitro*, recovered in lysis buffer after the indicated post-IR interval, and subjected to immunoblot detection of phospho-CHK1, CHK1 protein irrespective of its phosphorylation status as a loading control. For comparison, a sample from an HT29 xenograft treated with 20 mg/kg CPT-11 by *i.v.* is shown as positive control (Ctrl⁺). (B) *In vivo* induction of CHK1 phosphorylation by radiotherapy. p53^{-/-} HCT116 tumour xenografts were treated by irradiation (10 Gy) and recovered at 15 min post-IR. Protein extracts were prepared and analyzed, with the same positive control as in (A).

absence allows cyclin B/CDK1 complexes to enter the nucleus and to catalyze a premature entry into mitosis.²⁶ The number of 14-3-3^{-/-} cells in the G₂-M phase after irradiation was reduced as compared with wild type cells (30% for 14-3-3^{-/-} HCT116 vs. 50% for WT HCT116) (compare Figs. 5B with 1C). Combined treatment with Chir-124 and IR cause a minor (non-significant) increase in the sub-G₁ population of 14-3-3^{-/-} HCT116 cells as compared to Chir-124 or IR alone. No significant polyploidy was observed in 14-3-3^{-/-} HCT116 cells after of the combined treatment with Chir-124 and IR. Likewise, clonogenic survival assays revealed that Chir-124 lost its radiosensitizing effect in 14-3-3^{-/-} cells (Table 1). Thus, because the G₂-M arrest has already been abrogated in 14-3-3^{-/-} cells (Chan et al. 1999), there is no further radiosensitizing effect by CHK1 inhibition. This kind of epistatic analysis hence suggests that the inhibition of CHK1 by Chir-124 exerts its radiosensitizing effect by a direct effect on cell cycle regulation.

Spindle assembly checkpoint protein Mad2 in CHK1 inhibition induced radiosensitivity. Heterozygous deletion of Mad2 (Mad2^{+/-}) in HCT116 cell line results in a partially downregulated Mad2 spindle checkpoint protein level and causes an impairment of the SAC.²⁷ We speculated that the simultaneous impairment of two checkpoints (SAC and G₂-M checkpoint) might result in maximal radiosensitization. In a clonogenic survival assay, we identified a dramatic radiosensitizing effect of Chir-124 on Mad2^{+/-} cells (Fig. 5 and Table 1). A clear increment in the sub-G₁ fraction was observed after the combined treatment with Chir-124 and IR, as compared to IR or Chir-124 alone. Moreover, the combination regiment caused an override of the G₂-M block after IR as shown for the WT HCT116 cell line. However, we did not find any augmentation of polyploid cells nor any increase of mitotic cells after Chir-124 plus IR in Mad2^{+/-} cells (Fig. 5).

In Figure 6C, we tried to summarize our findings on simultaneous inhibition of CHK1 and proteins implicated in checkpoints regulation. Concomitant Mad2, p53, p21 or CHK2 inhibition increase cell killing induced by the CHK1 inhibitor. This was not the case for 14-3-3 deficient cells, suggesting that G₂-M checkpoint deficiency arising from CHK1 and 14-3-3 inhibition alter common mechanisms.

CHK1 inhibition increases radiosensitivity of tetraploid cancer cells. We have previously shown²⁸ that CHK1 inhibition or depletion killed tetraploid cancer cells yet had only minor effects on their diploid counterparts.²⁹ We tested two sub clones of tetraploid HCT116 cells that were exposed to 10 Gy IR and Chir-124. Like their diploid counterparts, these tetraploid clones arrested in G₂ upon IR, and Chir-124 treatment of tetraploid cells abolished the IR-induced G₂ arrest, consistent with abrogation of the G₂ checkpoint. A clear increase in the sub-G₁ population was observed after the combination of Chir-124 and IR, as compared to IR or Chir-124 alone (Fig. 6C). Clonogenic survival assays further confirmed that Chir-124 could enhance the radiation-mediated killing of tetraploid HCT116 cells (SF2 16% for Chir-124 pretreated tetra1 sub clone tetraploid cells vs. 36% for control tetraploid tetra1 cells, shown in Fig. 6D).

Discussion

Many conventional anticancer treatments including: ionizing radiation, alkylating agents, DNA topoisomerase inhibitors, and platinum compounds, induce DNA damage. Frequently, cancer cells are defective in the G₁ checkpoint and hence must rely on the G₂ checkpoint more heavily than normal cells.² This provides a therapeutic opportunity for combining conventional cytotoxic anticancer agents with G₂ checkpoint inhibitors. CHK1 is a potential target for such a combination approach. Various novel specific

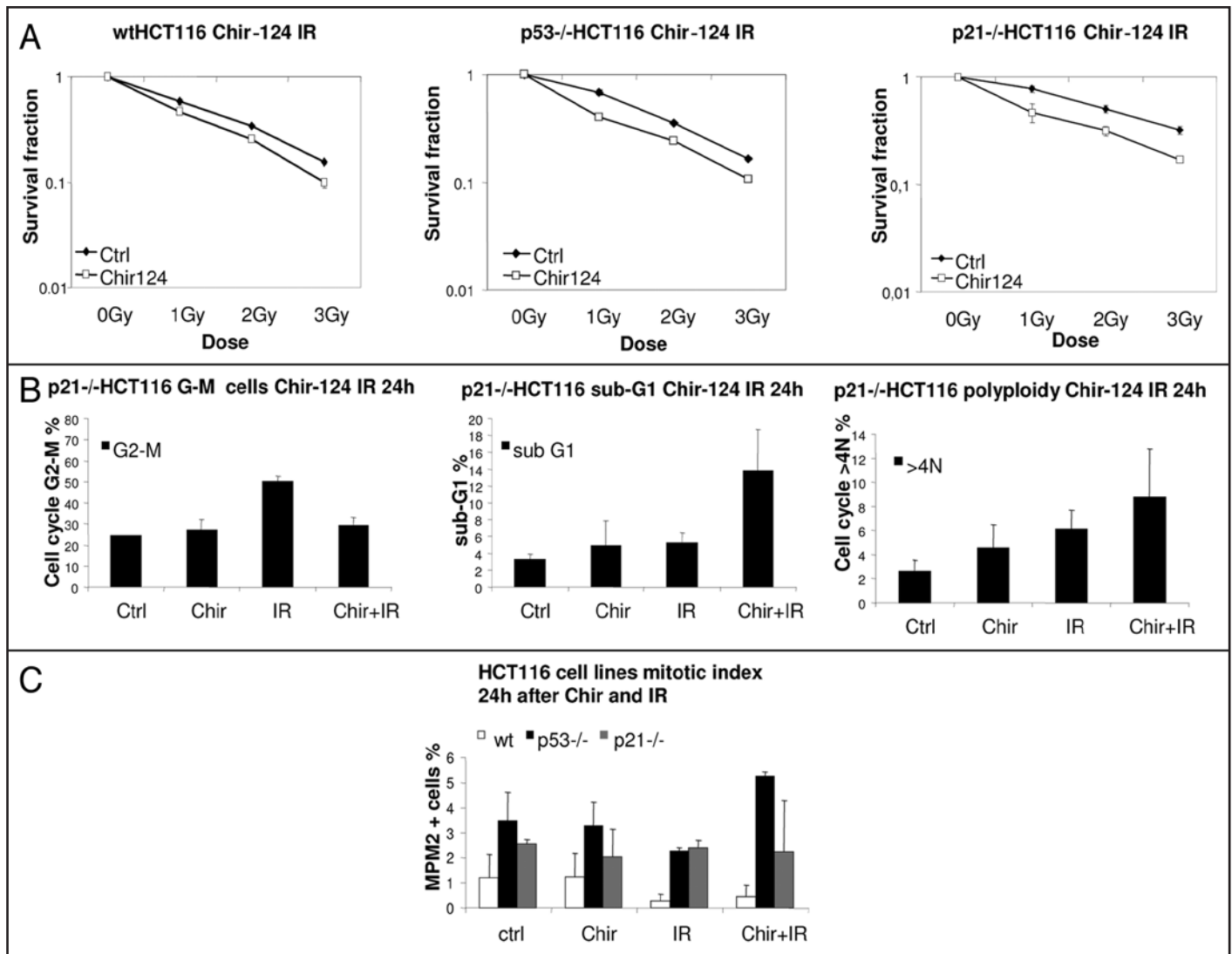


Figure 3. (A) Radio-sensitization by Chir-124 as assessed by measuring clonogenic survival following irradiation in WT, p53^{-/-} and p21^{-/-} HCT116 cell lines. 250 nM Chir-124 was given 1 h before different dose (0, 1, 2, 3 Gy) of IR, and the number of clones was evaluated after 10–14 days incubation. Data represent the mean \pm SD of three independent experiments in triplicate. (B) Cell cycle analysis in p21^{-/-} HCT116 cells after treatment with 1 h 250 nM Chir-124 followed by 10 Gy irradiation. Quantification of the summed FACS data of three experiments and histograms of percentage of cells in sub-G₁, with >4N DNA content and in G₂-M phase are shown here. Error bars represent SEM of three independent experiments. (C) Cytofluorometric detection of mitosis index (marked by MPM2 in FACS) change after 1 h 250 nM Chir124 followed by 10 Gy IR for a further 24 h in p53^{-/-}, p21^{-/-} and WT HCT116.

CHK1 kinase inhibitors including CEP-3891, PF-00477736 are being developed by the pharmaceutical industry.³⁰ Recently, a potent and selective CHK1 kinase inhibitor Chir-124 has been shown to potentiate the cytotoxicity of topoisomerase I poisons *in vitro* and *in vivo*.²² In the present report, we reveal that Chir-124 potentiates the cytotoxicity of radiation in various HCT116 cell lines with different genetic backgrounds, enabling us to outline the contributions of different cell cycle checkpoints to irradiation-induced cell death.

Because p53 functions in several checkpoint pathways, the loss of p53 might leave tumor cells more vulnerable to pharmacological inhibition of the remaining checkpoint components.

It has been proposed that when two or several checkpoints (such as the G₁-S and G₂-M checkpoints) are subverted at the same time, cells would be more sensitive to DNA damaging cytotoxic agents including IR.¹ We explored whether CHK1 inhibition selectively sensitizes p53 deficient cells to DNA damage.¹⁸ Chir-124 treatment of p53^{-/-} and WT HCT116 cells induced a marked reduction in IR-induced G₂ arrest consistent with abrogation of the G₂ checkpoint, irrespective of the p53 status. Clonogenic survival assays revealed a radiosensitizing effect of Chir-124 in both p53^{-/-} and p53-sufficient cells. The enhanced radiation effect by Chir-124 has been further confirmed by quantifying the frequency of apoptotic sub-G₁ cells. We did

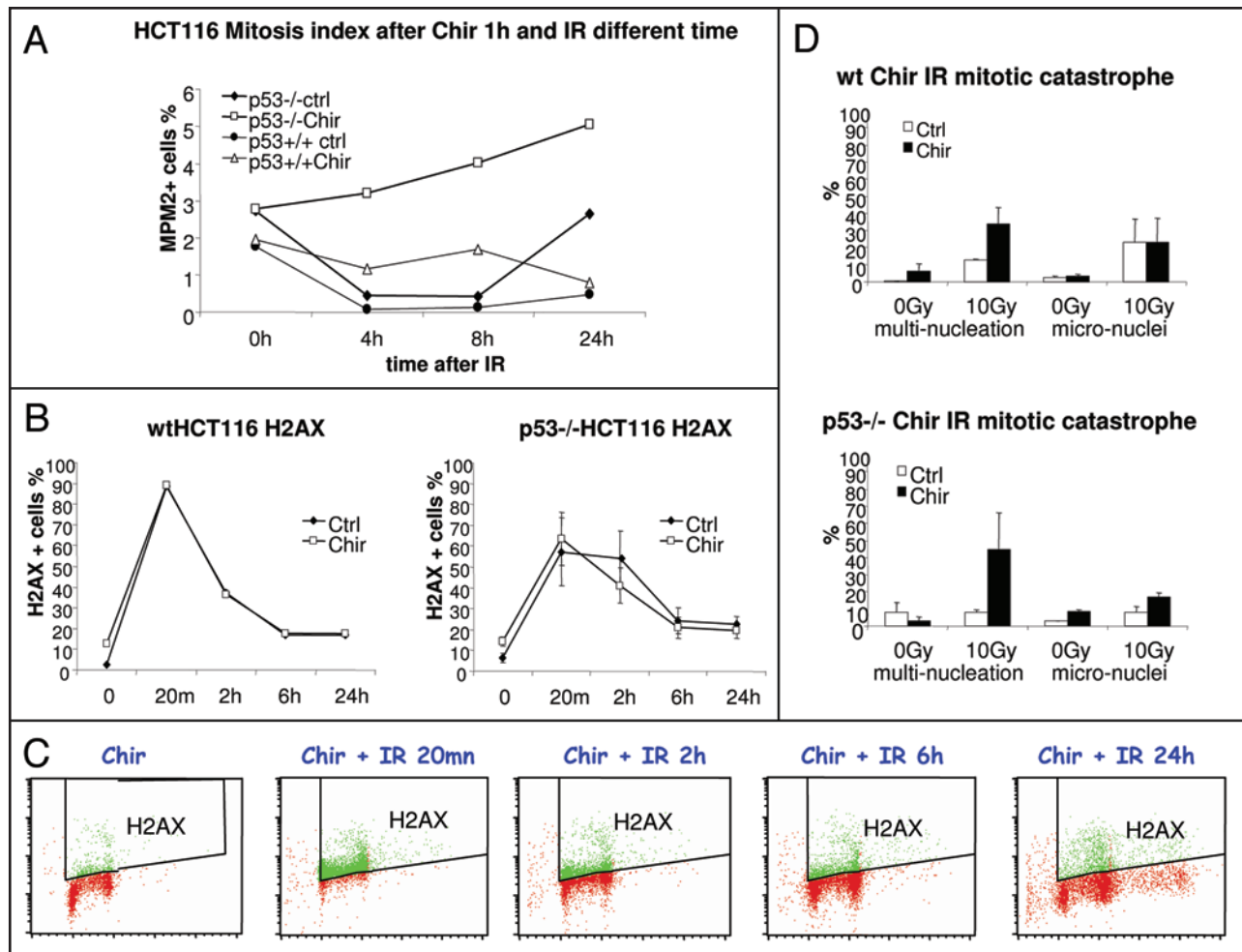


Figure 4. (A) Cytofluorometric detection of the mitotic index after 1 h pretreatment with 250 nM Chir124 (or DMSO as control) plus IR 10 Gy in p53^{-/-} and WT HCT116 cell lines. Data represent the mean \pm SEM of at least three independent experiments. (B) Quantification of γ H2Ax by FACS analysis after treatment with Chir-124 (250 nM) and IR (10 Gy) in p53^{-/-} (right) and WT HCT116 cells (left). (C) Representative FACS pictograms after γ H2Ax immunofluorescence of p53^{-/-} HCT116 cells subjected to the indicated treatment. (D) Frequency of cells that manifest morphological hallmarks of mitotic catastrophe (micronuclei or multi-nucleation) after IR (10 Gy) and/or Chir-124. Data represent the mean of three independent experiments, $X \pm$ SEM.

not find any difference in the pro-apoptotic effect of Chir-124 between wild type cells and p53 deficient cells. WT HCT116 cells accumulated in G₁ after IR even if CHK1 was inhibited with Chir-124 (Fig. 1A). This contrasts with results in which HCT116 cells were treated with the topoisomerase I inhibitor SN38 that causes a G₁ arrest, which can be overcome by addition of the CHK1 inhibitor 7-hydroxystaurosporine (UCN-01).³¹ However, UCN-01 has a relatively low specificity for CHK1 and may hence subvert the G₁ checkpoint by inhibiting other kinases.³² Additional experiments that focused on the G₂/M arrest yielded important differences between WT and p53^{-/-} HCT116 cells. We observed a dramatic difference in the proportion of mitotic (MPM2-positive) cells after IR alone and/or Chir-124 as a function of the p53 status. This could reflect the essential role of p53 in the p21-dependent G₁/S checkpoint in line with the observation that p21^{-/-} cells phenocopied p53^{-/-} HCT116 cells. This difference might also be attributed to the implication of p53 in the mainte-

nance of the G₂-M checkpoint and in the regulation of cyclin B/CDK1 activity through transcriptional activation and regulation of GADD45 and 14-3-3 σ . In p53-deficient tumor cells, CHK1 is probably particularly important for the integrity of the G₂/M checkpoint. We noted that cells that were forced to enter mitosis by treatment with Chir124, accumulated in mitosis in a time-dependent manner, consistent with another recent report.³³ This observation raised the possibility that the spindle assembly checkpoint might be activated when cells enter mitosis upon IR before cell death occurs and may explain how cells can remain in mitosis for up to 5 hours. We found that the frequency of polyploid cells, as well as that of cells with aberrant nuclear morphologies (micronuclei or multinucleation), increased dramatically after concomitant treatment with Chir-124 and IR in p53^{-/-} cells when compared to IR alone, whereas the same comparison yielded clearly less important differences in WT HCT116 cells. This suggests that Chir-124 can radiosensitize p53-deficient and p53-sufficient cells through

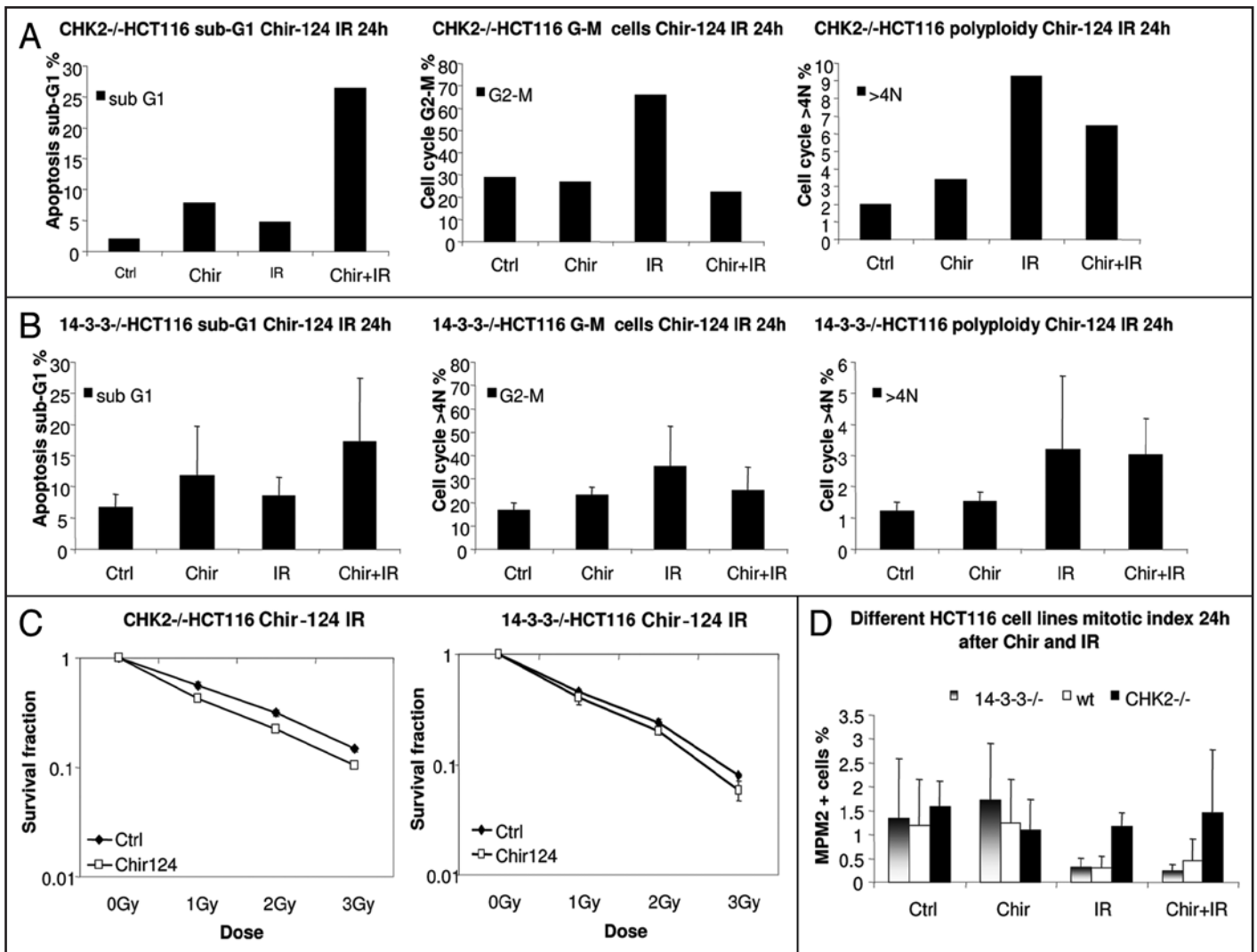


Figure 5. Cell cycle analysis of CHK2^{-/-} (A) and 14-3-3σ^{-/-} (B) HCT116 cells after treatment with 1 h 250 nM Chir-124 followed by 10 Gy irradiation. Quantification of the summed FACS data of three experiments and histograms of percentage of cells in sub-G₁, with >4N DNA content and in G₂-M phase are shown here. Error bars represent SEM of three independent experiments. (C) Clonogenic survival curves for CHK2^{-/-} (left) and 14-3-3σ^{-/-} (right) HCT116 cells pretreated for 1 h with 250 nM Chir-124 before IR. Data represent the mean ± SD of three independent experiments in triplicate. D: Cytofluorometric detection of the mitotic index (by staining for the detection of MPM2) after 1 h 250 nM Chir-124 followed by 10 Gy IR for a further 24 h in CHK2^{-/-} and 14-3-3σ^{-/-} HCT116. Data represent means ± SEM of at least two independent experiments.

Table 1 **SF2 and DER by Chir-124 in wild type and isogenic HCT116 cell lines**

Cell lines	SF2 control	SF2 Chir124	DER
WT HCT116	0.338 ± 0.011	0.256 ± 0.011	1.32
p53 ^{-/-}	0.374 ± 0.01	0.277 ± 0.026	1.35
p21 ^{-/-}	0.507 ± 0.04	0.316 ± 0.03	1.60
14-3-3 ^{-/-}	0.254 ± 0.012	0.219 ± 0.013	1.16
CHK2 ^{-/-}	0.315 ± 0.021	0.222 ± 0.002	1.42
Mad2±	0.36 ± 0.033	0.2 ± 0.025	1.77

distinct pathways, namely mitotic catastrophe in p53^{-/-} cells, and another yet-to-be-elucidated pathway in WT cells. p53^{-/-} cells may be more susceptible to the induction of mitotic catastrophe by Chir-124 plus IR because CHK1 might exert a particularly important “gatekeeper” function in the p53-deficient context. This hypothesis is consistent with our recent report concerning Aurora kinases inhibition on radiosensitivity.^{34,35}

The demonstration that homozygous deletion of 14-3-3σ in HCT116 colon cancer cells compromises the G₂ checkpoint provides novel insights into a potential mechanism for the selective pharmacological inhibition of the G₂ checkpoint.²⁶ In normal cells, DNA damage leads to a p53-dependent accumulation of 14-3-3σ, which binds to and sequesters cyclin B1/CDK1 complexes in the cytoplasm. 14-3-3σ^{-/-} cells are more sensitive to IR than WT HCT116

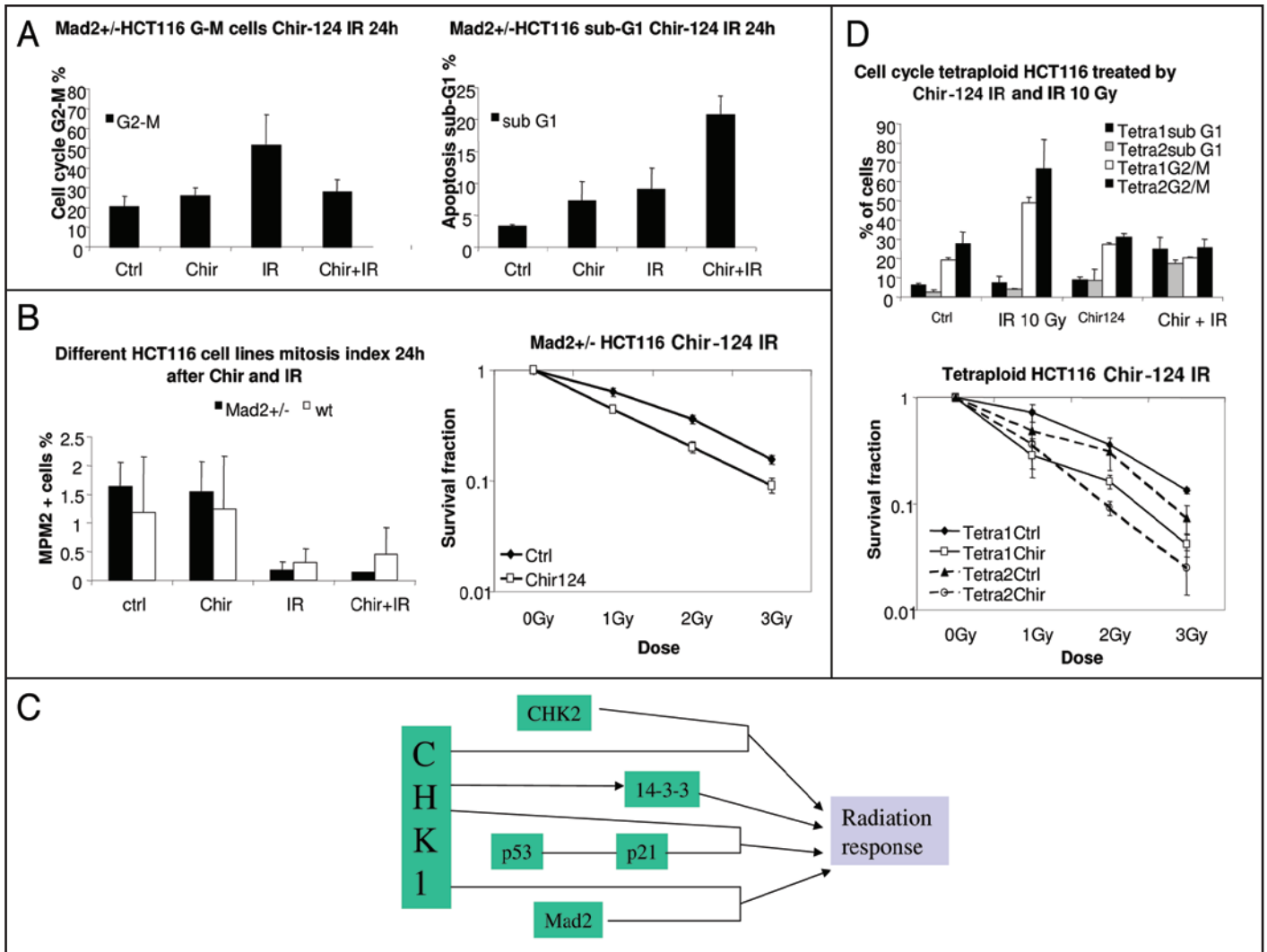


Figure 6. Influence of Mad2 and tetraploidy on the radiosensitizing effects of Chir-124. (A) Cell cycle analysis of Mad2^{+/-} HCT116 cells after treatment with 250 nM Chir-124 for 1 h, followed by 10 Gy irradiation. The percentage of cells in sub-G₁ (right) and in G₂-M phase (left) are shown. Error bars represent SEM of three independent experiments. (B) Cytofluorometric detection of the mitotic index (MPM2 positivity) after 1 h 250 nM Chir-124 followed by 10 Gy IR for a further 24 h in Mad2^{+/-} HCT116. Data represent the means ± SEM of at least three independent experiments. Right, Clonogenic survival curves for Mad2^{+/-} HCT116 pretreated 1 hour with 250 nM Chir-124 1 h before different IR. Data represent the means ± SD of three independent experiments in triplicate. (C) The schema shows CHK1 interaction in radiation response with other proteins implicated in the checkpoint. Concomitant Mad2, p53, p21 or CHK2 inhibition increase cell killing induced by the CHK1 inhibitor. This was not the case for 14-3-3 deficient cells, suggesting that G₂-M checkpoint deficiency arising from CHK1 and 14-3-3 inhibition alter common pathways. (D) Top: Cell cycle analyses of two clones of tetraploid HCT116 cells (Tetra1 and Tetra2) after treatment with 1 h 250 nM Chir-124 followed by 10 Gy irradiation, Error bars represent SD of two independent experiments. Bottom: Clonogenic survival curves for tetraploid HCT116 (Tetra1 and Tetra2) that were pretreated for 1 h with 250 nM Chir-124 before IR. Data represent the mean ± SD of two independent experiments in triplicate.

cells. However, in 14-3-3^{-/-} HCT116, Chir-124 did not increase G₂ cells accumulation after IR. Moreover, both clonogenic assays and measurement of sub-G₁ DNA levels revealed that Chir-124 led to a moderate radiosensitizing effect. This suggests an overlapping effect of CHK1 and 14-3-3 on the G₂-M checkpoint.

In sharp contrast to the results observed in 14-3-3^{-/-} deficient cells, Chir-124 could radiosensitize CHK2-deficient cells. CHK2 is structurally unrelated to CHK1 although the two proteins seem to share overlapping functions.⁷ Current data are consistent with the model that CHK1 is the main checkpoint kinase (the ‘workhorse’), which is also required in unperturbed cell cycles,

whereas CHK2 (the ‘amplifier’) has an important modulatory, complementary role in regulating cell cycle progression after certain types of DNA damage, particularly DSBs.⁷ In contrast to CHK1 inhibition, CHK2 inhibition alone did not have any chemosensitization effect.¹ Our data suggests that dual CHK1 and CHK2 inhibition might be an effective approach for radiosensitization.

We attempted to determine if spindle assembly checkpoint-defective cells would be radiosensitized by Chir-124. Mad2^{+/-} cells are spindle checkpoint defective, as indicated by a dramatically reduced mitotic arrest in response to spindle damage. Moreover, the

induction of cell death was significantly impaired in spindle checkpoint-compromised Mad2^{+/-} cells in response to topoisomerase inhibitors.³⁶ We observed that spindle checkpoint-compromised Mad2^{+/-} cells were marginally resistant to IR, an effect that was effectively overcome by Chir-124. Thus, CHK1 inhibition may overcome the partial radio-resistance mediated by SAC deficiency. Upon abrogation of the G₂-M checkpoint by UCN-01, p53-deficient tumor cells enter mitosis in the presence of DNA damage and activate a mitotic checkpoint that requires the spindle checkpoint proteins as well as the chromosomal passenger proteins leading to a sustained mitotic arrest.³³ Mitotic cell death is considered to be associated with SAC activation. However in our study, cell death induced by radiation plus CHK1 inhibition was not compromised by SAC deficiency. This could be explained by a threshold for cell death induction that, in HCT116 cells, is independent of mitotic events and rather involves alternative lethal mechanisms.³⁷

In conclusion, CHK1 inhibition by Chir-124 sensitizes cancer cells to radiation. While this effect is not strictly dependent on the p53 status, the modalities underlying lethality of p53-defective cells tends to be different than in p53-sufficient control cells. In addition, our data suggest that inhibition of CHK1 together with other checkpoint-relevant molecular targets such as CHK2 or SAC component could act synergistically to mediate radiosensitization.

Materials and Methods

Cell lines. HCT116 human colorectal cancer cell lines—p53 wild type (WT), p53^{-/-},³⁸ p21^{-/-}, CHK2^{-/-},³⁹ and 14-3-3 σ ^{-/-},²⁶ were kind gifts by B. Vogelstein. MAD2^{+/-} cells were kindly provided by R. Benezra.²⁷ HCT116 cells were maintained in McCoy's 5a medium (Gibco) supplemented with 10% fetal bovine serum (ATGC), 1% PS (Gibco), 1% L-glutamine (Eurobio), 1 mM sodium pyruvate (Gibco) and 10 mM HEPES (Sigma), in humidified atmosphere containing 5% CO₂ at 37°C. Tetraploid cells were generated from diploid WT HCT116 diploid precursor as previously described²⁸ and were grown in McCoy's 5A medium supplemented with 10% FCS.

Cell cycle analysis. Control and 10 Gy-irradiated cells with/without drug exposure were harvested by trypsinization at the indicated time after irradiation, washed with ice-cold PBS, fixed in 70% ethanol and stored at -20°C. Prior to DNA analysis, DNA content was labeled with 0.1 mg/ml propidium iodide (PI) and 1 mg/ml RNase.³ The cell cycle analysis was performed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

Mitotic index analysis. Cells were washed with PBS once—fixed in 70% Ethanol and stored at -20°C. Then, they were washed with PBS and incubated in 5 μ g/ml MPM2 in 40 μ l staining solution (0.5% Tween + 20 mM EDTA in 1% BSA/PBS) for 1 h at room temperature, washed once with PBS and incubated with the appropriate dose of Alexa 488-conjugated goat anti-mouse IgG (Clinisciences), DNA content was labeled with 0.1 mg/ml propidium iodide (PI) and 1 mg/ml RNase. Analysis was performed by flow cytometry.

Cytofluorometric detection of γ H2AX foci. Ethanol-fixed cells (as above) were washed in PBS and permeabilized in 1 ml

0.5 % Triton/PBS per tube for 5 min in ice, washed with PBS and incubated with 0.5 μ l γ H2AX mouse antibody per tube for 2 h in room temperature, washed three times and incubated with Alexa 488-conjugated anti-mouse IgG (0.5 μ l per tube for 1 h), and finally counterstained with PI (50 μ g/ml) plus RNase (100 μ g/ml) and 20 mM EDTA in PBS at 37°C for 1 h, then 4°C for 1 h to 24 h before cytofluorometric analysis.

Annexin V staining analysis in FACS. Cells were collected by centrifugation and resuspended in Annexin V FITC (BD Biosciences) and incubated in the dark for 15 min. PI (0.1 μ g/ml) was then added to the cell suspension, followed by cytofluorometric analysis on a FACS Vantage SE (BD Biosciences).³⁴

Western blotting. Total CHK1 expression and serine 345-phosphorylated CHK1 (CHK1-Pho) was detected by immunoblot on irradiated cell lines or human xenografts. Female athymic nude mice 6–8 weeks of age, (Janvier CERT 53940, Le Genest St. Isle, France) were kept at the Institut Gustave Roussy under the Animal Care license C94-076-11 (Ministère de l'Agriculture). 3 x 10⁶ p53^{-/-} HCT116 cells were subcutaneously implanted in the right flank of each mouse. Treatment began when the tumor was at least 5 mm in diameter. p53^{-/-} HCT116 tumour xenografts were treated by irradiation (10 Gy) and were recovered at 15 min, 30 min, 1 and 3 h. The gel 4–20% (NuPAGE[®] Bis-tris Gel Invitrogen) was used for the in vivo phosphor-CHK1 protein migration. The following antibodies were used: rabbit polyclonal anti-phosphor-CHK1 (1:200, phospho-CHK1 Ser 345, Cell Signaling Technology[®]) mouse monoclonal anti-CHK1 (1:500), mouse monoclonal anti β -tubulin (1:200, catalog no. ATN01-A; Cytoskeleton, Denver, CO, USA) was used for comparison of protein deposit quantity.

Clonogenic survival assays. Cells were seeded in triplicate into 6-well plates or 25 cm² flask in a range of 100 to 80,000 cells/well depending on radiation dose that the cells received, the test condition and different cell lines so as to yield 20–200 colonies per flask or well. A single dose of photon irradiation with/without drug was applied, once cells were attached. Cells were cultured in a 37°C, 5% CO₂ incubator for 10–14 days (Tao et al. 2008). Individual colonies (>50 cells/colony) were fixed and stained with a solution containing crystal violet and methanol for 20 min. The plating efficiency (PE) represents the percentage of cells seeded that grow into colonies under a specific culture condition of a given cell line. The survival fraction, expressed as a function of irradiation, was calculated as follows: Survival fraction (SF) at 2 Gy (SF2) = colonies counted of 2 Gy/(cells seeded of 2 Gy * PE/100). In the clonogenic survival curve, we normalized the different conditions according to the control. The radiation Dose Enhancement Ratio (DER) by Chir-124 was calculated using the following formula: DER = (SF at an indicated dose of radiation alone)/(SF at an indicated dose of radiation Chir-124), and we use here SF2 to calculate DER. Radiosensitization is defined as the term used when Chir-124 increases the sensitivity of cells to radiation (as assessed by clonogenic inhibition or apoptosis). This is calculated as per the formula listed above and represented in the form of DER. Thus, DER is defined as the ratio of surviving cells with radiation alone compared with a combination of radiation and Chir-124 exposures.³⁴ 200 KV X ray at a dose rate of 0.66 Gy/min was used for

the *in vivo* experiments and 137 Cesium source at a dose rate of 1.85 Gy/min was used for *in vitro* experiments.

CHK1 inhibitor. Chir-124 developed by Chiron Corporation (Novartis) and provided by Servier is readily soluble in dimethyl sulfoxide (DMSO), and conserved aliquot in -20°C at a concentration of 5 mM. The duration of cells incubation with Chir-124 *in vitro* is 24 h if not specified differently. Control cultures were treated with DMSO. The final concentration of DMSO did not exceed 0.1%.

Immuno(cyto)chemistry, detection of micronuclei (MN) and multinuclei. Cells that were fixed with 4% paraformaldehyde (Sigma) were stained for the detection of MN and multinuclei. For fluorescence microscopy, nuclei were stained with Hoechst 33342 (1 mg/ml, Molecular Probes). MN and multinuclei were detected by fluorescence microscopy and morphologically classified according to standard criteria. More than 200 cells were scored for each data point in at least three separate experiments.

Acknowledgements

We are grateful to Dr. Bert Vogelstein, Johns Hopkins University, for kindly providing the wild-type, $p53^{-/-}$, $p21^{-/-}$, $CHK2^{-/-}$ and $14-3-3\sigma^{-/-}$ HCT116 cell lines. We also thank Dr. Robert Benezra, Memorial Sloan-Kettering Cancer Center for kindly providing $Mad^{+/-}$ HCT116 cells. We thank Servier for kindly providing Chir-124 for experimental studies. We thank Safietou Mansaly for her assistance in the experiments. This study was supported by a grant from Association pour la Recherche sur le Cancer (to E.D.), as well as by grants from Ligue contre le Cancer (équipe labellisée), Agence Nationale pour la Recherche, Institut National du Cancer, Cancéropole Ile-de-France and European Union (Active $p53$, Apop-Train, Apo-Sys, RIGHT) to G.K.

References

- Zhou BB, Bartek J. Targeting the checkpoint kinases: chemosensitization versus chemoprotection. *Nat Rev Cancer* 2004; 4:216-25.
- Kastan MB, Bartek J. Cell cycle checkpoints and cancer. *Nature* 2004; 432:316-23.
- Lobrich M, Jeggo PA. The impact of a negligent G_2/M checkpoint on genomic instability and cancer induction. *Nat Rev Cancer* 2007; 7:861-9.
- Pawlik TM, Keyomarsi K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys* 2004; 59:928-42.
- Harvey SL, Charlet A, Haas W, Gygi SP, Kelloff DR. Cdk1-dependent regulation of the mitotic inhibitor Wee1. *Cell* 2005; 122:407-20.
- Booher RN, Holman PS, Fattacy A. Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. *J Biol Chem* 1997; 272:22300-6.
- Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* 2003; 3:421-9.
- Zachos G, Gillespie DA. Exercising restraints: role of Chk1 in regulating the onset and progression of unperturbed mitosis in vertebrate cells. *Cell Cycle* 2007; 6:810-3.
- Chen MS, Ryan CE, Pwnica-Worms H. Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. *Mol Cell Biol* 2003; 23:7488-97.
- Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Pwnica-Worms H, et al. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science* 1997; 277:1497-501.
- Lee J, Kumagai A, Dunphy WG. Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. *Mol Biol Cell* 2001; 12:551-63.
- Krämer A, Mailand N, Lukas C, Syljuäsen RG, Wilkinson CJ, Nigg EA, et al. Centrosome-associated Chk1 prevents premature activation of cyclin-B-Cdk1 kinase. *Nat Cell Biol* 2004; 6:884-91.
- Schmitt E, Boutros R, Froment C, Monsarrat B, Ducommun B, Dozier C. CHK1 phosphorylates CDC25B during the cell cycle in the absence of DNA damage. *J Cell Sci* 2006; 119:4269-75.
- Kramer A, Lukas J, Bartek J. Checking out the centrosome. *Cell Cycle* 2004; 3:1390-3.
- Zachos G, Black EJ, Walker M, Scott MT, Vagnarelli P, Earnshaw WC, et al. Chk1 is required for spindle checkpoint function. *Dev Cell* 2007; 12:247-60.
- Waldman T, Kinzler KW, Vogelstein B. p21 is necessary for the p53-mediated G_1 arrest in human cancer cells. *Cancer Res* 1995; 55:5187-90.
- Chen Z, Xiao Z, Gu WZ, Xue J, Bui MH, Kovar P, et al. Selective Chk1 inhibitors differentially sensitize p53-deficient cancer cells to cancer therapeutics. *Int J Cancer* 2006; 119:2784-94.
- Tse AN, Schwartz GK. Potentiation of cytotoxicity of topoisomerase I poison by concurrent and sequential treatment with the checkpoint inhibitor UCN-01 involves disparate mechanisms resulting in either p53-independent clonogenic suppression or p53-dependent mitotic catastrophe. *Cancer Res* 2004; 64:6635-44.
- Koniaras K, Cuddihy AR, Christopoulos H, Hogg A, O'Connell MJ. Inhibition of Chk1-dependent G_2 DNA damage checkpoint radiosensitizes p53 mutant human cells. *Oncogene* 2001; 20:7453-63.
- Syljuäsen RG, Sorensen CS, Nylandsted J, Lukas C, Lukas J, Bartek J. Inhibition of Chk1 by CEP-3891 accelerates mitotic nuclear fragmentation in response to ionizing Radiation. *Cancer Res* 2004; 64:9035-40.
- Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006; 444:756-60.
- Tse AN, Rendahl KG, Sheikh T, Cheema H, Aardalen K, Embry M, et al. CHIR-124, a novel potent inhibitor of Chk1, potentiates the cytotoxicity of topoisomerase I poisons *in vitro* and *in vivo*. *Clin Cancer Res* 2007; 13:591-602.
- Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* 1999; 104:263-9.
- Kim JJ, Tannock IF. Repopulation of cancer cells during therapy: an important cause of treatment failure. *Nature Rev Cancer* 2005; 5:516-25.
- Tao W, South VJ, Zhang Y, Davide JP, Farrell L, Kohl NE, et al. Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage. *Cancer Cell* 2005; 8:49-59.
- Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999; 401:616-20.
- Michel LS, Liberal V, Chatterjee A, Kirchwegger R, Pasche B, Gerald W, et al. MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature* 2001; 409:355-9.
- Castedo M, Coquelle A, Vivet S, Vitale I, Kauffmann A, Dessen P, et al. Apoptosis regulation in tetraploid cancer cells. *EMBO J* 2006; 25:2584-95.
- Vitale I, Galluzzi L, Vivet S, Nanty L, Dessen P, Senovilla L, et al. Inhibition of Chk1 kills tetraploid tumor cells through a p53-dependent pathway. *PLoS ONE* 2007; 2:1337.
- Blasina A, Hallin J, Chen E, Arango ME, Kraynov E, Register J, et al. Breaching the DNA damage checkpoint via PF-00477736, a novel small-molecule inhibitor of checkpoint kinase 1. *Mol Cancer Ther* 2008; 7:2394-404.
- Levesque AA, Fanous AA, Poh A, Eastman A. Defective p53 signaling in p53 wild-type tumors attenuates p21waf1 induction and cyclin B repression rendering them sensitive to Chk1 inhibitors that abrogate DNA damage-induced S and G_2 arrest. *Mol Cancer Ther* 2008; 7:252-62.
- Senderowicz AM. Small-molecule cyclin-dependent kinase modulators. *Oncogene* 2003; 22:6609-20.
- Vogel C, Hager C, Bastians H. Mechanisms of mitotic cell death induced by chemotherapy-mediated G_2 checkpoint abrogation. *Cancer Res* 2007; 67:339-45.
- Tao Y, Zhang P, Frascogna V, Lecluse Y, Auferin A, Bourhis J, et al. Enhancement of radiation response by inhibition of Aurora-A kinase using siRNA or a selective Aurora kinase inhibitor PHA680632 in p53-deficient cancer cells. *Br J Cancer* 2007; 97:1664-72.
- Tao Y, Zhang P, Girdler F, Frascogna V, Castedo M, Bourhis J, et al. Enhancement of radiation response in p53-deficient cancer cells by the Aurora-B kinase inhibitor AZD1152. *Oncogene* 2008; 27:3244-55.
- Vogel C, Kienitz A, Muller R, Bastians H. The mitotic spindle checkpoint is a critical determinant for topoisomerase-based chemotherapy. *J Biol Chem* 2005; 280:4025-8.
- Gascoigne KE, Taylor SS. Cancer cells display profound intra- and interline variation following prolonged exposure to antimetabolic drugs. *Cancer Cell* 2008; 14:111-22.
- Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, et al. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. *Science* 1998; 282:1497-501.
- Jallepalli PV, Lengauer C, Vogelstein B, Bunz F. The Chk2 tumor suppressor is not required for p53 responses in human cancer cells. *J Biol Chem* 2003; 278:20475-9.