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Cutting edge: Chk1 directs senescence and mitotic catastrophe in recovery from G_2 checkpoint arrest

Angela Poehlmann [a, *, #], Caroline Habold [a, b, #], Diana Walluscheck [a], Kathrin Reissig [a], Khuloud Bajbouj [a], Oliver Ullrich [a, c], Roland Hartig [d], Hala Gali-Muhtasib [e], Antje Diestel [a, f], Albert Roessner [a], Regine Schneider-Stock [a, g]

[a] *Department of Pathology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany*
[b] *IPHC-CNRS/UDS, Strasbourg, France*
[c] *Institute of Anatomy, Faculty of Medicine, University of Zurich, Zurich, Switzerland*
[d] *Department for Molecular and Clinical Immunology, Otto-von-Guericke University Magdeburg, Magdeburg, Germany*
[e] *Department of Biology, American University of Beirut, Beirut, Lebanon*
[f] *Department of Pediatric Cardiology, Charité Universitätsmedizin Berlin, Berlin, Germany*
[g] *Experimental Tumour Pathology, Department of Pathology, University of Erlangen, Erlangen, Germany*

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Abstract

Besides the well-understood DNA damage response *via* establishment of G_2 checkpoint arrest, novel studies focus on the recovery from arrest by checkpoint override to monitor cell cycle re-entry. The aim of this study was to investigate the role of Chk1 in the recovery from G_2 checkpoint arrest in HCT116 (human colorectal cancer) wt, $p53^{-/-}$ and $p21^{-/-}$ cell lines following H_2O_2 treatment. Firstly, DNA damage caused G_2 checkpoint activation *via* Chk1. Secondly, overriding G_2 checkpoint led to (*i*) mitotic slippage, cell cycle re-entry in G_1 and subsequent G_1 arrest associated with senescence or (*ii*) premature mitotic entry in the absence of $p53/p21^{WAF1}$ causing mitotic catastrophe. We revealed subtle differences in the initial Chk1-involved G_2 arrest with respect to $p53/p21^{WAF1}$: absence of either protein led to late G_2 arrest instead of the classic G_2 arrest during checkpoint initiation, and this impacted the release back into the cell cycle. Thus, G_2 arrest correlated with downstream senescence, but late G_2 arrest led to mitotic catastrophe, although both cell cycle re-entries were linked to upstream Chk1 signalling. Chk1 knockdown deciphered that Chk1 defines long-term DNA damage responses causing cell cycle re-entry. We propose that recovery from oxidative DNA damage-induced G_2 arrest requires Chk1. It works as cutting edge and navigates cells to senescence or mitotic catastrophe. The decision, however, seems to depend on $p53/p21^{WAF1}$. The general relevance of Chk1 as an important determinant of recovery from G_2 checkpoint arrest was verified in HT29 colorectal cancer cells.

Keywords: DNA damage • G_2 checkpoint • recovery • checkpoint-kinase 1 (Chk1) • senescence • mitotic catastrophe

Introduction

The development of cancer results from dysregulated proliferation or an incompetence of cells to undergo apoptotic cell death. Hence, an important issue emerging in drug discovery is to target anticancer treatments on cell cycle checkpoints that are responsible for the control of cell cycle phase progression or on apoptosis, eliminating defective cells [1]. Because damage to DNA might be the common underlying mechanism for the positive outcome of chemotherapy, reactive oxygen species (ROS)-generating anticancer drugs have raised clinical interest [2, 3]. In response to DNA damage, the G_2 checkpoint is activated to halt cell cycle progression, preventing cells from entering mitosis. Its activation proceeds through maintenance of the Thr14/Tyr15 inhibitory phosphorylations on cdc2, realized by the protein kinases Wee1 and Myt1, respectively [4, 5].

[#]These authors contributed equally.
*Correspondence to: Angela POEHLMANN,
Department of Pathology, Otto-von-Guericke University,
Leipziger Str. 44, 39120 Magdeburg, Germany.
Tel.: 49-391-6715488
Fax: 49-391-6715818
E-mail: Angela.Poehlmann@med.ovgu.de

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ATR indirectly modulates the phosphorylation status of these sites by activating the downstream protein kinase Chk1 *via* phosphorylation on Ser317 and Ser345 [6–9]. Activated Chk1 phosphorylates the dual specificity phosphatase cdc25C on Ser216, thus creating a binding site for 14–3-3 proteins [10]. The 14–3-3/cdc25C protein complexes are sequestered in the cytoplasm, thereby preventing cdc25C from activating cdc2 through removal of the inhibitory phosphorylations. This results in the maintenance of the cdc2-cyclin B1 complex in its inactive state and blocks entry into mitosis [11]. Conceptually, besides checkpoint initiation, the delay comprises two additional phases: maintenance during repair and termination to allow cell cycle progression. As much information has been gained regarding the signalling pathways involved in establishing a G_2 checkpoint arrest, novel studies focus on the question of how checkpoint signalling is maintained [12] and overcome to allow cell cycle re-entry [13, 14], especially if the drug is removed. p53 plays a critical role in maintaining G_2 checkpoint arrest. At least one half of the tumours are p53-deficient, and some also show mutations or altered expressions of other components of the G_2 checkpoint [15]. With this, induction of mitotic catastrophe as a result of checkpoint deficiency appears to be a desirable goal in cancer treatment [16]. In addition, permanently arresting tumour cell growth through the induction of senescence also seems to be an attractive treatment approach [17, 18].

Collectively, senescence [19–21] and mitotic catastrophe [22, 23] are two major effects desired in drug treatment, although many studies report a separate role of Chk1 in DNA damage response [24, 25]. This has encouraged us to link the single findings to a general model regulating oxidative DNA damage in colorectal cancer cells, firstly using HCT116 wt, $p53^{-/-}$ and $p21^{-/-}$ cells. We intended (*i*) to highlight recovery from arrest monitoring cell cycle re-entry and (*ii*) to find an underlying signalling pathway upstream of the long-term DNA damage responses. Secondly, we confirmed the general relevance of Chk1 as an important determinant of recovery from G_2 checkpoint arrest also in the HT29 colorectal cancer cell line.

We have recently shown that H_2O_2-induced DNA damage establishes a G_2/M arrest in HCT116 cells through epigenetic $p21^{WAF1}$ regulation [26]. To obtain information on how checkpoint signalling is overcome to initiate cell cycle re-entry, we again used short-term, high bolus H_2O_2 exposure to efficiently damage DNA, a prerequisite for monitoring long-term DNA damage responses. In the present study, we observed different desired cell fates that are in line with long-term signalling in therapy following Chk1-involved checkpoint initiation, including senescence or mitotic catastrophe. The decision, however, seems to depend upon balance on $p53/p21^{WAF1}$. Importantly, we did not consider the cellular outcomes as single events, but we found them linked to one upstream signalling as the first response: an activation of the Chk1 pathway. Moreover, Chk1 knockdown showed that the upstream Chk1 signalling seems to navigate cells to cell cycle re-entry in the G_1 or M phases, which finally led to senescence or mitotic catastrophe.

Material and methods

Cell culture and treatment

Colorectal cancer cell lines HCT116 wt, HCT116 $p53^{-/-}$, HCT116 $p21^{-/-}$ and HT29 were maintained in RPMI or DMEM (cell culture media) with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C. Cells were treated with 30 mM H_2O_2 for 3 min., because a majority of studies have reported that H_2O_2 was added directly to the cells as a bolus [27, 28]. Therefore, cells were initially exposed to high H_2O_2 concentrations. Cells were collected after 1, 6, 24, 48 and 72 hrs following treatment. More details regarding cell lines are given in the Supporting Information.

Flow cytometric analysis of DNA content

One day before treatment, cells were seeded into Petri dishes (90 mm diameter) at a density of 1.2–2.0 $\times$ 10^6 cells per dish. For cell cycle analysis following Chk1 siRNA transfection, cells were seeded in 6-well plates at a density of 1.5–2.4 $\times$ 10^5 cells per well. After the indicated times, the supernatants were collected and combined with cells that were harvested by trypsin, washed twice with phosphate-buffered saline, fixed with 70% ethanol, treated with 1% RNase and finally stained with a hypotonic propidium iodide solution (50 μg/ml). Distribution of cell cycle phases with different DNA contents was determined using a flow cytometer (Calibur, Becton-Dickinson, CA, USA). Cells whose DNA was less intensively stained than that of G_1 cells (Pre-G_1 cells) in flow cytometric histograms were considered apoptotic cells. Analysis of cell cycle distribution and the percentage of cells in the Pre-G_1, G_1, S and G_2/M phase of the cell cycle were determined using the software CellQuest Pro (Becton-Dickinson).

Western blotting

Proteins were prepared as described previously [26]. For Western blot analysis, we used antibodies to the following proteins: Chk1, phospho-cdc25C^{Ser216} (Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase 3, cdc2, phospho-cdc2^{Thr14}, phospho-cdc2^{Tyr15}, cyclin D1, phospho-H3^{Ser10} (Cell Signaling, Danvers, MA, USA), phospho-Chk1^{Ser317} (Novus, Littleton, CO, USA), cdc25C (Acris, Herford, Germany), cyclin B1 (Novo Castra, Newcastle upon Tyne, UK), p53, $p21^{WAF1}$ (Calbiochem, Gibbstown, NJ, USA), phospho-p53^{Ser15} (Abcam, Cambridge, UK), phospho-H2AX^{Ser139} (γ-H2AX, Millipore, Bedford, MA, USA), poly (ADP-ribose) polymerase (PARP) (Alexis Biochemicals, Lausen, Switzerland) and β-actin (Sigma-Aldrich, Munich, Germany). As Chk1 was activated 1 hr after H_2O_2 treatment in HCT116 wt, $p53^{-/-}$ and $p21^{-/-}$ cells, and HT29 cells, the 1 hr timepoint was used as an internal control for investigations regarding long-term DNA damage responses following Chk1 activation.

Fluorescence immunostaining analysis

The γ-H2AX and cyclin B1 subcellular localization was investigated in isogenic HCT116 cells treated with 30 mM H_2O_2 for 3 min. and further recovery for 1 hr or 24 hrs on slides. γ-H2AX was stained with anti-H2AX^{Ser139} (Millipore), cyclin B1 with anti-cyclin B1 (Novo Castra) and the nucleus with 4,6-diamidino-2-phenylindole (DAPI). Slides were

examined under a fluorescence microscope Axioplan2 imaging from ZEISS (Jena, Germany) using Isis V 3.4.0 software and appropriate filters.

Comet assay

To estimate DNA damage, we performed the CometAssay (Trevigene, Gaithersburg, MD, USA), according to the protocol of the supplier. Evaluation was performed with a fluorescence microscope (Axioplan2, ZEISS) equipped with appropriate filter sets. Images were acquired using Isis V 3.4.0 software. More details are given in the Supporting Information.

Senescence β-galactosidase staining

The histochemical detection of senescence-associated β-galactosidase activity at pH 6.0 was performed with the senescence β-galactosidase staining kit (Cell Signaling) according to the instructions of the manufacturer.

siRNA transfection

Chk1 knockdown was performed according to the manufacturer's instructions (Santa Cruz Biotechnology) as described previously [29], reaching a p-Chk1^{Ser317} protein down-regulation of at least 60%. More details are given in the Supporting Information.

Results

H_2O_2 induces establishment of Chk1-involved G_2 checkpoint arrest

Recently, we have shown that short-term, high bolus H_2O_2 exposure induces G_2/M arrest in HCT116 wt and p53$^{-/-}$ cells through epigenetic regulation of the p21^{WAF1} promoter [26]. Due to the known essential function of Chk1 in DNA damage response, we further investigated Chk1 signalling following H_2O_2 treatment in HCT116 wt and p53$^{-/-}$ cells. Here, we show that H_2O_2 also activated the G_2 checkpoint (Fig. 1A): Chk1 was phosphorylated in both cell lines 1 hr after treatment. Subsequently, there was an increase in the Chk1 target p-cdc25C^{Ser216} after 24 hrs. Interestingly, the extent of G_2 arrest after 24 hrs *via* p-cdc25C^{Ser216} did correlate with inhibitory phosphorylation on cdc2 on Thr14, but not with that on Tyr15. Chk1 knockdown showed 36% and 14% reduced G_2 checkpoint arrest at 24 hrs in wt and p53$^{-/-}$ cells, respectively (Fig. 1B and C). Consequently, the establishment of G_2 checkpoint arrest in HCT116 wt and p53$^{-/-}$ cells involves Chk1.

G_2 checkpoint override causes Chk1-dependent long-term DNA damage responses

To further study cell fates following H_2O_2-induced, Chk1-involved G_2 checkpoint arrest, we elucidated long-term DNA damage signalling in HCT116 wt and p53$^{-/-}$ cells (48, 72 hrs). We found a regressive H_2O_2-induced G_2 checkpoint arrest after 48 hrs in both cell lines, followed by G_1 arrest exclusively in wt cells 72 hrs after treatment (Fig. 1B and C). In addition, wt cells underwent apoptosis starting at 24 hrs, although early apoptosis resistance in p53$^{-/-}$ cells could be overcome by prolongating the recovery phase up to 72 hrs (Fig. 1B–D). Thus, G_2 checkpoint override caused both apoptosis and cell cycle re-entry, leading to G_1 arrest, in wt cells or delayed apoptosis in p53$^{-/-}$ cells. Importantly, the results of Chk1 knockdown (Fig. 1B and C) were as follows: (*i*) 52% and 57% reduced G_2 checkpoint arrest at 48 hrs in wt and p53$^{-/-}$ cells, respectively and (*ii*) abrogation of G_1 arrest at 72 hrs in wt cells and 10% less cells in the G_2/M phase at 72 hrs in p53$^{-/-}$ cells. Notably, a reduction in the cell numbers in the G_1 or G_2/M phases of HCT116 wt or p53$^{-/-}$ at 72 hrs following Chk1 siRNA transfection caused enhanced cell death reflected by increased Pre-G_1 cell populations (Fig. 1B and C). In summary, the override of the G_2 checkpoint arrest in HCT116 wt and p53$^{-/-}$ cells is Chk1 dependent.

Timing of Chk1-involved G_2 arrest impacts on the release back into the cell cycle

To unravel subtle differences in the initial Chk1-involved cell cycle arrest with respect to the p53 status, we associated the subcellular localization of cyclin B1 with G_2 arrest modality. Localization of cyclin B1 in the cytoplasm or the nucleus has been linked to G_2 arrest or an arrest just at the onset of mitosis (late G_2 arrest), respectively [30]. Immunofluorescence analysis of cyclin B1 in H_2O_2-treated wt cells confirmed that in 82% of cells cyclin B1 was located in the cytoplasm (cyt), indicating G_2 arrest (Fig. 1E). In contrast, cyclin B1 was found in the nucleus in 90% of p53$^{-/-}$ cells, allowing for the conclusion that p53$^{-/-}$ cells accumulated in late G_2 just before mitosis (Fig. 1E). To link the timing of G_2 arrest to downstream cell cycle re-entry, we studied the expression of cell cycle regulators (Fig. 2). According to G_2 arrest noticed in wt cells 24 hrs after H_2O_2 treatment, cyclin B1 and cdc2 were up-regulated. However, 72 hrs after H_2O_2 treatment, cells showed up-regulated G_1 arrest marker cyclin D1, whereas the expression of key mitotic markers cyclin B1 and cdc25C was completely abolished, and cdc2 was markedly reduced. In addition, accumulation of p53, p-p53^{Ser15} and p21^{WAF1} especially after 48 and 72 hrs accounted for G_1 arrest. Because we could not observe increased levels of mitosis-specific p-H3^{Ser10}, we concluded that wt cells had not entered mitosis. These results suggest that after Chk1-involved G_2 arrest, wt cells may have re-entered the cell cycle in G_1 phase through mitotic slippage.

In contrast, p53$^{-/-}$ cells did not completely repress key mitotic regulators cyclin B1 and cdc25C at 72 hrs, indicating mitotic entry, which was confirmed by p-H3^{Ser10} immunoblotting (Fig. 2). Altogether, p53$^{-/-}$ cells recovered from Chk1-involved late G_2 arrest by re-entering the cell cycle in the M phase, and damaged cells proceeded through mitosis to apoptosis, resulting in mitotic catastrophe. This idea is also supported by low levels of p21^{WAF1}.

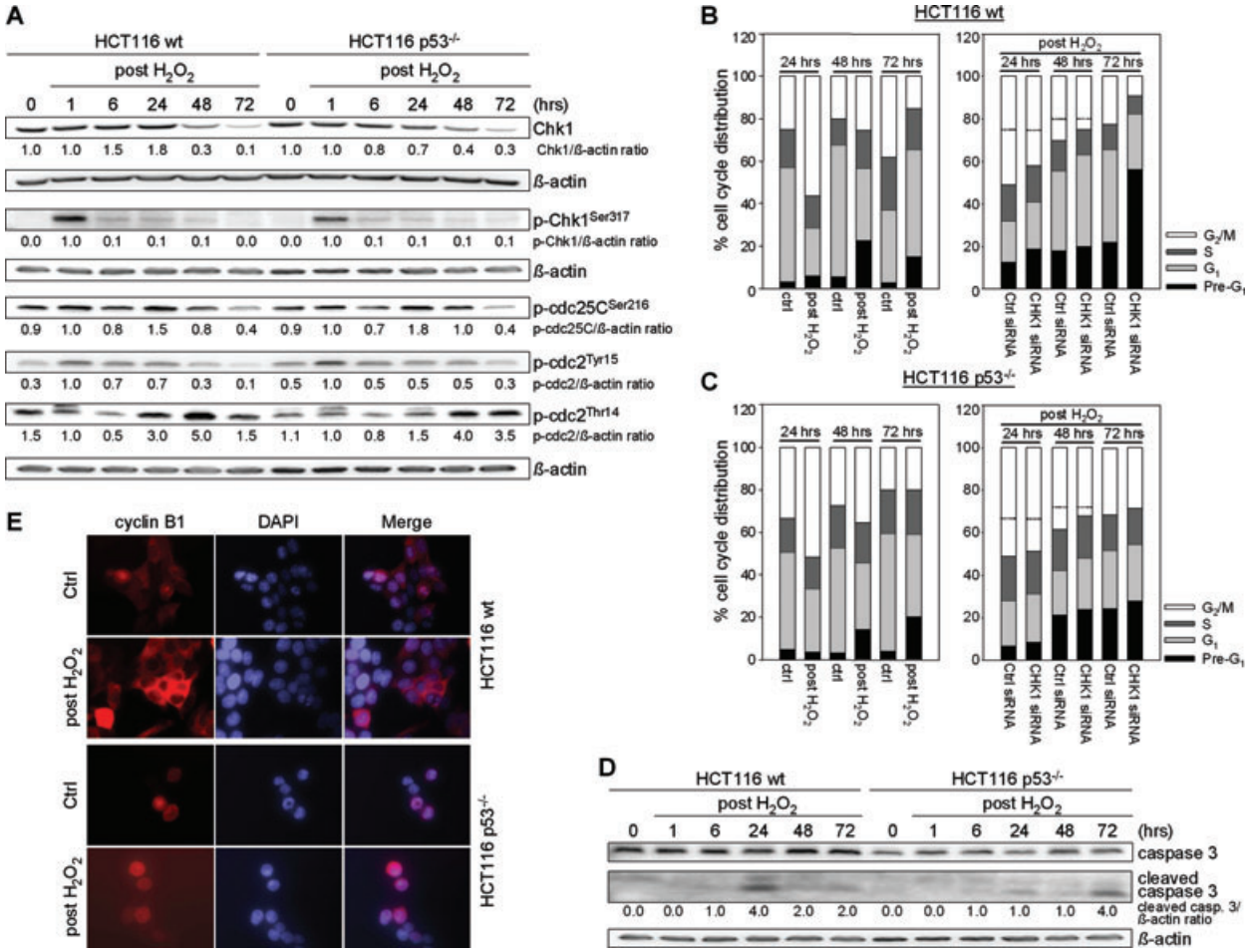

Fig. 1 H_2O_2 treatment induces establishment and override of Chk1-involved G_2 checkpoint arrest in HCT116 wt and p53$^{-/-}$ cells. (**A**) After H_2O_2 treatment (30 mM, 3 min.), G_2 checkpoint arrest is established *via* Chk1 involvement reflected by the accumulation of active p-Chk1^{Ser317}, inactive p-cdc25C^{Ser216} and p-cdc2^{Thr14}. Whole cell lysates were processed for Western blot analysis and probed with indicated antibodies. β-actin served as loading control. Fold expression changes are given below the blots. (**B**, **C**) G_2 checkpoint override causes Chk1-dependent long-term DNA damage responses in HCT116 wt (**B**, G_1 arrest) and p53$^{-/-}$ cells (**C**, apoptosis). FACS analyses were performed at 24, 48 and 72 hrs after treatment. Twenty-four hours after transfection with Chk1 siRNA, cells were treated with 30 mM H_2O_2 for 3 min. and grown for 24, 48 or 72 hrs. A control siRNA was used as a negative control for targeted siRNA transfection. Differentially gated cell populations were counted; their percentage in the total cell populations was calculated and presented in the diagram. Dashed lines contribute to cell cycle analysis without H_2O_2 treatment to mark H_2O_2-induced G_2 arrest as well as reduced G_2 arrest following Chk1 siRNA transfection. Data are means of three independent experiments. (**D**) Whole cell lysates were subjected to caspase 3 Western blot analysis. β-actin served as loading control. Fold expression changes are given below the blots. (**E**) Analysis of cyclin B1 localization 24 hrs after treatment revealed its dominant cytoplasmic localization in wt cells (G_2 arrest) and its dominant nuclear localization in p53$^{-/-}$ cells (late G_2 arrest). Cells were fixed, subsequently stained with anti-cyclin B1, and counterstained with DAPI.

Role of p53 in Chk1-dependent long-term DNA damage responses

To link the observed long-term cellular responses to the extent of DNA damage, we placed emphasis on the question of how the damage sensor p53 deals with the initial signal over time. For this, H_2O_2-induced single strand DNA breaks (SSB) and double strand DNA breaks (DSB) were analysed in HCT116 wt and p53$^{-/-}$ cells using the comet assay. Cells treated with H_2O_2 showed clearly visible comet tails of DNA fragments indicative of broken DNA strands, independently of the p53 status as early as 1 and 24 hrs after H_2O_2 treatment (Fig. 3A). In addition, we observed remarkable nuclear γ-H2AX foci formation in both cell lines already after 1 hr [31, 32], (Fig. 3B). However, γ-H2AX immunoblotting showed accumulated DNA damage until 48 hrs in wt cells, whereas it decreased at 72 hrs, suggesting DNA repair (Fig. 3C). In accordance, we observed accumulation of p-p53^{Ser15} in wt cells in response to DNA damage [33], (Fig. 2).

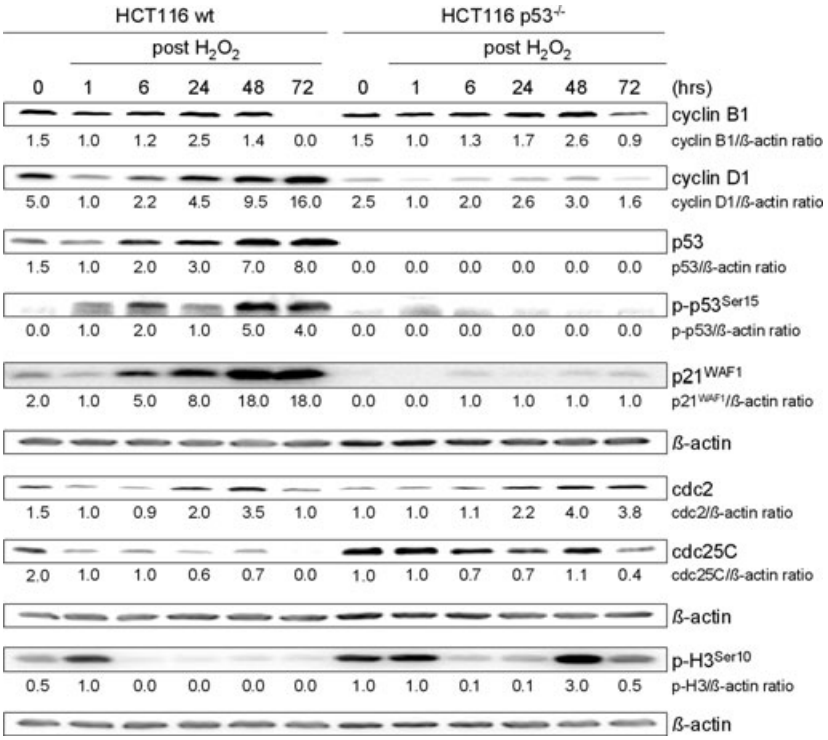

Fig. 2 H_2O_2 treatment alters expression of cell cycle regulatory proteins in HCT116 wt and $p53^{-/-}$ cells. Cells were treated with 30 mM H_2O_2 for 3 min. and further grown for 1 hr up to 72 hrs. Whole cell lysates were analysed by Western blot and probed with indicated antibodies. β-actin was used to control protein loading. Fold expression changes are given below the blots.

In contrast, $p53^{-/-}$ cells accumulated DNA damage over time as indicated by processed expression of γ-H2AX (Fig. 3C). In this context, we also observed PARP cleavage (Fig. 3C), which reflects an inactivation of the enzyme, destroying its ability to respond to DNA strand breaks. Furthermore, we observed a dramatic increase in the amount of oxidative damage to proteins in $p53^{-/-}$ cells (Fig. 3D), which is in accordance with the observation that these cells failed to repair DNA damage. Overall, $p53^{-/-}$ cells undergo premature mitosis as their DNA is largely damaged, which drives these cells into mitotic catastrophe, whereas wt cells with repairable DNA damage slip through mitosis to finally arrest in G_1.

Contribution of $p21^{WAF1}$ to Chk1-dependent long-term DNA damage responses

To refer the G_2 regulator $p21^{WAF1}$ a role in recovery from G_2 checkpoint arrest, we investigated H_2O_2-induced signalling in $p21^{-/-}$ cells. We also observed G_2 checkpoint activation (Fig. 4A): Chk1 was activated 1 hr after treatment, cdc25C was phosphorylated at Ser216 at 24 hrs, and subsequently, $pcdc2^{Tyr15}$ and $pcdc2^{Thr14}$ accumulated at 24 hrs. Importantly, $p21^{-/-}$ cells did not arrest 48 hrs after H_2O_2 treatment (Fig. 4B), suggesting that $p21^{WAF1}$ may retain cells in G_2. Apoptosis induction could be observed from 24 to 72 hrs (Fig. 4A and B). Simultaneously, mitotic key regulators cdc2, cdc25C and cyclin B1 were not completely down-regulated at 72 hrs in $p21^{-/-}$ cells (Fig. 4C). Chk1 knockdown revealed abrogation of G_2 checkpoint arrest at 24 hrs and 11% and 12% fewer cells in the G_2/M phase along with increased Pre-G_1 cell populations at 48 and 72 hrs (Fig. 4B).

Investigating cyclin D1 and $p\text{-}H3^{Ser10}$ levels, we confirmed that $p21^{-/-}$ cells did not enter G_1 phase but mitotic prophase at 48 hrs and obviously stayed there, which might result in apoptotic mitosis (Fig. 4C). Immunofluorescence analysis of cyclin B1 revealed its dominant nuclear localization 24 hrs after H_2O_2 treatment (Fig. 4D, late G_2 arrest). Collectively, these data demonstrate that premature mitotic entry cumulates in mitotic catastrophe on the basis of Chk1-dependent late G_2 arrest in the presence of accumulated DNA damage as shown by γ-H2AX immunoblotting (Fig. 4C).

Chk1 navigates senescence and mitotic catastrophe during recovery from G_2 checkpoint arrest

We found that wt cells establish a G_1 arrest following Chk1-involved G_2 arrest, which was associated with a senescent phenotype as shown by staining for β-galactosidase activity (Fig. 5A). In addition, wt cells showed the characteristic flattened and enlarged morphology in the majority of cells (Fig. 5A) together with prolonged expression of p53 and $p21^{WAF1}$ (Fig. 2). In contrast, the absence of $p53/p21^{WAF1}$ promotes premature mitosis after Chk1-involved or Chk1-dependent late G_2 arrest, and therefore, cells entered apoptosis. According to this, microscopic examination revealed H_2O_2-induced formation of multinucleated $p53^{-/-}$ and $p21^{-/-}$ cells, which are characteristic of the mitotic catastrophic state (Fig. 5A). However, both cellular outcomes followed an upstream Chk1

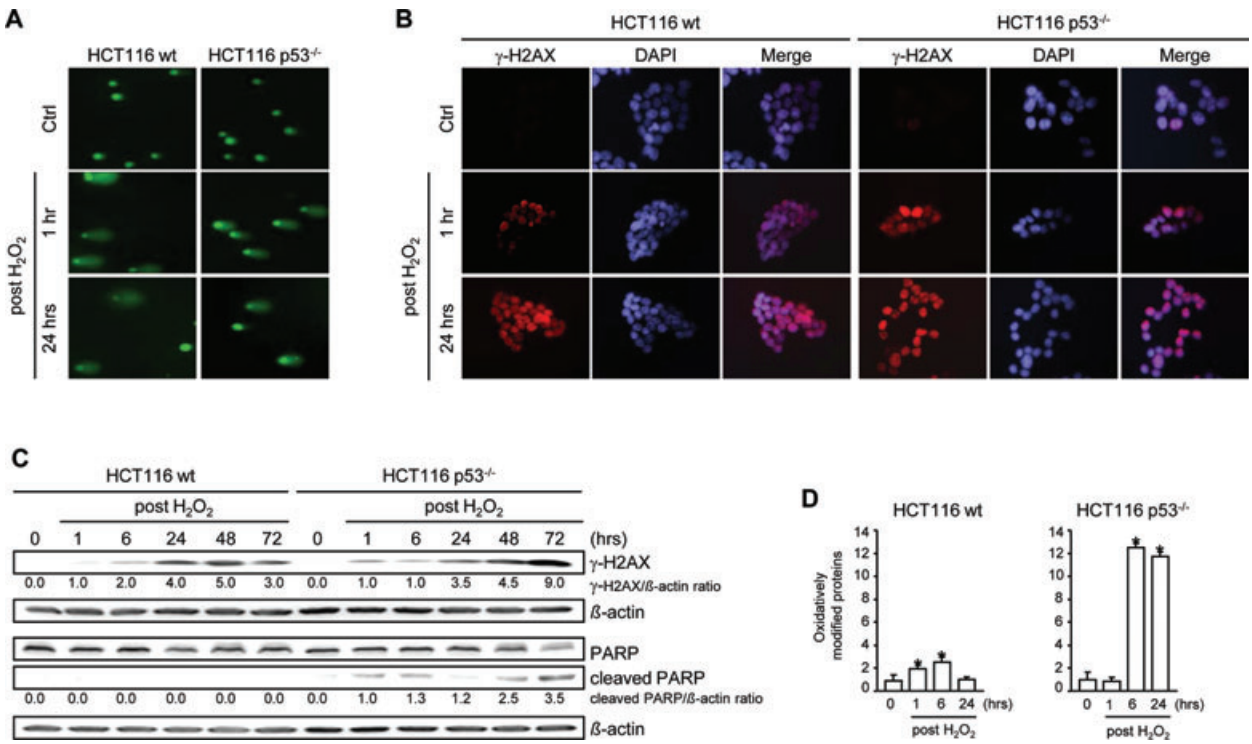

Fig. 3 Role of p53 in Chk1-dependent long-term DNA damage responses. (**A**) Comet assay analysis of nuclear DNA in HCT116 wt and p53$^{-/-}$ cells revealed DNA strand breaks in cells exposed to 30 mM H_2O_2 for 3 min. and further grown for 1 hr and 24 hrs. (**B**) Formation of γ-H2AX foci in H_2O_2-treated wt and p53$^{-/-}$ cells. Cells were fixed and subsequently stained with anti-γ-H2AX and counterstained with DAPI. (**C**) Accumulation of γ-H2AX in H_2O_2-treated wt and p53$^{-/-}$ cells and PARP cleavage in p53$^{-/-}$ cells. Whole cell lysates were subjected to Western blot analysis. β-actin was used to control protein loading. Fold expression changes are given below the blots. (**D**) Effect of H_2O_2 treatment on protein modification in HCT116 wt and p53$^{-/-}$ cells. Cells were treated with 30 mM H_2O_2 for 3 min. Proteins having undergone oxidative modifications were detected after 1, 6 and 24 hrs after H_2O_2. Data are means ± S. D. of three independent experiments. *, $P < 0.05$ *versus* untreated cells.

activation, suggesting that mitotic catastrophe and senescence may be functionally linked with upstream Chk1 signalling. To mechanistically investigate if the upstream Chk1 pathway directs mitotic catastrophe and senescence, we performed knockdown experiments of Chk1 in all three isogenic cell lines. Our results show that Chk1 knockdown significantly decreased the expression of p-Chk1^{Ser317} in wt cells 1 hr after H_2O_2 treatment (Fig. 5B). Remarkably, the late expression (72 hrs) of G_1 arrest-associated p-p53^{Ser15}, p21^{WAF1} and cyclin D1 was significantly reduced following Chk1 knockdown (Fig. 5B). Consequently, as G_1 arrest was shown to be Chk1 dependent (Fig. 1B), Chk1 knockdown reverses the senescent phenotype of wt cells (Fig. 5E). Interestingly, abrogation of G_1 arrest at 72 hrs was accompanied by increased Pre-G_1 cell population (Fig. 1B). However, as no increase in cleaved caspase 3 could be observed following Chk1 knockdown, we suggest caspase-independent cell death at 72 hrs (Fig. 5B). In summary, we speculate that it is Chk1 that determines downstream senescence in wt cells through activating p53, which induces p21^{WAF1}.

Analogically, Chk1 knockdown also reduced the expression of p-Chk1^{Ser317} in p53$^{-/-}$ cells 1 hr after H_2O_2 treatment and caused time-delayed down-regulation of mitotic p-H3^{Ser10}, cdc2 and cyclin B1 after 72 hrs (Fig. 5C). In this context, cell cycle analysis revealed fewer cells in the G_2/M phase at 72 hrs following Chk1 siRNA transfection (Fig. 1C). In addition, inhibition of Chk1 by siRNA resulted in an increase in cell death as seen by 1.2-fold increase in Pre-G_1 cell population (Fig. 1C) and by 2.5-fold increase in caspase 3 cleavage (Fig. 5C). In summary, we suppose that upstream Chk1 activation directs mitotic catastrophe in the absence of p53.

Interestingly, Chk1 knockdown reduced the expression of p-Chk1^{Ser317} in p21$^{-/-}$ cells, but significantly increased the expression of mitotic p-H3^{Ser10}, cdc2, cyclin B1 and cleaved caspase 3 (Fig. 5D). Cell cycle analysis showed fewer cells in the G_2/M phase, but 1.7-fold increased Pre-G_1 cell population (Fig. 4B). Thus, we presume that upstream Chk1 activation protects cells lacking p21^{WAF1} from stronger mitotic catastrophe, which would result in increased cell death. Indeed, p21$^{-/-}$ cells showed increased multinucleation following Chk1 siRNA transfection, whereas Chk1 knockdown reverses the multinucleated phenotype of p53$^{-/-}$ cells (Fig. 5E).

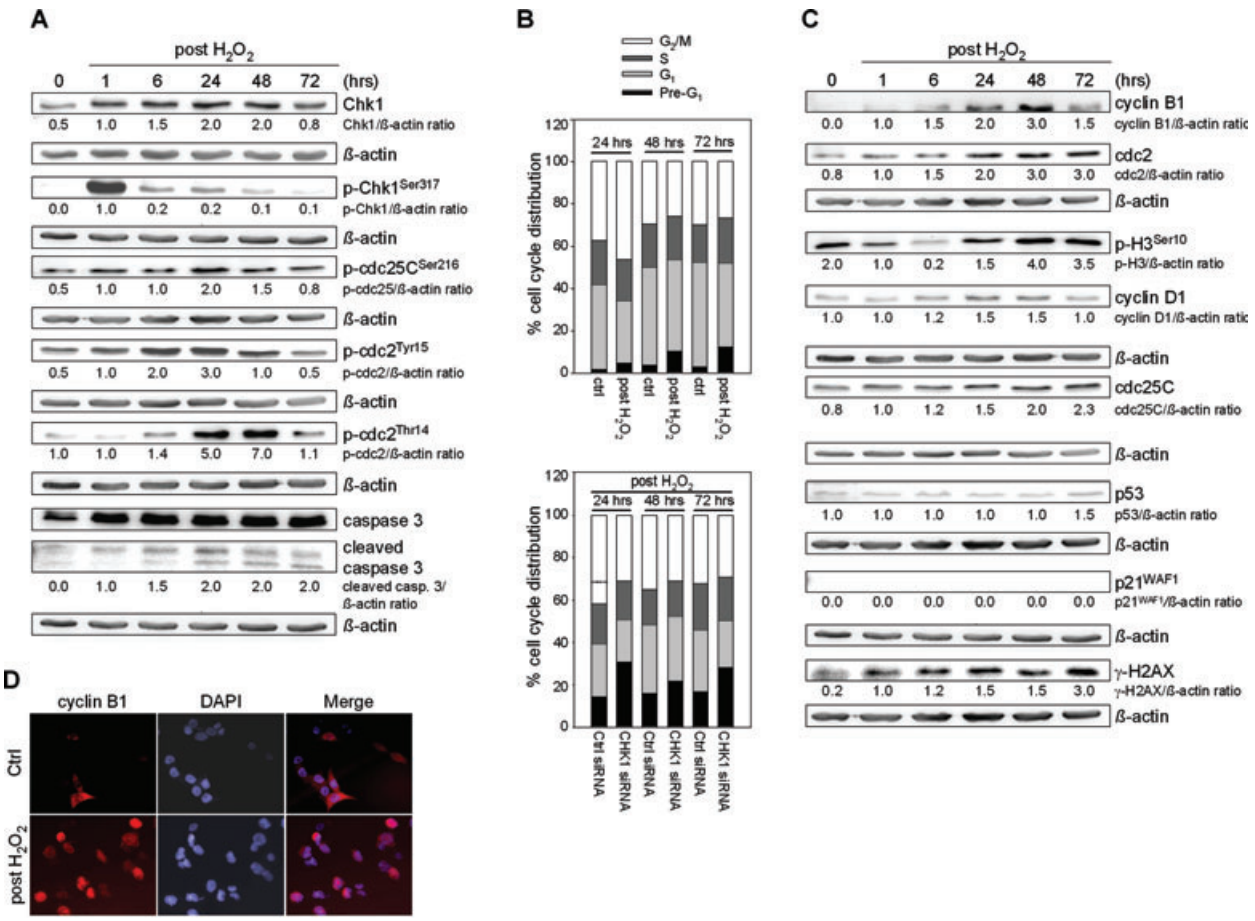

Fig. 4 Analysis of establishment and override of Chk1-dependent G_2 checkpoint arrest in HCT116 $p21^{-/-}$ cells. (**A**) H_2O_2 treatment (30 mM, 3 min.) induces establishment of G_2 checkpoint arrest in $p21^{-/-}$ cells *via* the Chk1 pathway. H_2O_2 also induces apoptosis as indicated by the expression level of cleaved caspase 3. Whole cell lysates were subjected to Western blot analysis. β-actin was used to control protein loading. Fold expression changes are given below the blots. (**B**) G_2 checkpoint override causes increased Chk1-dependent apoptosis as indicated by Pre-G_1 cell population. FACS analyses were performed at 24, 48 and 72 hrs after treatment. Twenty-four hours after transfection with Chk1 siRNA, cells were treated with 30 mM H_2O_2 for 3 min. and grown for 24, 48 or 72 hrs. A control siRNA was used as a negative control for targeted siRNA transfection. Differentially gated cell populations were counted; their percentage in the total cell populations was calculated and presented in the diagram. The dashed line contributes to cell cycle analysis without H_2O_2 treatment to mark H_2O_2-induced G_2 arrest as well as abrogation of G_2 arrest following Chk1 siRNA transfection. Data are means of three independent experiments. (**C**) H_2O_2 treatment alters expression of cell cycle regulatory proteins in $p21^{-/-}$ cells. Whole cell lysates were subjected to Western blot analysis. β-actin was used to control protein loading. Fold expression changes are given below the blots. (**D**) Analysis of cyclin B1 localization 24 hrs after H_2O_2 revealed its dominant nuclear localization in $p21^{-/-}$ cells (late G_2 arrest). Cells were fixed and subsequently stained with anti-cyclin B1 and counterstained with DAPI.

Chk1 regulates long-term cell fate in recovery from G_2 checkpoint arrest also in HT29 cells

In order to strengthen the conclusion that Chk1 directs recovery from G_2 checkpoint arrest and therefore cell cycle re-entry, we performed key experiments also in the p53 mutant colorectal cancer cell line HT29. We found G_2 checkpoint activation (Fig. 6A): Chk1 was activated 1 hr after treatment, and cdc25C was phosphorylated at Ser216 at 1, 24, 48 and 72 hrs. Consequently, we observed accumulation of $pcdc2^{Tyr15}$ and $pcdc2^{Thr14}$. Cell cycle analysis revealed the establishment of G_2 checkpoint arrest at 24 hrs, which was regressive at 48 and 72 hrs, but was still maintained until 6 days (Fig. 6B). Thus, the override of the first G_2 checkpoint arrest was not complete in HT29 cells, suggesting biphasic G_2 checkpoint arrest. As Chk1 knockdown revealed no reduced G_2 checkpoint arrest at 24 hrs (Fig. 6B), we presume that the establishment of the first G_2 checkpoint arrest does not proceed under Chk1 participation despite Chk1 activation. Notably, Chk1 siRNA transfection showed 17%, 24% and 30% reduced G_2 checkpoint arrest at later time-points (48, 72 hrs, 6 days),

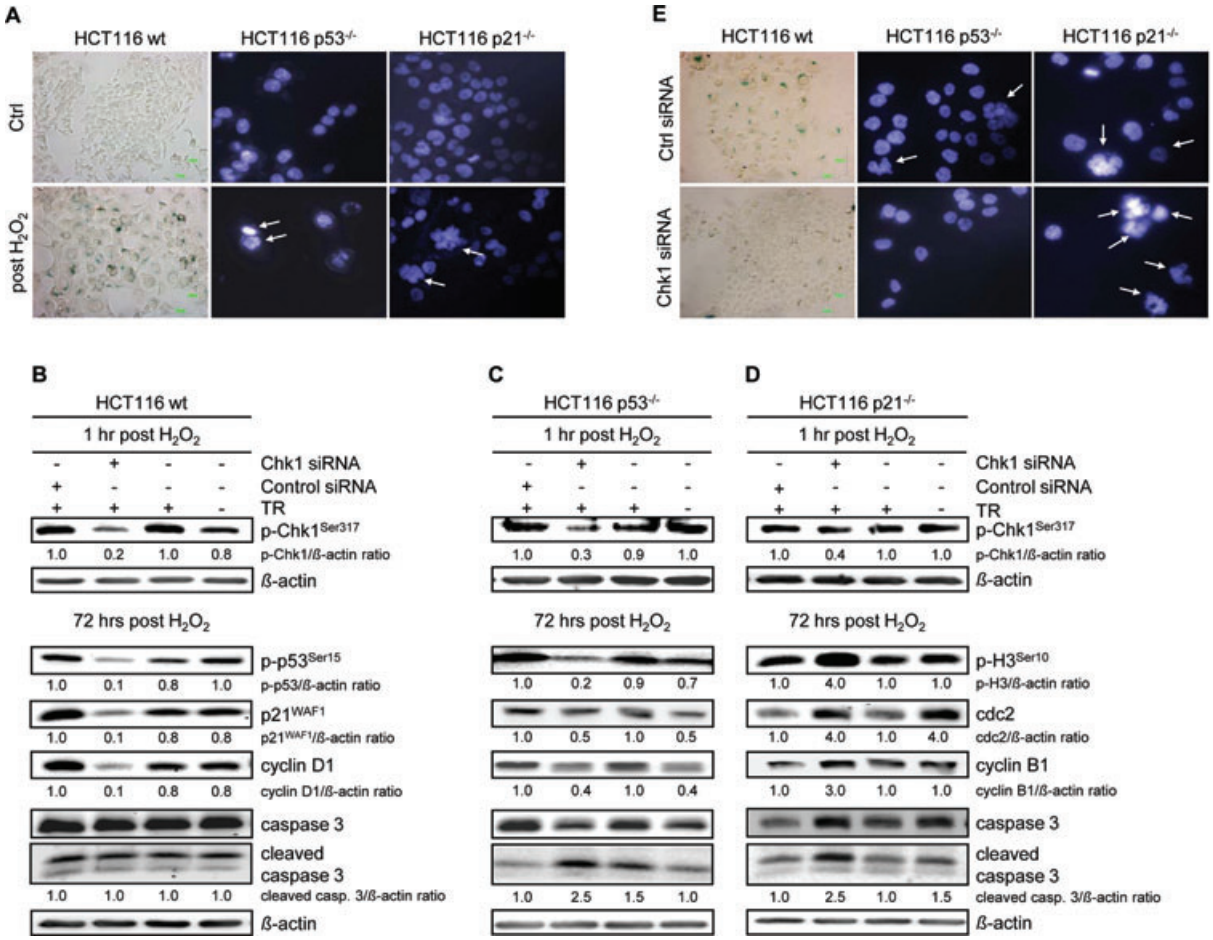

Fig. 5 Chk1 directs downstream senescence and mitotic catastrophe in HCT116 wt and $p53^{-/-}$ or $p21^{-/-}$ cells after H_2O_2 treatment. (**A**) HCT116 wt cells became senescent after they were treated with 30 mM H_2O_2 for 3 min. and grown for 72 hrs. Cells were fixed and subsequently stained for β-galactosidase activity. Cells grew larger, assumed a flattened shape and expressed senescence-associated β-galactosidase (blue areas). In contrast, $p53^{-/-}$ and $p21^{-/-}$ cells went into mitotic catastrophe 72 hrs after treatment. Cells were fixed and stained with DAPI. Multinucleation is marked. (**B**)–(**D**) Twenty-four hours after transfection with Chk1 siRNA, cells were treated with H_2O_2 and further analysed 1 and 72 hrs after H_2O_2 treatment. (**B**) Chk1 navigates HCT116 wt cells to senescence. Down-regulation of p-$Chk1^{Ser317}$ in wt cells causes time-delayed down-regulation of senescence-associated G_1 arrest markers p-$p53^{Ser15}$, $p21^{WAF1}$ and cyclin D1 after 72 hrs. (**C**) Chk1 navigates HCT116 $p53^{-/-}$ cells to mitotic catastrophe. Down-regulation of p-$Chk1^{Ser317}$ in $p53^{-/-}$ cells causes down-regulation of mitotic markers p-$H3^{Ser10}$, cdc2 and cyclin B1 after 72 hrs. (**D**) Chk1 navigates HCT116 $p21^{-/-}$ cells to mitotic catastrophe. Down-regulation of p-$Chk1^{Ser317}$ in $p21^{-/-}$ cells causes up-regulation of mitotic markers p-$H3^{Ser10}$, cdc2 and cyclin B1 after 72 hrs. The transfection medium alone (TR) and a control siRNA were used as negative controls for targeted siRNA transfection. Whole cell lysates were subjected to Western blot analysis. Fold expression changes are given below the blots. β-actin was immunoblotted to control protein loading. (**E**) Chk1 knockdown affects cell morphology of H_2O_2-treated HCT116 cells. Twenty-four hours after transfection with Chk1 siRNA, cells were treated with 30 mM H_2O_2 for 3 min. and grown for 72 hrs. Cells were fixed and subsequently stained for β-galactosidase activity (blue areas: wt cells) or with DAPI (multinucleation is marked: $p53^{-/-}$, $p21^{-/-}$ cells). A control siRNA was used as a negative control for targeted siRNA transfection.

respectively (Fig. 6B). Thus, both incomplete recovery from first G_2 checkpoint arrest and second, prolonged G_2 checkpoint arrest are Chk1 dependent in HT29 cells. Focusing on recovery from first G_2 checkpoint arrest, apoptosis induction (Fig. 6A and B) and multinucleation (Fig. 6C) could be observed at 72 hrs. In addition, mitotic key regulators cdc2, cyclin B1 and p$H3^{Ser10}$ were not completely down-regulated at later time-points (Fig. 6A). Thus, we presume that DNA-damaged HT29 cells enter premature mitosis in recovery from first G_2 checkpoint arrest, which cumulates in mitotic catastrophe. This process may also be directed through Chk1. Indeed, following Chk1 siRNA transfection, we observed that (*i*) multinucleation as a sign of mitotic catastrophe (Fig. 6C) and (*ii*) expression of mitotic markers (Fig. 6D) at 72 hrs were Chk1 dependent. In summary, in HT29 cells, Chk1 also directs recovery from G_2 checkpoint arrest and therefore the long-term DNA damage response mitotic catastrophe.

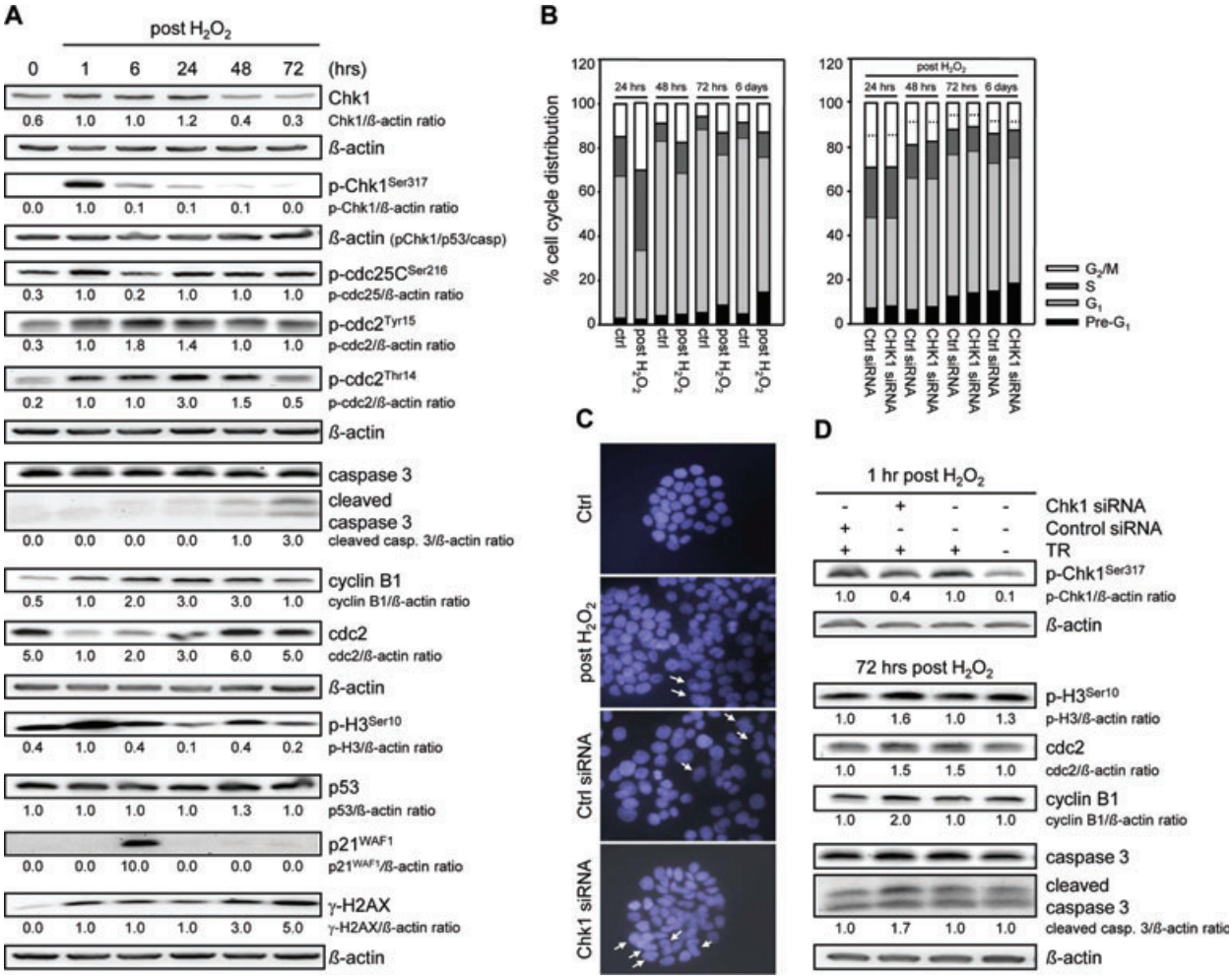

Fig. 6 Chk1 regulates long-term DNA damage response in HT29 cells. (**A**) H_2O_2 treatment causes activation of the Chk1 pathway, apoptosis induction and altered expression of cell cycle regulatory proteins in HT29 cells. Whole cell lysates were processed for Western blot analysis and probed with indicated antibodies. β-actin served as loading control. Fold expression changes are given below the blots. (**B**) G_2 checkpoint override causes Chk1-dependent apoptosis induction in HT29 cells. FACS analyses were performed at 24, 48, 72 hrs, and 6 days after treatment. Twenty-four hours after transfection with Chk1 siRNA, cells were treated with 30 mM H_2O_2 for 3 min. and grown until 6 days. A control siRNA was used as a negative control for targeted siRNA transfection. Differentially gated cell populations were counted; their percentage in the total cell populations was calculated and presented in the diagram. Dashed lines contribute to cell cycle analysis without H_2O_2 treatment to mark H_2O_2-induced G_2 arrest as well as reduced G_2 arrest following Chk1 siRNA transfection. Data are means of three independent experiments. (**C**) Chk1 knockdown affects cell morphology of H_2O_2-treated HT29 cells. Twenty-four hours after transfection with Chk1 siRNA, cells were treated with 30 mM H_2O_2 for 3 min. and grown for 72 hrs. Cells were fixed and subsequently stained with DAPI. Multinucleation is marked. A control siRNA was used as a negative control for targeted siRNA transfection. (**D**) Chk1 navigates HT29 cells to mitotic catastrophe. Down-regulation of p-Chk1^{Ser317} in HT29 cells causes up-regulation of mitotic markers p-H3^{Ser10}, cdc2 and cyclin B1 after 72 hrs. The transfection medium alone (TR) and a control siRNA were used as negative controls for targeted siRNA transfection. Whole cell lysates were subjected to Western blot analysis. Fold expression changes are given below the blots. β-actin was immunoblotted to control protein loading.

Discussion

The cellular response to DNA damage involves both activation of signalling pathways, known as checkpoints, and repair of DNA lesions. The G_2 checkpoint prevents mitotic entry in the presence of DNA damage. Conceptually, DNA damage checkpoint arrest can be divided into three phases: initiation, maintenance during repair and release back to the cell cycle. Although many studies concentrate on checkpoint initiation, there is increasing interest in the question of how the checkpoint is maintained and then switched off. Therefore, we focused on the role of Chk1 in recovery from DNA damage-induced G_2 checkpoint arrest monitoring cell cycle re-entry. Our approach regarding short-term, high bolus H_2O_2

exposure allowed us, besides epigenetic investigations [26], to dissect and unravel cell responses, such as senescence and mitotic catastrophe, in recovery from G_2 checkpoint arrest. At first glance similarly, exposure of HCT116 wt cells to the anticancer agents doxorubicin and irinotecan induced senescence, whereas absence of p53/p21^{WAF1} led to mitotic catastrophe [34, 35]. However, Chang *et al.* demonstrated the establishment of these treatment responses rather during drug exposure than after drug removal. One should keep in mind that long-term drug exposure without additional repeat could be equal to a catabolized substance, thus resembling the observation of recovery from checkpoint arrest. However, both studies did not link the observed treatment responses to Chk1 signalling. In addition, Macip *et al.* demonstrated a role of Chk1 in oxidative stress-induced G_2 checkpoint arrest atypically associated with a senescent-like phenotype in p53-null human bladder cancer cells [36]. However, our data demonstrate a classic Chk1-mediated G_1 arrest associated with senescence in recovery from G_2 checkpoint arrest. Recently, it has been reported that inhibition of heat shock protein HSP90 by geldanamycin pushed irradiated p53 signalling-defective HCT116 cells into premature mitosis [37]. In this context, depletion of the major heat shock protein HSP72 led to defects in Chk1 activation and thus to induction of the senescence program [38]. However, here we show that both mitotic catastrophe and senescent signalling are Chk1 dependent.

Collectively, we could bridge single observations, such as senescence and mitotic catastrophe, in the process of DNA damage response in colorectal cancer cells. We linked them to functional upstream Chk1 activation, which suggests a comprehensive role of Chk1 also in recovery from checkpoint arrest. Moreover, we discovered a role of Chk1 as a 'track builder' for the long-term DNA damage responses, although levels of p53 and p21^{WAF1} finally decide about the fate of the cell. The absence of p21^{WAF1} had a marked effect on DNA damage response, because p21$^{-/-}$ cells fail to maintain G_2 arrest. Therefore, the levels of p21^{WAF1} seem to arrange the duration of the G_2/M arrest supported by p-Chk1^{Ser317}. In addition, p53 plays a key role in DNA repair as shown by the reduced formation of γ-H2AX. According to Banáth *et al.* [39], the inability of the comet assay to detect significant DNA damage at 48 and 72 hrs after H_2O_2 (Fig. S1) suggests that many residual γ-H2AX foci may not be associated with a physical break.

Timing of the upstream Chk1-involved G_2 arrest impacts on long-term DNA damage responses

The checkpoint effector kinase Chk1 mediates temporal cell cycle arrest and allows for successful completion of DNA repair before progressing into mitosis. Initially, in all three isogenic cell lines, H_2O_2 activated the upstream Chk1 pathway as indicated by phosphorylation of cdc25C, thus preventing dephosphorylation of cdc2, holding the cells in the G_2 phase. Interestingly, cdc2 was maintained in its inactive state by Tyr15-phosphorylation, possibly by Wee1 kinase in p21$^{-/-}$ cells, whereas in wt and p53$^{-/-}$ cells, cdc2 was phosphorylated mainly at Thr14, indicating participation of the Myt1 pathway. In addition, we observed down-regulation of un-phosphorylated Chk1 in HCT116 wt and p53$^{-/-}$ cells at later time-points, whereas Chk1 protein levels retained nearly unchanged in p21$^{-/-}$ cells. Thus, we suggest a p21^{WAF1}-dependent Chk1 down-regulation as it was reported from Gottifredi *et al.* [40]. Moreover, we observed a transient decrease of the levels in the Chk1 pathway proteins pcdc25C^{Ser216}, pcdc2^{Tyr15} and pcdc2^{Thr14} at 6 hrs in wt and p53$^{-/-}$ cells, and this was paralleled by increased p21^{WAF1} levels. As increased levels of Chk1 pathway proteins were found in p21$^{-/-}$ cells, we suggest that if G_2 checkpoint activation proceeds *via* p21^{WAF1}, it may negatively regulate not only Chk1 expression [40] but also Chk1 signalling. Indeed, initiating G_2 checkpoint arrest seems to be exclusively mediated *via* p21^{WAF1} in wt cells [26], although we observed Chk1-dependent G_2 checkpoint arrest at 6 hrs in p21$^{-/-}$ cells (data not shown).

Although Chk1 was activated rapidly in all three cell lines, the duration of activation was different. p21$^{-/-}$ cells showed sustained levels of key mitotic regulators cdc2 and cdc25C. As a consequence, cells re-entered the cell cycle in the M phase after Chk1-dependent late G_2 arrest as indicated by nuclear cyclin B1 accumulation. p53$^{-/-}$ cells retained moderate levels of the key mitotic proteins until 72 hrs, and accumulated cyclin B1 in the nucleus, also indicating mitotic re-entry after Chk1-involved late G_2 arrest. Obviously, mitotic catastrophe requires the activation of cdc2, and it is currently assumed that premature entry of active cdc2-cyclin B1 complex into the nucleus suffices to cause premature chromatin condensation and apoptosis [41, 42]. In accordance with this, our data imply that promotion of cyclin B1 nuclear localization after H_2O_2-induced DNA damage resulting in late G_2 arrest is one of the mechanisms responsible for the recovery from G_2 checkpoint arrest causing re-entry into mitosis. Here, we link this knowledge to upstream Chk1 activation, which directs cells into mitotic catastrophe following premature mitotic re-entry. In contrast, wt cells nearly completely repressed key mitotic regulators at later time-points and did not show p-H3^{Ser10} protein accumulation. This suggests that they did not enter mitosis, but re-entered and arrested in G_1 in association with senescence, although this long-term DNA damage response was shown to be also Chk1 dependent. Collectively, the G_2 arrest modality during checkpoint initiation had an impact on the subsequent release back into the cell cycle, whereas classic G_2 arrest correlated with senescence, and late G_2 arrest caused mitotic catastrophe. Thus, the timing of Chk1-involved G_2 arrest determines recovery from oxidative DNA damage-induced G_2 checkpoint arrest to allow cell cycle re-entry in the G_1 or M phases, which finally led to senescence or mitotic catastrophe.

Recovery from oxidative DNA damage-induced G_2 arrest requires Chk1

Performing Chk1 knockdown, we revealed that Chk1 defines long-term DNA damage responses. In the case of senescence, Chk1 is required for recovery and re-entry to the cell cycle following H_2O_2-induced G_2 arrest, and the further establishment of the classic

senescent arrest in G_1 also seems to be dependent at least in part on an intact Chk1-dependent checkpoint, because p-Chk1^{Ser317} down-regulation markedly reduced G_1-associated arrest markers p-p53^{Ser15}, p21^{WAF1} and cyclin D1. Consequently, as G_1 arrest is associated with senescence, cell morphology of wt cells could be restored following Chk1 siRNA transfection. Therefore, we presume that Chk1 may determine senescence in wt cells through transmitting the DNA damage signal to downstream G_1 checkpoint by phosphorylating p53 at Ser15, which induces p21^{WAF1}, thereby inhibiting cdk2-cyclin E complexes.

As p53 function is often lost in human cancers, it still needs to be clarified which role Chk1 plays in the scenario of long-term DNA damage responses following G_2 checkpoint override in the absence of p53/p21^{WAF1} after upstream late G_2 arrest. Here, we show that the upstream Chk1 activation protects p53$^{-/-}$ cells from increased cell death by navigating cells into mitotic catastrophe, as p-Chk1^{Ser317} down-regulation markedly reduced the expression of mitotic markers p-H3^{Ser10}, cyclin B1 and cdc2. Consistently, Chk1 knockdown reversed the multinucleated phenotype of p53$^{-/-}$ cells, whereas cleavage of caspase 3 was increased. Interestingly, p-Chk1^{Ser317} down-regulation caused increased expression of p-H3^{Ser10}, cyclin B1, cdc2 and caspase 3 in p21$^{-/-}$ cells. In accordance with this, p21$^{-/-}$ cells showed increased multinucleation following Chk1 knockdown. Therefore, upstream Chk1 activation seems to protect p21$^{-/-}$ cells from stronger mitotic catastrophe.

Collectively, the last few years have seen the development of a large variety of chemical inhibitors to the checkpoint kinase Chk1, reflecting its central function in DNA damage response. Because H_2O_2 mimics ROS-generating DNA-damaging anticancer drugs, it may serve as a basal model for chemotherapy studies. In fact, here we show that knockdown of Chk1 resulted in increased cell death in colorectal cancer cell lines HCT116 wt, p53$^{-/-}$ and p21$^{-/-}$, and HT29 after H_2O_2. In this context, we have recently shown that the pro-oxidant, plant-derived drug thymoquinone induces ROS generation and DNA damage in HCT116 cells, which contributed to apoptosis [29]. In accordance, Chk1 knockdown in HCT116 p53$^{-/-}$ cells sensitized them to thymoquinone-induced apoptosis [29]. This supports the conclusion that Chk1 inhibition is a promising strategy to improve chemotherapy treatment. In addition, we could show that HCT116 wt, p53$^{-/-}$ and p21$^{-/-}$ cells, as well as the p53 mutant HT29 cell line, responded to the cytostatic drug 5-fluorouracil (5-FU) by activating Chk1 (Fig. S2A). γ-H2AX immunoblotting confirmed DNA damage caused by 5-FU. Apoptosis induction could be observed in each cell line (Fig. S2A). As DNA damage is the underlying mechanism of 5-FU's impact, Chk1 knockdown caused increased apoptosis induction (Fig. S2B). Therefore, Chk1 inhibition is a powerful tool to improve also the efficiency of 5-FU.

Proposed model

Recently, we have investigated the establishment of G_2 checkpoint arrest in HCT116 wt and p53$^{-/-}$ cells [26]. In this study, greater emphasis was placed on recovery from arrest by checkpoint override to monitor cell cycle re-entry. The results presented here may answer the question of how Chk1 regulates even long-term DNA damage response in colorectal cancer cells (Fig. 7A and B). H_2O_2-induced DNA damage causes upstream Chk1 stimulation, which activates three DNA damage signalling axes: (*i*) Chk1-involved G_2 arrest, (*ii*) apoptosis induction and (*iii*) a classic senescent arrest in G_1. Importantly, p53 and p21^{WAF1} are not necessary for the G_2 arrest to occur, but p53 and p21^{WAF1} play an important role in (*i*) the blockage of the cell cycle either in G_2 or late G_2, (*ii*) the repair of DNA damage and (*iii*) the fate of long-term DNA damage responses in recovery from G_2 checkpoint arrest, namely mitotic catastrophe or senescence. It is significant that Chk1 activation is the underlying signalling pathway upstream of the long-term DNA damage responses senescence (Fig. 7A) and mitotic catastrophe (Fig. 7B). Chk1 knockdown experiments have shown that re-entry of DNA-damaged cells into the cell cycle is Chk1 dependent, whereas the phase of re-entry is determined by the levels of p53/p21^{WAF1} as follows:

Functional p53/p21^{WAF1}

Firstly, Chk1 knockdown revealed that colorectal cancer cells with functional p53/p21^{WAF1} showed reduced establishment of G_2 checkpoint arrest. Secondly, but most importantly, they failed to arrest in G_1. Thus, in the presence of functional p53/p21^{WAF1}, Chk1 directs re-entry of cells in G_1, and they arrest there and go into senescence as long-term DNA damage response (Fig. 7A: HCT116 wt).

Absence of p53

In the absence of p53, Chk1 knockdown led to reduced establishment, but most notably to significant reduced override of G_2 checkpoint arrest. Conceptually, in the absence of Chk1, cells may not re-enter the cell cycle in the M phase due to missing upstream Chk1-dependent G_2 checkpoint override, but undergo cell cycle death from G_2 phase. Thus, in p53$^{-/-}$ cells, Chk1 directs re-entry of cells in mitosis, which causes mitotic catastrophe as long-term DNA damage response (Fig. 7B: HCT116 p53$^{-/-}$).

Absence of p21^{WAF1}

In the absence of p21^{WAF1}, G_2 checkpoint arrest is realized by Chk1. Thus, Chk1-deficient cells fail to arrest cell cycle progression at first. Then, as a consequence, they go into premature mitosis. This drives DNA-damaged cells into significant mitotic catastrophe. Conceptually, as there is no upstream cell cycle arrest following Chk1 knockdown, cells cannot re-enter the cell cycle. Thus, in the absence of p21^{WAF1}, Chk1 delays and reduces mitotic catastrophe as long-term DNA damage response by upstream G_2 checkpoint arrest (Fig. 7B: HCT116 p21$^{-/-}$).

Presence of mutated p53

To further strengthen our conclusion that Chk1 directs long-term DNA damage responses in colorectal cancer cells, we performed key experiments in the p53 mutant colorectal cancer cell line HT29

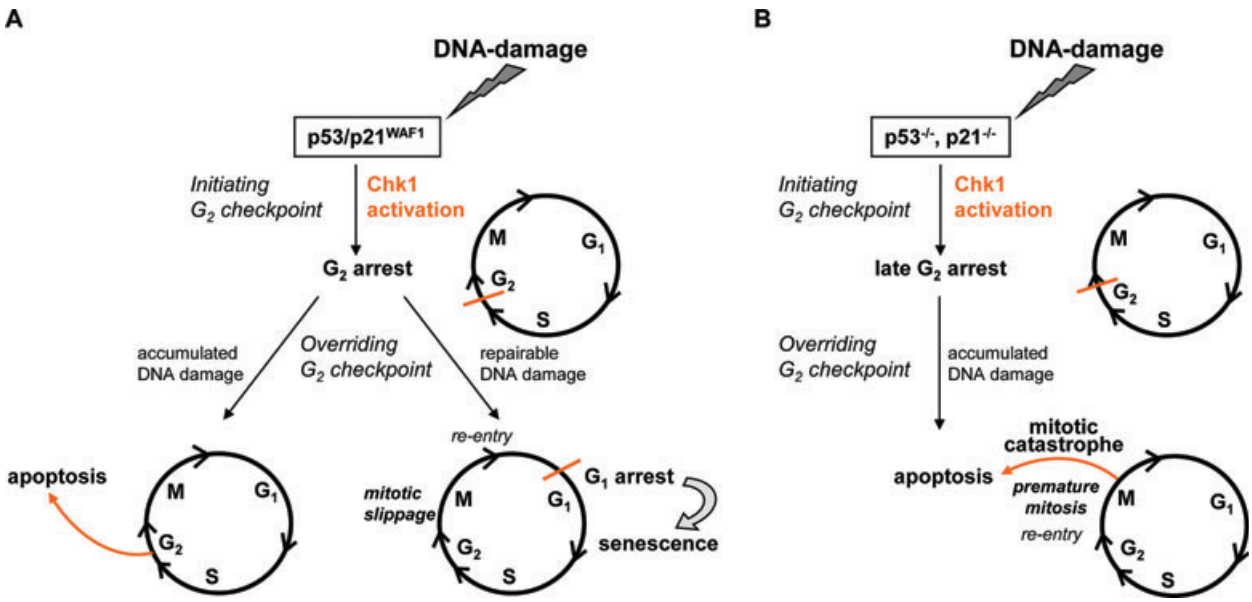

Fig. 7 Proposed model of how Chk1 regulates oxidative DNA damage in HCT116 colorectal cancer cells. (**A**) H_2O_2-induced DNA damage activates Chk1 in wt cells, which prevents progression into mitosis *via* G_2 arrest. Cells with non-repairable DNA damage go into apoptosis, whereas cells with repairable DNA damage slip through mitosis and arrest in G_1 associated with senescence. (**B**) H_2O_2-induced DNA damage activates Chk1 in $p53^{-/-}$/ $p21^{-/-}$ cells, preventing progression into mitosis *via* late G_2 arrest. Because of a lack or low levels of $p53/p21^{WAF1}$, respectively, cells undergo premature mitosis with their DNA largely unrepaired, which drives these cells into mitotic catastrophe. Importantly, Chk1 is required for recovery from G_2 checkpoint arrest, leading to long-term senescence (**A**) and mitotic catastrophe (**B**).

[43]. Our results show that G_2 checkpoint arrest seems to be biphasic, whereas the first phase is not established *via* Chk1, but the second. Due to increased $p21^{WAF1}$ level at 6 hrs, we propose that the establishment of the first G_2 checkpoint arrest is $p21^{WAF1}$ mediated. However, we observed an incomplete override of the first phase which led to G_2 to M transit and cell death during mitosis in the process of mitotic catastrophe (Fig. 7B). Most importantly, both the incomplete G_2 checkpoint override and the reduced, but prolonged, second G_2 checkpoint arrest were Chk1 dependent. Thus, Chk1 protects HT29 cells from stronger mitotic catastrophe by means of downstream Chk1 dependent, prolonged G_2 checkpoint arrest. In conclusion, Chk1 directs recovery from G_2 checkpoint arrest also in HT29 cells, whereas (*i*) the establishment of the first G_2 checkpoint arrest proceeds without involvement of Chk1, (*ii*) the override is not complete, but Chk1 dependent and (*iii*) the second, prolonged G_2 checkpoint arrest is Chk1 dependent.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting information may be found in the online version of this article:

Fig. S1. Comet assay analysis of nuclear DNA in HCT116 wt and $p53^{-/-}$ cells at 48 and 72 hrs. Cells were exposed to 30 mM H_2O_2 for 3 min. and further grown for 48 or 72 hrs.

Fig. S2. Chk1 knockdown improves 5-FU treatment in colorectal cancer cell lines. (**A**) 5-FU (5 μM) induces Chk1-phosphorylation due to DNA damage as shown by increased γ-H2AX expression in HCT116 wt, $p53^{-/-}$ and $p21^{-/-}$ cells, and HT29 cells. 5-FU also induces apoptosis as indicated by the expression level of cleaved caspase 3. Whole cell lysates were subjected to Western blot analysis. Fold expression changes are given below the blots. β-actin was immunoblotted to control protein loading. (**B**) Twenty-four hours after transfection with Chk1 siRNA, cells were treated with 5 μM 5-FU for 48 or 72 hrs. The transfection medium alone (TR) and a control siRNA were used as negative controls for targeted

siRNA transfection. Whole cell lysates were subjected to Western blot analysis. Fold expression changes are given below the blots. β-actin was immunoblotted to control protein loading.

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