

A novel mechanism of checkpoint abrogation conferred by Chk1 downregulation

Zhan Xiao^{*1}, John Xue¹, Thomas J Sowin¹, Saul H Rosenberg¹ and Haiying Zhang^{*1}

¹Cancer Research, Abbott Laboratories, 100 Abbott Park Rd., Abbott Park, IL 60064-6101, USA

Chk1 is the major mediator in the activation of cell-cycle checkpoints in response to a variety of genotoxic stresses. We have previously shown that inhibition of Chk1 sensitizes tumor cells to topoisomerase inhibitors such as camptothecin and doxorubicin through abrogation of cell-cycle arrest (S or G2/M checkpoints). However, it was not clear whether inhibition of Chk1 could potentiate antimetabolites, a mainstay of cancer therapy, which confer genotoxic stress through a different mechanism than topoisomerase inhibitors. 5-Fluorouracil (5-FU) is the most widely used antimetabolite in the treatment of colorectal, breast and other major types of cancers. Here we demonstrate that 5-FU activates Chk1 and induces an early S-phase arrest. Chk1 downregulation abrogates this arrest and dramatically sensitizes tumor cells to the cytotoxic effects of 5-FU. 5-FU confers S-phase arrest through Chk1-mediated Cdc25A proteolysis leading to inhibition of Cdk2. Chk1 elimination stabilizes the Cdc25A protein and results in the abrogation of the S checkpoint and resumption of DNA synthesis, which leads to excessive accumulation of double-stranded DNA breaks. As a result, downregulation of Chk1 potentiates 5-FU efficacy through induction of premature chromosomal condensation followed by apoptosis. Interestingly, the profiles of various cell-cycle markers indicate that cells progress to early Mphase to induce apoptosis after checkpoint abrogation. Yet, cells fail to increase their DNA content to 4N as revealed by FACS analysis, probably due to the dramatic induction of double-stranded DNA breaks and chromosomal fragmentation. This is significantly different from the cell-cycle profiles observed in the potentiation of topoisomerase inhibitors by Chk1 siRNA, which showed mitotic progression with 4N DNA content leading to mitotic catastrophe after abrogation of the S or G2 checkpoint. Thus, our results illustrate a novel mode of checkpoint abrogation and cell death conferred by Chk1 inhibition. Additionally, we show that Chk1 deficiency potentiates 5-FU efficacy through the preferential induction of the caspase-8 pathway and subsequent caspase-3 activation. In conclusion, we have clearly demonstrated that inhibition of Chk1 not only potentiates the toxicity of conventional DNA-damaging agents such as ionizing radiation and topoisomerase inhibitors, but

also enhances the toxicity of antimetabolites in cancer cell lines. This discovery reveals novel scope of checkpoint abrogation and will significantly broaden the potential application of Chk1 inhibitors in cancer therapy if they do not potentiate the toxicity of 5-FU in normal cells.

Oncogene (2005) 24, 1403–1411. doi:10.1038/sj.onc.1208309
Published online 20 December 2004

Keywords: 5-fluorouracil (5-FU); antimetabolites; phospho-histone H2A.X; Chk1; premature chromosomal condensation (PCC); mitotic catastrophe

Introduction

5-Fluorouracil (5-FU) is one of the most widely used antimetabolites in clinical cancer therapy. The mechanism of cytotoxicity of 5-FU is largely through inhibition of the nucleotide synthesis enzyme thymidylate synthase (TS). The 5-FU metabolite FdUMP binds to the nucleotide-binding site of TS, forming a stable complex with the enzyme, thereby blocking the binding of the normal substrate dUMP. This suppresses dTMP synthesis leading to inhibition of DNA replication. Thus cells treated with 5-FU will display replication fork arrest and S-phase block. In addition, two other active metabolites of 5-FU, FUTP and FdUTP, compete with normal pool of nucleotides and incorporate into RNA or DNA to compromise the normal functions of these macromolecules (Longley *et al.*, 2003; Sampath *et al.*, 2003).

Clinically, 5-FU is widely used in the treatment of a range of cancers, including colorectal, breast and the aerodigestive tract cancers. Although 5-FU-based chemotherapy improves survival of patients with certain cancers, the response rates to 5-FU as a first-line treatment for advanced colorectal cancer are only 10–15% (Johnston and Kaye, 2001). The combination of 5-FU with newer chemotherapies such as irinotecan and oxaliplatin has improved the overall response rates to 40%. However, this is achieved at the high expense of increased morbidity and early mortality rates (Longley *et al.*, 2003). Additionally, the combination therapy of interferon- γ with 5-FU and leucovorin has shown signs of success in early stages of clinical trials (Schwartzberg *et al.*, 2002). Despite all these improvements, it is obvious that novel and more effective therapeutic strategies are still urgently needed to enhance the clinical efficacy of 5-FU.

*Correspondence: H Zhang; E-mail: haiying.zhang@abbott.com;

Z Xiao; E-mail: zhan.xiao@abbott.com

Received 15 July 2004; revised 8 October 2004; accepted 20 October 2004; published online 20 December 2004

Checkpoint kinase 1 (Chk1) was first identified in *Schizosaccharomyces pombe* as a mediator of the G2/M checkpoint. It was later found to be a major checkpoint kinase in mammals. Upon DNA damage, Chk1 is activated by ATM/ATR-mediated phosphorylation. Activated Chk1 phosphorylates and inactivates the protein phosphatase Cdc25, hence preventing the activation of its downstream target, the Cdc2 kinase, that is responsible for G2/M transition (Peng *et al.*, 1997; Sanchez *et al.*, 1997). Although Chk1 homozygous knockout is embryonic lethal (Liu *et al.*, 2001), somatic cells with Chk1 knockout are viable and cell-cycle profile does not appear to be perturbed (Chen *et al.*, 2003; Zachos *et al.*, 2003). This suggests that the cellular requirement for Chk1 may be different at various developmental stages.

Recently, we and others have demonstrated that Chk1 mediates both S- and G2-phase checkpoints by targeting Cdc25A to proteolysis following DNA damage (Zhao *et al.*, 2002; Sorensen *et al.*, 2003; Xiao *et al.*, 2003; Zhou and Bartek, 2004), and inhibition of Chk1 potentiates the cytotoxicity of γ -irradiation (IR) and topoisomerase inhibitors such as camptothecin (CPT) and doxorubicin (Dox) (Koniaras *et al.*, 2001; Eastman *et al.*, 2002; Zhao *et al.*, 2002; Xiao *et al.*, 2003). However, it is not clear whether an antimetabolite such as 5-FU, which confers genotoxic stress through a completely distinct mechanism from conventional DNA-damaging agents, can activate the Chk1 pathway, and whether Chk1 inhibition can potentiate the cytotoxicity of 5-FU. Here we report that 5-FU induces Chk1 phosphorylation and activation. Chk1 downregulation enhances 5-FU efficacy by conferring premature chromosomal condensation (PCC) and apoptosis via a novel and nonclassical mode of checkpoint abrogation.

Results

5-FU induces double-stranded DNA breaks and causes Chk1 phosphorylation

In order to ascertain the effect of 5-FU on the Chk1 pathway, we treated HeLa cells with 5-FU for different time points and immunoblotted the cell lysates for Chk1 S317P/S345P (the sites of Chk1 phosphorylation by ATR/ATM) and phospho-H2A.X (a marker for double-stranded DNA breaks) (Sedelnikova *et al.*, 2003) (Figure 1a). As a control, we also treated the cells with etoposide, a topoisomerase II inhibitor that is known to confer double-stranded DNA breaks resulting in the induction of Chk1 phosphorylation and activation. Phosphorylation of Chk1 and H2A.X was observed in cells treated with etoposide as early as 6 h, indicating a rapid induction of the Chk1 pathway and concomitant DNA damage response (Figure 1a, lane 1). Cells treated with 5-FU display generally weaker responses. Only at the 18 h time point did we observe a significant activation of Chk1 and phosphorylation of H2A.X, consistent with the mode of indirect DNA damage conferred by 5-FU.

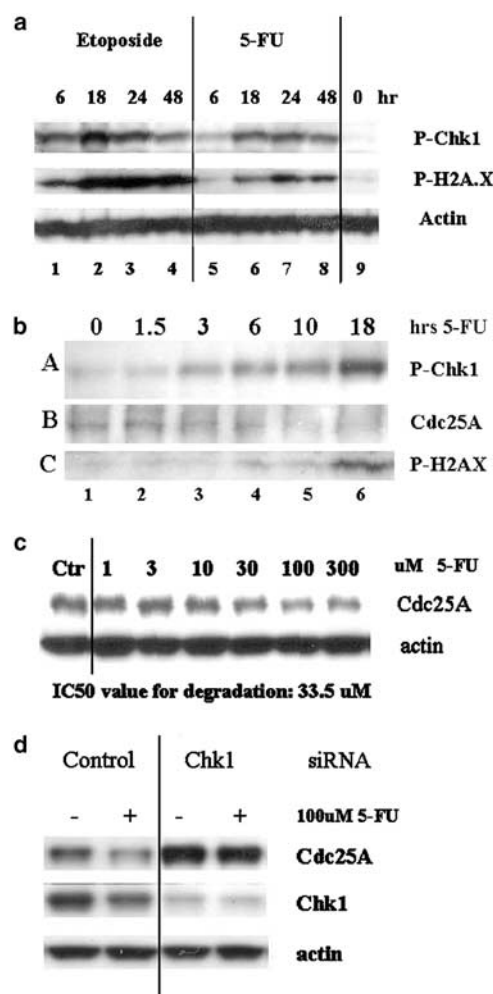


Figure 1 (a, b) 5-FU induces Chk1 phosphorylation, Cdc25A degradation and double-stranded DNA breaks sequentially. (a) HeLa cells were treated with 10 μ M etoposide or 50 μ M 5-FU for 6, 18, 24 and 48 h. Cell lysates were immunoblotted for Chk1 S345P and phospho-histone H2A.X. Actin was used as a loading control. (b) HeLa cells were treated with 50 μ M 5-FU for 0, 1, 3, 6, 10 and 18 h. Cell lysates were immunoblotted for Chk1 S345P, Cdc25A and phospho-histone H2A.X. Actin was used as a loading control. (c, d) 5-FU mediates Cdc25A degradation in a dose-dependent manner, which is abrogated by Chk1 downregulation. (c) H1299 cells were transfected with a small amount of Cdc25A-expressing vector (0.03 μ g/well) to facilitate detection of Cdc25A protein and then treated with the indicated doses of 5-FU for 24 h. Cdc25A level was immunoblotted. (d) H1299 cells expressing Cdc25A were treated with 100 μ M 5-FU for 24 h in the presence of either scrRNA or Chk1 siRNA. Cell lysates were probed for Chk1 and Cdc25A levels

Since 5-FU induces both Chk1 phosphorylation and phospho-H2AX in a similar time frame (significant induction starting at 18 h), it is possible that Chk1 phosphorylation is not a direct effect of 5-FU treatment but rather an indirect consequence of 5-FU-induced DNA damages as revealed by phospho-H2AX induction. To ascertain the exact time course of Chk1 phosphorylation vs phospho-H2AX induction, we additionally analysed more detailed time points of 5-FU treatment: 0, 1, 3, 6, 10 and 18 h (Figure 1b).

Comparison of Chk1 phosphorylation time course with that of phospho-H2AX indicated that the former process occurs more rapidly than the latter event, proving that 5-FU confers Chk1 phosphorylation earlier than DNA damage response.

Since Chk1 phosphorylation may not directly correspond to Chk1 activation, we investigated the downstream effect of 5-FU treatment in HeLa cells to further prove that 5-FU activates the Chk1 pathway. One of the most intrinsic functions of Chk1 in response to genotoxic stress is to promote the ubiquitin-mediated proteolysis of Cdc25A leading to cell-cycle arrest (Zhao *et al.*, 2002; Sorensen *et al.*, 2003; Xiao *et al.*, 2003). To investigate whether Chk1 phosphorylation induced by 5-FU treatment results in Chk1 activation, we analysed the stability of Cdc25A in the same treatment process (Figure 1b, middle panel). It is apparent that Chk1 phosphorylation leads to a corresponding decrease of Cdc25A abundance starting at 3 h time point, indicating that 5-FU not only confers Chk1 phosphorylation but also Chk1 activation.

To ascertain whether the above observations also apply to other cancer cell lines, we treated H1299 cells with increasing doses of 5-FU for 24 h and the cell lysates were analysed for Cdc25A (Figure 1c). Because of the lower endogenous level of Cdc25A, H1299 cells were first transfected with a very small amount of Cdc25A-expressing vector (0.03 μ g/well) to facilitate the detection of Cdc25A protein and then treated with the indicated doses of 5-FU for 24 h. Under this condition, Cdc25A protein was overexpressed about twofold higher than the endogenous level and this does not cause any obvious perturbations of cell cycle or other processes within our experimental time frame. We observed a dose-dependent degradation of the expressed Cdc25A, suggesting that 5-FU induces Chk1 activity to promote the turnover of Cdc25A in various cell lines. Since Cdc25A is the major activator of Cdk2, the kinase responsible for the S-phase transition, the degradation of Cdc25A is expected to induce S-phase arrest. Indeed this is shown to be the case in Figure 4a (lower panels). Furthermore, Chk1 siRNA protects Cdc25A from degradation (Figure 1d), indicating that Chk1 is responsible for the observed proteolysis of Cdc25A. Thus, Chk1 downregulation is expected to abrogate S-phase arrest induced by 5-FU (Figure 4a, upper panels).

Chk1 siRNA enhances 5-FU efficacy

We have demonstrated that 5-FU activates Chk1 resulting in S-phase arrest. To determine whether Chk1 inhibition could sensitize tumor cells to 5-FU toxicity through abrogation of the checkpoint, we treated HeLa cells with either 5-FU or Chk1 siRNA alone, or a combination of both (Figure 2a). Neither Chk1 siRNA alone nor 5-FU alone at the used dose conferred significant extent of cell death as demonstrated by the lack of condensed nuclei (7 and 5%, respectively). In contrast, the combination of 5-FU and Chk1 siRNA caused a dramatic induction of nuclear condensation and cell death (62% of cells with

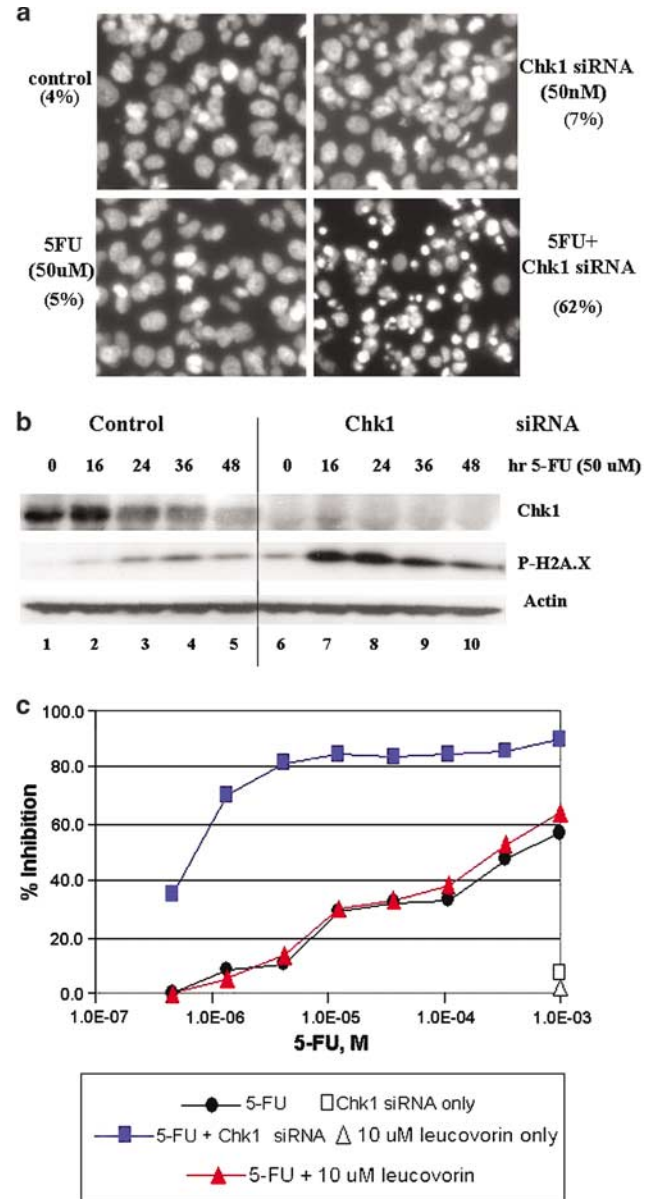


Figure 2 Chk1 siRNA sensitizes HeLa cells to 5-FU toxicity. (a) HeLa cells were treated with DMSO (control), 5-FU (50 μ M), Chk1 siRNA or the combination of 5-FU and siRNA for 48 h. Cells were fixed and stained with Hoescht to visualize nuclear morphology. Apoptotic cells with condensed nuclei were counted and their percentage was calculated. (b) HeLa cells transfected with Chk1 siRNA or control siRNA were treated with 50 μ M 5-FU for different time points. Cell lysates were immunoblotted for phospho-H2A.X. Actin was probed as a loading control. (c) MTS assay was carried out to quantify the enhancing effect of Chk1 siRNA on the cytotoxicity of 5-FU in HeLa cells. Cells in 96-well plates were transfected with either control siRNA (black curve) or Chk1 siRNA (blue curve) and then treated with increasing doses of 5-FU for 48 h. The calculated EC_{50} values for 5-FU toxicity are approximately 452 μ M with control siRNA and 0.81 μ M with Chk1 siRNA

condensed nuclei). This phenomenon is reminiscent of PCC, which describes the abnormal phenomenon of chromosomal condensation in the presence of incomplete DNA replication or unreparable DNA damage

(Nghiem *et al.*, 2001; Chini and Chen, 2003). Consistent with this, we observed a dramatic increase of phospho-histone H2A.X signal in Chk1 siRNA-transfected cells but not in control cells after 16 h of 5-FU treatment, indicating that abrogation of the S checkpoint caused excessive double-stranded DNA breaks, which probably overwhelmed the cellular repair machinery and thereby driving the cells to PCC followed by apoptosis (Figure 2b). Intriguingly, Chk1 knockout alone induced a noticeable elevation of phospho-H2A.X signal in the absence of 5-FU (compare lane 6 with 1).

We further used the cell proliferation assay (MTS assay) to quantify the extent of potentiation of 5-FU by Chk1 siRNA (Figure 2c). Chk1 elimination decreased the EC_{50} value of 5-FU from 452 to $0.81 \mu\text{M}$ thereby achieving a potentiation of over 500-fold. In the clinic, 5-FU is usually used in combination with leucovorin to enhance its efficacy (Longley *et al.*, 2003). Thus, we tested the effect of leucovorin in the same assay as a comparison to Chk1 siRNA (Figure 2c). Leucovorin at $10 \mu\text{M}$ fails to confer any significant potentiation of 5-FU with an EC_{50} value of $238 \mu\text{M}$ achieving only 1.9-fold of potentiation. Even at a concentration of $30 \mu\text{M}$, which far exceeds the clinically relevant dosage, it still fails to sensitize HeLa cells to 5-FU (data not shown). Additionally, the combination of Chk1 siRNA with leucovorin confers a similar extent of sensitization as Chk1 siRNA alone (data not shown). This may be explained by the observation that Chk1 siRNA has already achieved the maximal degree of potentiation detectable in this system. Therefore, Chk1 siRNA is far more potent than leucovorin, the benchmark sensitizer for 5-FU potentiation. This result is also reproduced in HeLa S3 cells, a subline derived from HeLa cells but with significantly different properties (data not shown). In addition, we have observed the potentiation of 5-FU by Chk1 inhibition in other cancer cell lines such as Colo205 (colon cancer line) and H1299 (non-small cell lung cancer line) (data not shown), indicating that the observed sensitization effect is not restricted to only HeLa cells.

Chk1 elimination abrogates 5-FU-induced S-phase arrest and confers cell death through a novel mode of checkpoint abrogation

To examine the underlying mechanisms of the potentiation of 5-FU by Chk1 siRNA, we probed the cellular profiles of various cell-cycle and DNA synthesis markers. HeLa cells were transfected with control or Chk1 siRNA and treated with 5-FU at $50 \mu\text{M}$ for 20 h followed with BrdU labeling. Cells were fixed and stained to examine BrdU incorporation, an indicator of DNA synthesis, and the profiles of cyclin B1 and phospho-histone H3, markers for cell-cycle progression. Cells transfected with control siRNA and treated with 5-FU did not display any BrdU incorporation, consistent with the expected S-phase arrest (Figure 3a and b, lower panel 4). However, cells transfected with Chk1 siRNA and treated with 5-FU show a significant amount of BrdU incorporation signifying resumption of DNA

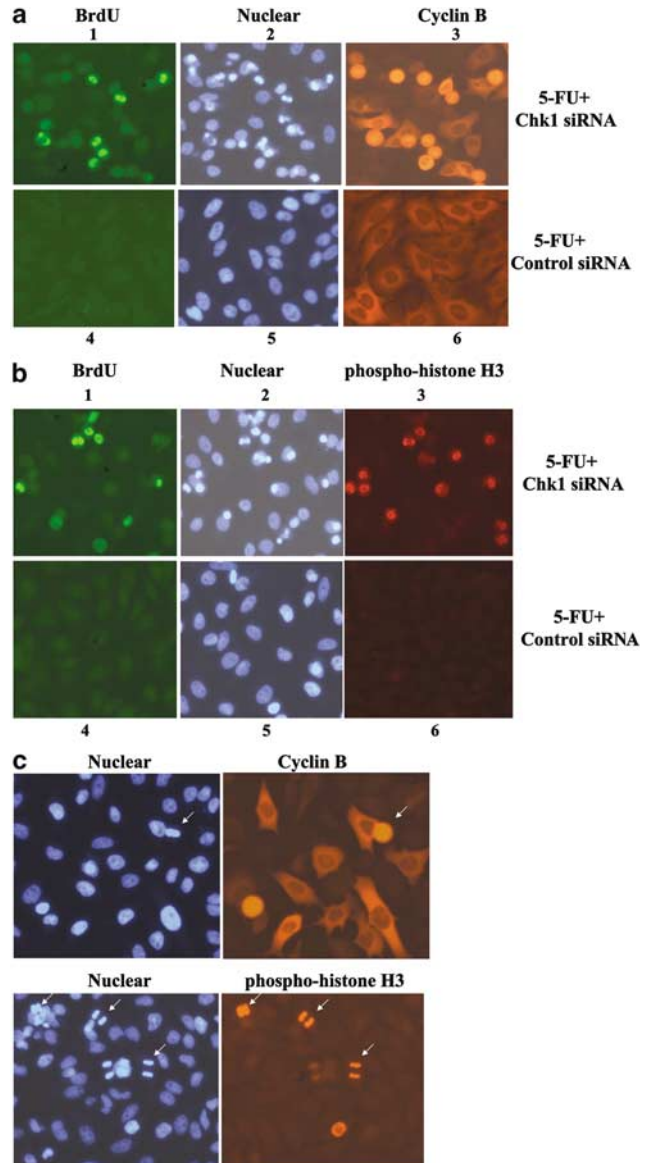


Figure 3 Cells resume DNA replication and progress to M phase after abrogation of 5-FU-induced checkpoint. (a) HeLa cells were transfected with Chk1 siRNA or control siRNA and then treated with 5-FU at $50 \mu\text{M}$ for 20 h. BrdU was added to the cells during the last 2 h of incubation. Cells were fixed and stained for BrdU, nuclei and cyclin B. (b) Cells were similarly treated as in panel a and stained for BrdU, nuclei and phospho-histone H3. (c) A population of asynchronously growing HeLa cells was fixed and stained for cyclin B or phospho-histone H3. Cells were also counterstained with DAPI to visualize the nuclear morphology. Representative mitotic cells are indicated with arrows

synthesis and the abrogation of S-phase checkpoint (Figure 3a and b, panel 1). Interestingly, there is a general correspondence between BrdU accumulation and nuclear condensation, indicating that excessive incorporation of 5-FU metabolites into the DNA strands led to cell death, presumably through the induction of double-stranded breaks (Figure 2b). Certain condensed nuclei did not show significant levels of BrdU, probably because the nuclear condensation

occurred before the addition of BrdU; thus, these nuclei had no chance to incorporate BrdU.

We next examined cyclin B profile to ascertain the status of cell-cycle progression after the checkpoint abrogation (Figure 3a, panels 3 and 6). Cyclin B is expressed in a cell-cycle-dependent manner: minimal to low expression in the cytoplasm during G0 and G1, and increased expression during S to G2 progression coupled with nuclear translocation, and achieving maximum accumulation in late G2 and early M phase (Morgan, 1997; Johnson and Walker, 1999). This cell-cycle-dependent profile was first confirmed using an asynchronous population of HeLa cells (Figure 3c, upper panels). A heterogeneous staining pattern was observed. While most of the cells showed a minimal level of cyclin B signal and are thus presumably in G1 phase, the cells showing mitotic properties as demonstrated by condensed nuclei (prophase) or chromosomal alignment (metaphase, arrow) displayed the highest signal level. In control siRNA-transfected cells, 5-FU treatment induced a uniform level of cyclin B in the cytoplasm (Figure 3a, panel 6), consistent with the 5-FU-induced S-phase arrest. In comparison, in cells treated with 5-FU and Chk1 siRNA, an elevated level of cyclin B1 and rounded morphology of cyclin B staining were revealed (Figure 3a, panel 3), reminiscent of mitotic cells with a 'popped-up' appearance. This indicates that cells migrate to early M phase after the checkpoint abrogation. This conclusion is additionally confirmed by phosphohistone H3 (P-H3) staining, which is a specific M-phase marker (as demonstrated in Figure 3c, lower panels). 5-FU treatment of control cells showed a complete suppression of P-H3 signal, again consistent with the S-phase arrest (Figure 3b, panel 6). In contrast, in cells transfected with Chk1 siRNA and treated with 5-FU, the checkpoint abrogation induced extensive formation of condensed nuclei, which showed both BrdU labeling and strong phospho-H3 staining (Figure 3b, panel 3). Collectively, these results compellingly established the fact that cells initiate apoptosis during M-phase progression after abrogation of the S checkpoint by Chk1 siRNA.

To corroborate the above findings, we use FACS analysis to study the cell-cycle profiles of cells treated with 5-FU in the presence or absence of Chk1 down-regulation. HeLa cells were treated with 5-FU at 50 μ M in the presence of Chk1 siRNA or control siRNA. In control cells (Figure 4a, lower panels), 5-FU confers a G1 to early S-phase arrest (G1 peak shifted toward G1/S border, and S-phase cells increases from 10.6 to 22.6%). However, consistent with the nuclear staining and MTS profiles (Figure 2), no significant cell death was observed as indicated by only a slight increase of sub-G1 population (from 3.1 to 6.7%), which usually corresponds to apoptotic cells. In contrast, Chk1 siRNA (Figure 4a, upper panels) greatly enhances the cytotoxic effect of 5-FU as demonstrated by a significant increase of the sub-G1 population (from 4.4 to 27.6%). In the meantime, S-phase arrest was significantly diminished (from 22.6 to 7.3%), suggesting an abrogation of the S checkpoint and sensitization of the tumor cells to 5-FU

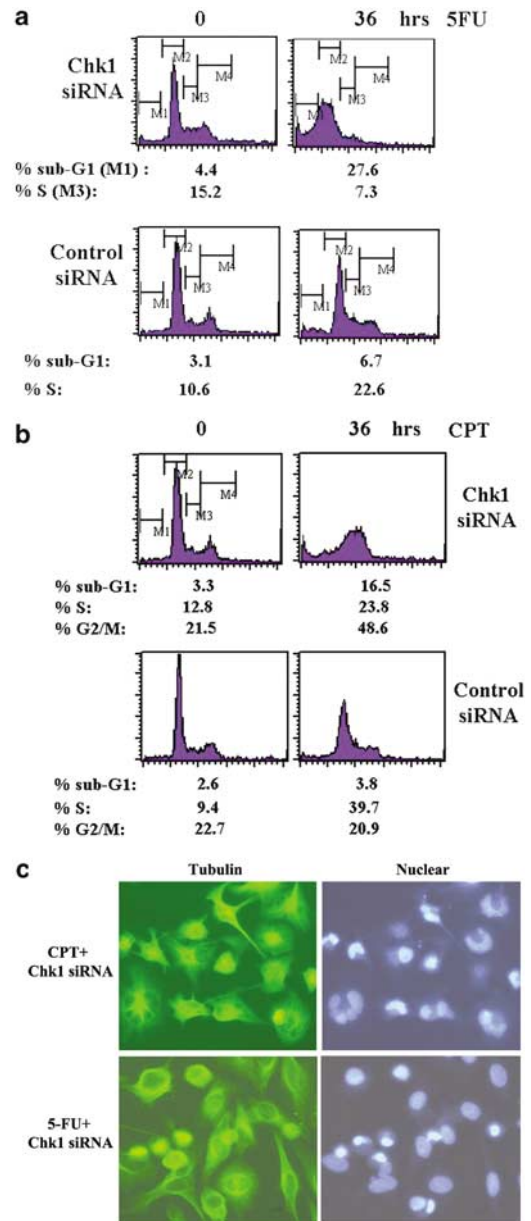


Figure 4 Chk1 knockout potentiates different chemotherapeutics through divergent process. (a) Chk1 siRNA abrogates 5-FU-induced S-phase arrest without conferring mitotic progression. HeLa cells were transfected with Chk1 siRNA or control siRNA and then treated with 50 μ M 5-FU for 36 h. Cells were stained with propidium iodide (PI) and processed for FACS analysis. Percentages of cells in S phase and sub-G1 (apoptotic) phase were obtained through the Cell-Quest program. (b) Chk1 knockout abrogates CPT-induced S-phase arrest and promotes premature mitosis/mitotic catastrophe. HeLa cells were transfected with Chk1 siRNA or control siRNA and then treated with 150 nM CPT for 36 h. Cells were stained with PI and processed for FACS analysis. Percentages of cells in S-, G2/M and sub-G1 (apoptotic) phases were obtained through the Cell-Quest program (BD Biosciences). (c) Chk1 siRNA confers mitotic catastrophe in combination with CPT but not 5-FU. HeLa cells were transfected with Chk1 siRNA and then treated with either 150 nM CPT (upper panels) or 50 μ M 5-FU (lower panels) for 24 h. Cells were fixed and stained for tubulin and counterstained with DAPI to reveal the nuclear morphology

toxicity. Most interestingly, we did not observe a progression of cells from S to G2/M phase since the cellular DNA content mostly remained at $2N$ and failed to increase to $4N$, suggesting an apparent ‘uncoupling’ effect between cell-cycle marker profiles and cellular DNA content. This is in sharp contrast with the FACS profile of HeLa cells treated with CPT and Chk1 siRNA, which demonstrated a clear example of mitotic progression after abrogation of the CPT-induced S-phase checkpoint (Figure 4b). Cell-cycle marker profiles also showed M-phase transition (data not shown). Therefore, it represents a ‘classical’ case of checkpoint abrogation where the increase of cellular DNA content is coupled with cell-cycle progression to M phase. We speculate that the reasons for the ‘uncoupling’ of DNA content with cell-cycle progression in the case of Chk1 siRNA’s abrogation of 5-FU-induced checkpoint are as follows: 5-FU suppresses DNA synthesis by depleting the pool of nucleotide substrates; hence, when the S checkpoint is abrogated and DNA replication is resumed, excessive incorporation of 5-FU will result, causing massive DNA damage and chromosomal fragmentation. Thereby, it hinders the completion of DNA replication and prevents the increase of DNA content from $2N$ to $4N$, and cells died with apparent $2N$ DNA content. Taken together, the cell-cycle analysis shows distinct cell-cycle profiles for the antimetabolites and the topoisomerase inhibitors in combination with Chk1 siRNA, even though Chk1 downregulation potentiates both kinds of S-phase arresting agents. In the case of CPT, the abrogation is coupled with an increase of DNA content to $4N$ and an obvious peak shift from S to G2/M in FACS analysis, thus fitting the classical profile of checkpoint abrogation. In contrast, with 5-FU, the abrogation fails to increase DNA content to $4N$ even though other cell-cycle markers indicate mitotic progression. Hence, the latter represents a form of nonclassical checkpoint abrogation.

This divergence in the mode of checkpoint abrogation is translated into dramatic morphological differences (Figure 4c). A hallmark of cells undergoing mitotic catastrophe is the presence of multiple micronuclei, usually a result of improper chromosomal segregation or aberrant cytokinesis (Hendry and West, 1997; Roninson *et al.*, 2001). As expected, Chk1 siRNA-transfected cells showed a striking feature of frequent multi-micronuclei formation when treated with CPT (Figure 4c, upper panels). The ring-shaped alignment of the multiple nuclei confers a distinctive horseshoe configuration. On the other hand, only small and condensed nuclei were observed upon treatment with 5-FU, and multinuclei formation is mostly absent (Figure 4c, lower panels). This suggests that for cells to reach mitotic catastrophe, they need to attain a $4N$ DNA content. Although we only presented the 24-h time-point comparisons, we have also checked prior and ensuing time points to confirm that the highly condensed nuclei in 5-FU-treated cells did not undergo a stage that would, in earlier time points, resemble the patterns generated by CPT + Chk1 siRNA.

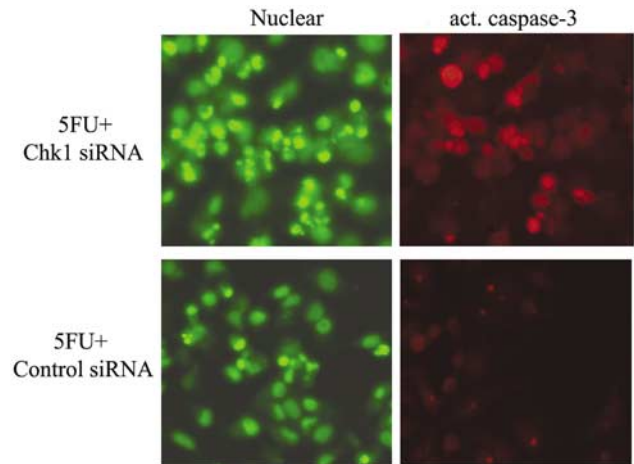


Figure 5 Chk1 downregulation potentiates caspase-3 activation in the presence of 5-FU. HeLa cells were transfected with Chk1 siRNA or control siRNA and then treated with $50\ \mu\text{M}$ 5-FU for 48 h. Cells were fixed and stained with antibody recognizing cleaved active caspase-3 and counterstained with DAPI to display the nuclear profile

Chk1 siRNA sensitizes tumor cells to 5-FU by induction of apoptosis, largely via activation of the caspase-8 pathway

To verify whether Chk1 inhibition sensitizes cells to 5-FU through the induction of apoptosis, we examine the treated cells for activated caspase-3. As shown in Figure 5, Chk1 siRNA enhances 5-FU efficacy by activating caspase-3. These results are consistent with the observed nuclear condensation (upper panels), a hallmark of apoptosis. With control siRNA, 5-FU treatment fails to induce significant caspase-3 activation and nuclear condensation (Figure 5, bottom panels).

Both the intrinsic (mitochondrial/caspase-9) and extrinsic (death receptor-mediated/caspase-8) apoptotic pathways converge on caspase-3 activation (Strasser *et al.*, 2000). To characterize which upstream pathway is mainly responsible for the observed induction of caspase-3, we first transfected HeLa cells with Chk1 siRNA or control siRNA and added 5-FU at various times and immunoblotted the cell lysates for caspase-8 and caspase-9. The antibodies used are able to recognize both the procaspase forms and the cleaved active species. We observed a clear induction of procaspase-8 cleavage with Chk1 siRNA alone (Figure 6b, upper part, compare lane 1 with 6). With control siRNA, 5-FU did not confer any procaspase-8 cleavage at any of the tested time points. However, with Chk1 siRNA, at least half of procaspase-8 has been converted into the 43-kDa cleaved form at the 16-h time point and, by 36 h, almost all of procaspase-8 is cleaved. Besides the 43-kDa cleaved form, we also observed an accumulation of the 18 kDa cleaved caspase-8 species (Figure 6b, lower part), which is believed to be the more active species (Boldin *et al.*, 1996; Muzio *et al.*, 1996). In contrast, procaspase-9 displayed a more delayed and much less significant cleavage. Only about 10% of procaspase-9 was converted into the active 35 kDa species at the maximal

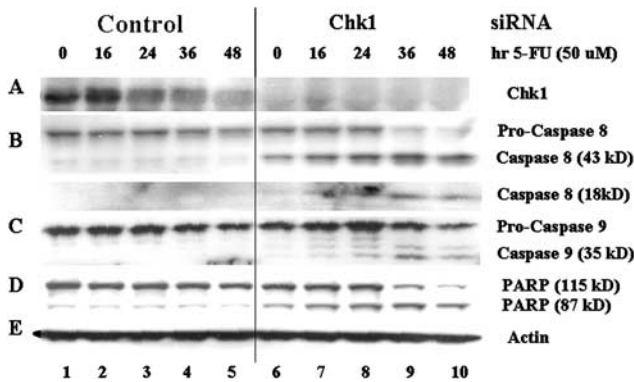


Figure 6 Chk1 siRNA potentiates 5-FU mainly through the induction of the caspase-8 pathway leading to caspase-3 activation and PARP cleavage. HeLa cells were transfected with Chk1 siRNA or control siRNA for 24 h and then treated with 50 μ M 5-FU for the indicated time points. Cell lysates were probed for the following apoptosis markers: caspase-8 (recognizing both the proform and the cleaved active forms), caspase-9 (recognizing both the proform and the cleaved active forms) and PARP

induction time achieved at 36 h (Figure 6c). This result suggests that Chk1 elimination potentiates 5-FU preferentially through induction of the caspase-8 pathway. We additionally probed for PARP, a major substrate for caspase-3. We found that PARP cleavage was clearly observed, especially at the last two time points, in cells transfected with Chk1 siRNA but not in cells transfected with control siRNA (Figure 6d). This is consistent with the previous result demonstrating caspase-3 activation in similarly treated cells (Figure 5).

Discussion

We demonstrate here that 5-FU, the most widely used antimetabolite in cancer therapy, activates Chk1 and arrests cells in early Sphase through Cdc25A proteolysis. Inhibition of Chk1 dramatically sensitizes tumor cells to 5-FU by protecting Cdc25A from degradation, abrogating the checkpoint and inducing PCC followed by apoptosis. This novel discovery is in addition to previous studies showing that Chk1 downregulation potentiates the conventional DNA-damaging agents such as IR and topoisomerase inhibitors. We further provide evidence that even though all of these agents activate Chk1 and cause cell-cycle arrest, the underlying mechanisms for their potentiation by Chk1 inhibition are distinct from one another. In this process, we illustrate a previously uncharacterized mode of checkpoint abrogation where the cellular DNA content is uncoupled from the cell-cycle status.

Chk1 downregulation potentiates chemotherapeutics through induction of either PCC or mitotic catastrophe

Mitotic catastrophe is a special type of cell death resulting from premature or abnormal mitosis, usually in the presence of unrepaired DNA damage or defective microtubule structures. A common defining feature of mitotic catastrophe is the multi-micronuclei morphology

(Roninson *et al.*, 2001; Figure 4c, upper panel). Multiple chemotherapeutics can induce mitotic catastrophe in tumor cells with defective cell-cycle checkpoints (Tounekti *et al.*, 1993; Lock and Stribinskiene, 1996; Torres and Horwitz, 1998). In the case of potentiation of CPT by Chk1 siRNA, DNA replication is completed after checkpoint abrogation. In this scenario, the cells continue to progress through the Sphase and into mitosis with damaged DNA leading to mitotic catastrophe and cell death with 4N DNA content.

In contrast, PCC denotes the phenomenon of chromosome condensation in the absence of complete DNA replication (Nghiem *et al.*, 2001). Apparently for the potentiation of 5-FU by Chk1 siRNA, the tremendous induction of DNA double-stranded breaks prevents the completion of DNA replication and produces a chromosomal abnormality similar to PCC. Taken together, what we have found here is an interesting dichotomy in cell death conferred by Chk1 elimination depending on whether checkpoint abrogation is coupled with complete DNA replication or not. Although inhibition of Chk1 invariably abrogates the S-phase checkpoints induced by CPT or 5-FU, DNA synthesis machinery is still functional in the case of CPT treatment, so the cells can progress through the Sphase, increase their DNA content and continue into mitosis. This unscheduled M-phase progression in the presence of DNA damage results in mitotic catastrophe, hence cells die with 4N DNA content. In contrast, in the case of antimetabolites such as 5-FU, the checkpoint abrogation fails to increase cellular DNA content due to a depletion of the cellular nucleotide pool (especially dTTP), which results in excessive incorporation of FdUTP, a 5-FU metabolite. This induces massive DNA damage and hinders completion of DNA replication. Thus, cells die from a PCC-like process with 2N DNA content. As a result, we observed dramatic differences in the cell-cycle profiles between Chk1 siRNA potentiation of CPT and 5-FU (Figure 4), which could be due to the different phases at which CPT and 5-FU initially arrest the cells (S/G2 for CPT and early G1/S for 5-FU) and the distinct mechanisms through which they exert these arrests. Our discovery thus presents an interesting case of ‘uncoupling’ between the completion of DNA replication and M-phase progression. It further illustrates the inherent danger of interpreting cell-cycle conditions solely on the basis of FACS analysis. Previously, Shi *et al.* (2001) showed that UCN-01 abrogated the S-phase arrest induced by gemcitabine, another antimetabolite, without cell-cycle progression. Since UCN-01 has many cellular targets besides Chk1, the observed effect may not be uniquely ascribed to Chk1 alone. More importantly, this conclusion was drawn purely on the basis of FACS analysis without corroborating cell-cycle marker study to uncover the underlying mechanism of the abrogation. In light of our current finding, we speculate that a similar uncoupling of cell-cycle progression and DNA content increase may also have occurred in Shi’s system.

In summary, depending on the genotoxic agents used, two types of checkpoint abrogation will be conferred by

Table 1 Fundamental differences between Chk1 siRNA's potentiation of CPT and 5-FU

<i>Classical S-phase checkpoint abrogation (CPT)</i>	<i>Nonclassical S-phase checkpoint abrogation (5-FU)</i>
Checkpoint abrogation with DNA synthesis and increase of DNA content to 4N Cells progress through G2 to M phase with highly accumulated cyclin B1 and phospho-histone H3 An apparent peak shift from S to G2/M after checkpoint abrogation by Chk1 siRNA Mitotic catastrophe with multi-micronuclei formation	Checkpoint abrogation with DNA synthesis and without increase of DNA content to 4N Cells progress through G2 to M phase with highly accumulated cyclin B1 and phospho-histone H3 Lack of an apparent peak shift from S to G2/M after checkpoint abrogation by Chk1 siRNA Condensed nuclei formation reminiscent of PCC

Chk1 downregulation. A detailed comparison of the differences is presented in Table 1.

Chk1 siRNA potentiates 5-FU preferentially through caspase-8 pathway

Another intriguing finding of ours is that Chk1 downregulation seems to potentiate 5-FU preferentially through the caspase-8 pathway. This is in line with an earlier study showing that interferon- γ enhances 5-FU/leucovorin clinical efficacy through upregulation of FAS signaling pathway (Schwartzberg *et al.*, 2002), of which caspase-8 is a downstream component. More interestingly, Chk1 elimination alone incurred a visible cleavage and presumably activation of caspase-8 (Figure 6b, compare lane 1 with 6). This may explain why the caspase-8 pathway plays a dominant role in the potentiation of 5-FU by Chk1 siRNA. The fact that downregulation of Chk1 induces caspase-8 activation is consistent with the finding of a previous report showing increased spontaneous apoptosis rate of lymphoma cells after Chk1 ablation (Zachos *et al.*, 2003). In comparison, we did not observe a similar effect with caspase-9 (Figure 6c).

DNA-damaging agents are the mainstays of cancer therapy and have achieved impressive clinical results. However, they are often plagued by various limitations, the most serious of which is drug resistance. Chk1 is the major cell-cycle checkpoint mediator and responds to DNA damages by initiating cell-cycle arrest, which provided time for the cell to repair the damage and evade apoptosis before any cell-cycle progression can resume. Thus, it constitutes a defense mechanism to circumvent the toxicity of DNA-damaging agents. Our current study combined with previous findings (Koniaras *et al.*, 2001; Eastman *et al.*, 2002; Chen *et al.*, 2003; Xiao *et al.*, 2003) has convincingly demonstrated that Chk1 downregulation remarkably potentiates anti-metabolites as well as topoisomerase inhibitors by depriving the cancer cells of this defensive mechanism. This greatly broadens the potential application of Chk1 inhibitors in cancer therapy.

Materials and methods

Cell culture

Human cervical cancer cell line HeLa and human lung cancer cell line H1299 were obtained from ATCC (Manassas, VA, USA). H1299 cells were grown in RPMI 1640 supplemented

with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% penicillin-streptomycin and 0.45% glucose at 37°C in a 5% CO₂ incubator. HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% penicillin-streptomycin and 0.45% glucose at 37°C in a 5% CO₂ incubator. Prior to transfection, the cells were switched to medium without any penicillin-streptomycin.

Antibodies

Antibodies against Chk1 and Cdc2 Y15P were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-Chk1 (Chk1 S345P), caspase-8 and caspase-9 antibodies were from Cell Signaling Technology (Watertown, MA, USA). Two Cdc25A antibodies were used: sc-7389 (F-6) monoclonal antibody (Santa Cruz Biotech, CA, USA) for detection of Cdc25A in HeLa cells, and antibody C-3 (Neomarker, Labvision, CA, USA) for detection in H1299 cells. PARP antibody was from BD Biosciences. Antibodies for phospho-histone H3 and phospho-H2A.X were obtained from Upstate Technology (Waltham, MA, USA). Tubulin antibody was obtained from Molecular Probes (Eugene, OR, USA).

Transfection

Human Chk1 siRNA was as described previously (Xiao *et al.*, 2003). GFP siRNA was used as control siRNA (5'-3' sequence: AACACTTGTCACACTTT CTC). siRNA transfection protocols were as described previously (Xiao *et al.*, 2003). The final concentration of the siRNA was 50 nM. For 96-well transfection used in the MTS assay, 0.5 μ l of 20 μ M stock siRNAs was used in a final volume of 200 μ l medium for each well, resulting in a final concentration of 50 nM.

Western blot analysis

Adherent cells in the tissue culture wells were rinsed with PBS and directly lysed in Laemmli sample buffer. Floating cells or cell fragments were also collected, lysed and combined with the above lysates. Samples were heated at 95°C for 5 min and resolved on the Novex minigel system (Invitrogen) under denaturing conditions and blotted to polyvinylidene difluoride membrane using a semidry transfer device (Amersham Biosciences). The membrane was blocked with 5% nonfat dry milk and probed with various antibodies. Enhanced chemiluminescent detection (ECL) was performed using ECL reagents according to the vendor's protocols (Santa Cruz Biotechnology).

Cell proliferation assay (MTS assay)

HeLa cells were seeded in 96-well plates and transfected with Chk1 siRNA or scrRNA with oligofectamine. The final siRNA concentration was maintained at 50 nM. At 16 h after transfection, the cells were treated with the indicated doses of 5-FU for 48 h. After treatment, MTS reagents that measure the

amount of live cells (Promega, Madison, WI, USA) were added to the cells and allowed to develop from, 20 min to 2 h. Colorimetric measurement was made at 490 nm on Spectra MAX 190 from Molecular Devices (Sunnyvale, CA, USA).

BrdU labeling assay

HeLa cells were transfected with Chk1 siRNA or control siRNA and treated with 5-FU for 20 h. Cells were then labeled with 10 μ M BrdU (Molecular Probes, Eugene, OR, USA) for 2.5 h. Cells were fixed and permeabilized for BrdU detection with monoclonal BrdU antibody according to the manufacturer's recommendations (Amersham Biosciences).

Cell-cycle analysis

HeLa cells after the indicated treatments were washed once in PBS and fixed in 70% ethanol. The fixed cells were washed again twice with PBS and treated with RNase A at 37°C for 30 min. Finally, the cells were stained with PI and incubated in the dark for 60 min or overnight before analysis. The samples were analysed through flow cytometry using fluorescence-activated cell sorting (FACS) manufactured by BD Bioscience (San Jose, CA, USA) using the Cell-Quest program.

References

- Boldin MP, Goncharov TM, Goltsev YV and Wallach D. (1996). *Cell*, **85**, 803–815.
- Chen Z, Xiao Z, Chen J, Ng SC, Sowin T, Sham H, Rosenberg S, Fesik S and Zhang H. (2003). *Mol. Cancer Ther.*, **2**, 543–548.
- Chini CC and Chen J. (2003). *J. Biol. Chem.*, **278**, 30057–30062.
- Eastman A, Kohn EA, Brown MK, Rathman J, Livingstone M, Blank DH and Gribble GW. (2002). *Mol. Cancer Ther.*, **1**, 1067–1078.
- Hendry JH and West CML. (1997). *Int. J. Radiat. Biol.*, **71**, 709–719.
- Johnson DG and Walker CL. (1999). *Annu. Rev. Pharmacol. Toxicol.*, **39**, 295–312.
- Johnston PG and Kaye S. (2001). *Anticancer Drugs*, **12**, 639–646.
- Koniaras K, Cuddihy AR, Christopoulos H, Hogg A and O'Connell MJ. (2001). *Oncogene*, **20**, 7453–7463.
- Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA and Elledge SJ. (2001). *Genes Dev.*, **14**, 1448–1459.
- Lock RB and Stribinskiene L. (1996). *Cancer Res.*, **56**, 4006–4012.
- Longley DB, Harkin DP and Johnston PG. (2003). *Nat. Rev. Cancer*, **3**, 330–338.
- Morgan DO. (1997). *Annu. Rev. Cell Dev. Biol.*, **13**, 261–291.
- Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME and Dixit VM. (1996). *Cell*, **85**, 817–827.
- Nghiem P, Park PK, Kim YS, Vazari C and Schreiber SL. (2001). *Proc. Natl. Acad. Sci. USA*, **98**, 9092–9097.
- Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS and Pwnica-Worms H. (1997). *Science*, **277**, 1501–1505.
- Roninson IB, Broude EV and Chang B-D. (2001). *Drug Resist. Updates*, **4**, 303–313.
- Sampath D, Rao VA and Plunkett W. (2003). *Oncogene*, **22**, 9063–9074.
- Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Pwnica-Worms H and Elledge SJ. (1997). *Science*, **277**, 1497–1501.
- Schwartzberg LS, Petak I, Stewart C, Turner PK, Ashley J, Tillman DM, Douglas L, Tan M, Billups C, Mihalik R, Weir A, Tauer K, Shope S and Houghton JA. (2002). *Clin. Cancer Res.*, **8**, 2488–2498.
- Sedelnikova OA, Pilch DR, Redon C and Bonner WM. (2003). *Cancer Biol. Ther.*, **2**, 233–235.
- Shi Z, Azuma A, Sampath D, Li YX, Huang P and Plunkett W. (2001). *Cancer Res.*, **61**, 1065–1072.
- Sorensen CS, Syljuasen RG, Falck J, Schroeder T, Ronnstrand L, Khanna KK, Zhou BB, Bartek J and Lukas J. (2003). *Cancer Cell*, **3**, 247–258.
- Strasser A, O'Connor L and Dixit VM. (2000). *Annu. Rev. Biochem.*, **69**, 217–245.
- Torres K and Horwitz SB. (1998). *Cancer Res.*, **58**, 3620–3626.
- Tounekti O, Pron G, Belehradek Jr J and Mir LM. (1993). *Cancer Res.*, **53**, 5462–5469.
- Xiao Z, Chen Z, Gunasekera AH, Sowin TJ, Rosenberg SH, Fesik S and Zhang H. (2003). *J. Biol. Chem.*, **278**, 21767–21773.
- Zachos G, Rainey MD and Gillespie DA. (2003). *EMBO J.*, **22**, 713–723.
- Zhao H, Watkins JL and Pwnica-Worms H. (2002). *Proc. Natl. Acad. Sci. USA*, **99**, 14795–14800.
- Zhou BB and Bartek J. (2004). *Nat. Rev. Cancer*, **4**, 216–225.

Immunofluorescence

HeLa cells were grown to 50% confluence on coverslips in six-well plates and transfected with Chk1 siRNA or scrRNA and treated as indicated. The cells were then fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.15% Triton X-100 for 10 min, blocked with 5% normal goat serum (Invitrogen) for 30 min and then stained with the appropriate dilution of the indicated antibody for 1 h at room temperature. After extensive washing, FITC-conjugated secondary antibody was added at a 1:500 dilution for 1 h (Molecular Probes). Before mounting, cells were stained with the nuclear stain Hoechst 33342 at 10 μ g/ml for 10 min. Fluorescence signals were visualized with a Zeiss inverted microscope equipped for epifluorescence using a 488-nm excitation filter and a 522/535-nm emission filter. Images were recorded with a Hamamatsu Orca CCD camera and analysed with Axiovert software.

Acknowledgements

We thank Dr Steve Fesik for his critical reading of the manuscript and for his many stimulating inputs. We also thank Mai Bui and Zehan Chen for help with the FACS analysis.