DUAL REGULATION OF CDC25A BY CHK1 AND p53-ATF3 IN DNA REPLICATION CHECKPOINT CONTROL

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Eukaryotic cells respond to DNA damage and stalled replication forks by activating signaling pathways that promote cell cycle arrest and DNA repair. A systematical screening of the protein kinase siRNA library reveals that Chk1 and ATR are the main kinases responsible for intra-S-phase checkpoint upon topoisomerase I inhibitor camptothecin (CPT) -induced DNA-damage. It is well known that ATR-Chk1-mediated protein degradation of Cdc25A protein phosphatase is a crucial mechanism conferring this checkpoint activation. Here we describe another mechanism underlying Cdc25A down-regulation in response to DNA damage which occurs at the transcriptional level. We show that activation of tumor suppressor p53 by DNA damage results in inhibition of Cdc25A transcription as a result of activation of transcriptional repressor ATF3 that directly binds to the Cdc25A promoter. In cells deficient in both Chk1 and p53, Cdc25A down-regulation upon CPT-induced DNA damage is completely abolished, leading to severe defects in cell cycle checkpoints and remarkable cell death in mitosis. Our findings reveal two independent mechanisms acting in concert in regulation of Cdc25A in DNA damage response. While Chk1 affects Cdc25A via rapid phosphorylation and protein turnover, inhibition of Cdc25A transcription by p53-ATF3 is required for the maintenance of cell cycle arrest.

Cells respond to DNA damage by halting cell-cycle progression or by undergoing programmed cell death (1). There are several strategies that arrest the cell cycle in response to DNA damage (2). The rapidly activated pathway is triggered by PI3 Kinase ATR or (and) ATM together with DNA-PK, which sense the DNA lesions and activate effector kinases Chk1 and Chk2 (3). Effector kinases further spread out the signal particularly by phosphorylating the key components of cell cycle progression, such as Cdc25 phosphatases A, B and C (4,5), leading to inactivation of cyclin E- and cyclin A-dependent protein kinases important for G1/S and G2/M transition (6,7). After DNA damage, Cdc25A undergoes inhibitory phosphorylation by Chk1 or Chk2 kinases, depending on the stress, and degrades via ubiquitin-mediated proteasome pathway, leading to transit inhibition of DNA replication (8)

Unlike the ATR/ATM-Chk1/2 pathway that is activated within minutes after DNA damage and is crucial in early
events of cell cycle arrest, stress-induced stabilization and activation of the tumor suppressor p53 is required for maintenance of cell cycle arrest, most likely through transcriptional activation or repression of its downstream target genes (9). Indeed, the functional roles of p53 in both G1 and G2/M checkpoints has been previously shown (10). Together with other defects in checkpoint control, inactivation of p53 leads to comprised checkpoint response and enhanced sensitivity to DNA damage (11,12). Given the protective role of cell cycle checkpoint in DNA damage response, abrogation of cell cycle checkpoint has been proposed to be a strategy for anti-cancer therapy (13). In particular, pharmacological inhibition of cell cycle checkpoint pathways has been shown to sensitize p53-deficient cells to chemotherapeutic agents (14,15). However, the detailed mechanism underlying this sensitization in p53-deficient cells is not completely understood.

Activating transcription factor 3 (ATF3), a member of ATF/CREB transcriptional factor family, is one of the direct targets of p53 (16,17). While the first member of the family – ATF (later ATF1) was initially described as a transcriptional activator (18), the other members (ATF3 and ATF2) seemed to function as transcriptional repressors (Chen et al., 1994). ATFs and CREB proteins share similar properties and bind to a consensus DNA sequence (TGACGTCA) in gene promoters for transcriptional regulation. Moreover, ATF3 is a stress inducible gene: its mRNA level greatly increases upon the exposure of cells to a variety of stress signals, including cytokines, hydrogen peroxide, ultraviolet light and ionizing radiation, as well as genotoxic agents such as Camptothecin (CPT), 5-Flurouracil (5-FU) and Doxorubicin (17,19,20).

The majority of anti-cancer drugs kill cancer cells by causing DNA damage and by taking the advantage of defective cell cycle checkpoint in cancer cells. Camptothecin is a topoisomerase I inhibitor that can stall DNA replication by causing single and double-strands DNA breaks and its action is specific to S-phase (21-23). In clinic, Camptothecin derivates, such as Irinotecan and Topotecan are key components of first- and second-line treatment for metastatic colon cancer and ovarian cancer, respectively (24,25) and thus CPT is widely used as a clinically relevant agent to study chemotherapy-induced DNA damage response.

Here, we report a novel mechanism of Cdc25A regulation in DNA damage response, which involves the transcriptional regulation by ATF3 in a p53-dependent manner. Through this mechanism, along with Chk1-regulated protein degradation, Cdc25A down-regulation upon DNA damage is well maintained in the presence of functional p53. Loss of this dual regulation leads to severe defects in DNA damage checkpoint, allowing cells to proceed through mitosis with damaged DNA and undergo mitotic cell death. Our results provide novel insight into the mechanism by which pharmacological inhibition of Chk1 selectively sensitizes p53-deficient tumor cells to chemotherapy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment-** The HCT116 human colon cancer cell lines (wt and p53-/−) were kindly provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD, USA). Cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) and maintained at 37°C with 5% CO2. Camptothecin (CPT) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

**RT-PCR Analysis-** Total RNA was extracted using RNeasy kit (Qiagen). All RT-PCR was performed using the LightCycler® RNA Master SYBR Green I Kit (Roche). Primer sequences were 5’-CTCCTGGGTCACTGGTGTT-3’ and 5’-TCTTACCTTCGAGGCTAAGCA-3’ for ATF3; 5’-TAGATTCTCTGGGGCCATTG-3’ and 5’-GCTGGAGCTACACAGGGAAG-3’ for
Cdc25A; 5’-CAAAAGTTGTCATGGATGACC-3’ and 5’-CCATGGAGAAGGCTGGGG-3’ for GAPDH.

**Flow Cytometry-** Cell cycle analysis was performed by DNA content quantification. The cells were fixed with 70% ethanol, washed twice in PBS and stained with PI solution (PBS with propidium iodide 5 μg/ml and 100 μg/ml RNase A) for 30 min at room temperature in the dark. The stained cells were analyzed by FACScalibur (BD Bioscience, San Diego, USA). Stained cells were analyzed by FACScalibur (BD Bioscience) and quantified by using CellQuest software (BD Bioscience). To measure caspase-3 activity, cells were fixed with Cytofix/Cytoperm solution (BD Bioscience) according to the manufacturer’s instructions and then stained with FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody (BD Bioscience) followed by FACS analysis. For phosphorylated H3 detection, 70% ethanol-fixed cells were washed twice in PBS and incubated for 15 min in PBS, 0.1% bovine serum albumin (BSA), and 0.25% Triton X-100. After centrifugation, cells were suspended in 100 μl of PBS containing 1% BSA and 20 μl of an anti-Histone H3 (pS28) antibody (BD Bioscience). After 1 h incubation at room temperature in the dark, cells were washed with PBS and stained with PI solution followed by FACS analysis. For S-phase cells detection, cells were labeled with BrdU 1h before harvesting, fixed and stained with anti-BrdU antibodies according to manufacturer instructions (BD Bioscience) followed by FACS analysis.

**Gene Silence by RNA Interference and over-expression-** The siRNA library of 780 human kinases was purchased from Dharmacon. The siRNA oligo targeting Chk1 was purchased from Cell Signalling (#6241), the siRNA targeting Cdc25A was from Dharmacon (Lafayette, CO, USA) and the siRNA targeting ATF3 was from Santa Cruz Biotechnology. Control duplex RNA (Dharmacon) corresponding to an unknown protein was used in controls. Transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. 48 hours after transfection, cells were washed with PBS before further treatment. To generate stable Chk1 knock-down cell lines, the hsRNA sequence (AACTGAAGAAGCATGCGCAGT) was cloned into the pSIREN-RetroQ retroviral expression vector (BD Bioscience) according to the manufacturer’s instruction. Virally infected cells were selected in a medium containing 2 μg/ml puromycin and individual drug-resistant clones were collected, pooled and expanded.

Human p53 and ATF3 cDNA was amplified by PCR from a HCT116 cDNA library and cloned into pcDNA3 vector (Invitrogen). Empty pcDNA3 vector was used in controls. Transfection was conducted overnight using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction.

**Western blotting-** Cells were harvested and lysed with RIPA buffer (50mM Tris-HCl, pH7.4, 1mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and proteinase inhibitors). 20-50 μg protein samples were separated by SDS-PAGE, and transferred onto an Immobilon membrane (Millipore). Proteins were detected with the ECL detection system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using anti-p53 (DO-1), anti-Chk1 (G-4), anti-ATF3 (C-19) (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti-Cdc25A (Ab-3, Lab Vision Corp, USA), anti-phospho-Chk1 (Ser345) (Cell Signaling Technology, Beverly, MA, USA); or anti-β-actin (Sigma-Aldrich).

**ChIP assays-** ChIP assays were performed as described previously (Zhao et al. 2005). HCT116 cells were treated with CPT (1 μM) for 24 h. Precleared chromatin from
HCT116 cells was immunoprecipitated with anti-ATF3 (C-17x, Upstate Biotechnology), and a nonspecific IgG (sc-2027, Santa Cruz Biotechnology). The ChIP-precipitated DNA and input DNA were subjected to PCR analysis. Primer sequences were 5'-CAAAGCTGGGGCTGAGATAG-3' and 5'-ATGCATTGTCGCACCCTTGA-3' for ATF/CREB-1; 5'-GAAAACGAAGCCGACCTACA-3' and 5'-ATTTGGATCCCCGCTCTTCTC-3' for ATF/CREB-2; 5'-CAAAATA CGGAGTCGTGTTTGTGTTTG-3' and 5'- CGGAGTCGTGTTTGTGTTTG-3' for control internal region.

RESULTS

ATR and Chk1 are the main protein kinases participating in CPT-induced checkpoint activation. Protein phosphorylation plays a key role in cell cycle checkpoint regulation. Several protein kinases, including ATR( ATM)-Chk1(Chk2), p38 MAPK, MAPKAP2 and PLK2 have been previously shown to be involved in S-phase checkpoint in various cellular contexts (26-28). Fig.1A shows that HCT116 cells treated with CPT underwent cell cycle arrest in S-phase, indicating the activation of S-phase checkpoint. As expected, this checkpoint induction was abrogated upon treatment with Chk1 siRNA. To systematically identify protein kinases participating in CPT-induced S-phase checkpoint, we performed a screening of SmartPool siRNA library of 780 human kinases (Dharmacon). To this end, HCT116 cells were transfected individually with each kinase siRNA for 48h, followed by CPT treatment at 200 nM for additional 24h, a condition that can arrest cells in intra-S-phase. Using this assay, we assessed the impact of each kinase siRNA on S-phase accumulation by calculating the ratio of cells in G2 over that in S-phase (Figure 1B). The G2/S ratios were further normalized to G2/S ratios of cells treated with negative control (NC)-siRNA. Three independent transfections were further performed to verify each Chk1-like phenotype that shows a G2/S ratio of 1.8 or greater. The screening results show that ATR and Chk1 are the primary kinases accounting for the S-Phase checkpoint activation in response to CPT treatment, though some other kinases such as PLK1, ERK8, and FGFR4 may also participate in this process but less profoundly. By contrast, ATM and Chk2 appear to be not essential in S-phase arrest under this experimental condition. We thus conclude that ATR-Chk1 pathway is central to CPT-induced S-phase arrest among all the kinases we have examined.

Depletion of both p53 and Chk1 completely abrogates the Cdc25A down-regulation in response to DNA replication stress. We next explored the role of Chk1-mediated checkpoint pathway in relation to other checkpoint defects in cancer cells. Recent reports indicate that inactivation of both Chk1 and p53 results in intense checkpoint abrogation in both S- and G2-phase (29-31). However, the mechanism underlying this effect is not clear. In this study we sought to determine the exact mechanisms that allow cancer cells to bypass checkpoints upon loss of these pathways. To facilitate the investigation, we generated stable cell lines derived from human colon cancer HCT116 cells that constitutively expressed a short hairpin RNA targeting Chk1 (shChk1) or a non-specific control shRNA (NC shRNA) in both p53 wild-type and p53-/- backgrounds. To characterize these cell lines, we first examined their ability to arrest the cell cycle by treating them with low (0.2 μM) or high (1 μM) doses of camptothecin (CPT). The roles of p53 and Chk1 in G1 and S-checkpoints activated by CPT have been shown previously (32,33). As expected from the loss of Chk1, cell cycle analysis by flow cytometry revealed that cells depleted of Chk1 (HCT116 shChk1) showed deficient S-phase arrest in response to 0.2 μM CPT for 24h but a strong accumulation in G2/M phase, while the wild-type cells or p53-/-HCT116 cells were strongly accumulated in S-phase (Figure 2A). Higher concentration
of CPT at 1μM resulted in strong G1 arrest in both wild-type and Chk1-depleted HCT116 cells but not in p53-/cells. These data suggest that low dose of CPT triggers mainly an intra-S-phase checkpoint that requires Chk1, whereas the high dose of CPT induces G1 arrest that requires p53. In cells lacking both Chk1 and p53, CPT treatment resulted in enhanced cell accumulation in sub-G1 at 48 h, indicating a strong commitment of such cells to apoptosis as a result of severe checkpoint defects (Figure 2A).

We next wished to investigate how Cdc25A expression is affected by the loss of Chk1, p53 or both. We treated the four cell lines with 1 μM CPT for 6 and 24 hours. Western blot analysis indicates that wild type HCT116 cells displayed a marked decrease in Cdc25A protein level in both 6 and 24 h following CPT treatment, which was attenuated in Chk1-depleted cells at 6h but not at 24h (Figure 2B, Supplementary Figure 1). By contrast, p53-/ cells showed a decrease in Cdc25A at the early time point (6h), which was back to the normal levels at 24 h. Furthermore, in cells lacking both Chk1 and p53, Cdc25A protein remained unchanged throughout the time course treatment. A similar result also observed in cells treated with CPT at 0.2 μM (Supplementary Figure 1). Together, these results indicate that Chk1 and p53 seem to act sequentially to maintain low levels of Cdc25A during the stress response: while Chk1 regulates Cdc25A in the early time point, p53 does so in the later phase. Loss of both results in a complete abrogation of Cdc25A down-regulation upon CPT treatment, as seen in cells lacking both p53 and Chk1.

High levels of Cdc25A enable cells to bypass G2/M checkpoint and result in massive cell death in mitosis. Numerous reports have shown that inactivation of Chk1 in p53-deficient cells resulted in abrogation of the G2 arrest and subsequent apoptosis (4,34-36) upon exposure to DNA damaging agents. However, the molecular mechanism underlying such phenotype changes have not been fully defined. We hypothesize that the increased cell death of p53-/shChk1 cells to CPT treatment is due to mitotic catastrophe, as a result of bypass of G2 checkpoint. To measure cells in mitotic phase, we used phosphorylated histone H3 as a mitotic marker. In wild type cells or cells lacking only Chk1 or p53, CPT treatment resulted in decreased cells accumulated in mitosis, indicating an effective G2 checkpoint. In a striking contrast to these cells, cells lacking both p53 and Chk1 displayed a dramatic increase in H3 phosphorylation, suggesting a strong accumulation in mitosis as a result of the defective G2 checkpoint (Figure 3A).

We next investigated whether resistance of Cdc25A down-regulation to CPT treatment is responsible for the G2 bypass seen above. Figure 3B shows that depletion of Cdc25A by siRNA-mediated gene silencing in p53-/shChk1 cells decreased the number of cells positive for H3 phosphorylation in response to CPT treatment. This result demonstrates that the high levels of Cdc25A indeed confer the defective G2 checkpoint in CPT-treated cells upon loss of both Chk1 and p53. Similar approach was used to study the role of Cdc25A in an intra-S-phase checkpoint. HCT116 p53-/shChk1 cells were transfected with Cdc25A or NC siRNA, treated with CPT for 24 h and analyzed for BrdU incorporation. Cdc25A depletion resulted in slowing down of S-phase progression in response to CPT treatment (Supplementary Figure 2). These data indicate that Cdc25A down-regulation is required for proper intra-S-phase checkpoint in response to DNA-replication stress.

To trace the cell fate following the defective S- and G2-checkpoint, the four cell lines were treated with 0.2 μM CPT for 24 and 48 hours and the activity of caspase-3 was measured by flow cytometry. As seen in Figure 3C, cells depleted for both p53 and Chk1 showed highest level of caspase-3 activation. To further assess the role of Cdc25A in this apoptosis induction, we performed the Cdc25A knockdown. Figure 3D shows that treatment of p53-/shChk1 cells with CPT resulted in an 8-fold
induction in caspase-3 activity, whereas only a 3-fold induction of caspase-3 activity was detected in these cells upon Cdc25A knockdown. Moreover, transfection of p53-/-shChk1 cells with Cdc25A siRNA also resulted in enhanced long term cell survival as assessed by focus formation assay, compared to cells treated with control siRNA (Figure 3E). Together, the data indicate that the high levels of Cdc25A in p53-/-shChk1 cells are responsible for the bypass of the G2 checkpoint and subsequent apoptosis in mitosis.

**P53 down-regulates Cdc25A expression through induction of ATF3.** We next investigated the mechanism by which p53 regulates Cdc25A expression in DNA damage response. qPCR analysis indicates that 1 μM CPT treatment for 24h resulted in a sharp decrease in Cdc25A mRNA level in HCT116 cells but not in HCT116 p53-/- cells. This indicates that CPT treatment down-regulates Cdc25A expression in a p53-dependent manner. p53 is activated after stress exposure and within hours induces or represses transcription of its target genes (37). Our previous efforts in search for p53 binding targets in a genome-wide study did not reveal p53 binding to Cdc25A loci (38), indicating that p53 might regulate Cdc25A expression indirectly.

To elucidate the mechanism underlying the transcriptional repression of Cdc25a by p53, we analyzed the Cdc25A promoter sequence. The sequence analysis revealed the presence of two ATF/CREB binding motifs in the Cdc25A promoter. Given previous reports that ATF3 is a stress responsive gene and is an inducible p53 target of p53 (16,17), we tested the possibility that p53 might repress Cdc25A expression through induction of ATF3. As expected, ATF3 mRNA level was rapidly increased after 1 μM CPT treatment in wild type HCT116 cells, but its induction was significantly impaired in p53-/- cells (Figure 4B). To test the hypothesis that ATF3 is a transcriptional repressor for Cdc25a, changes of Cdc25a mRNA levels after CPT treatment were monitored in HCT116 cells transfected with ATF3 siRNA. In such cells ATF3 siRNA nearly completely ablated the ATF3 induction at 6 h following CPT treatment, despite a continued increase at 24 h (Figure 4C). In ATF3 siRNA transfected cells Cdc25a mRNA levels were elevated and maintained a higher level that that in the control cells after CPT treatment (Figure 4D). Thus, ATF3 levels are negatively associated with Cdc25a, which supports our hypothesis that ATF3 activation results in transcriptional down-regulation of Cdc25a.

Repressor ATF3 belongs to CREB/ATF family of transcriptional factors and the Cdc25A promoter region contains several putative binding sites for CREB (tgacctca) at positions -259/-254, -330/-324 and -2440/-2433 relative to the transcriptional start site (Figure 4E). To test whether ATF3 directly binds to the Cdc25a promoter, we performed chromatin immunoprecipitation (ChIP) assay using an anti-ATF3 antibody and PCR to analyze the ATF3 binding enrichment in genomic region encompassing the proximal or distal ATF/CREB binding sites. The results showed strong enrichment with ATF3 protein to the Cdc25a promoter at both sites after CPT treatment (Figure 4F). As a negative control, no binding was detected in a control sequence within the first exon of Cdc25a or in samples pull down with non-specific IgG. This result confirms that ATF3 regulates Cdc25a expression through direct binding to its promoter. As expected, ATF3 binding was lower in HCT116 p53-/- cells (Figure 4G).

To investigate whether ectopic expression of p53 or ATF3 suppresses Cdc25a expression, we transfected HCT116 cells with a p53- or ATF3-expressing plasmid or an empty vector. Surprisingly, no detectable changes in Cdc25a levels were observed, indicating that p53 or ATF3 alone was insufficient to cause the repression of Cdc25a expression (Figure 5A). However, the exogenous ATF3 was able to bind to the Cdc25a promoter without CPT, as demonstrated by ChIP assay (Figure 5B). It is known that CREB transcriptional factors
activate transcription only after Ser133 phosphorylation (39). To examine if ATF3 inhibition of Cdc25A transcription requires stress signals, we transfected p53 and ATF3 into HCT116 p53-/- cell line in which 1 μM CPT treatment failed to decrease Cdc25A at 24 h (see Figure 2B). As shown in Figure 5C, while CPT treatment did not result in a decrease in Cdc25A in p53-/- cells transfected with an empty vector, the same treatment caused a marked down-regulation of Cdc25A in cells over-expressing p53 or ATF3. In order to demonstrate the effect of ATF3 knockdown on late-stage Cdc25A protein down-regulation, HCT116 shChk1 cells were chosen for ATF3 siRNA transfection to exclude possible proteasomal degradation caused by Chk1. Figure 5D shows that depletion of ATF3 in these cells rescued the down-regulation of Cdc25A protein after CPT treatment for 24h. Moreover, knockdown of ATF3 enhanced apoptotic response to CPT treatment, which was more profound in HCT116 shChk1 cells (Figure 5E). Thus, loss of ATF3 recapitulates at least partially the effect of p53 loss on both Cdc25A accumulation and apoptosis. These data provide further evidence supporting an important role of ATF3 accumulation in down-regulates Cdc25A and checkpoint activation in response to genotoxic stress. Furthermore, our data indicate that ATF3 binding alone is insufficient to inhibit Cdc25A transcription but requires other stress-inducible factors or post-translational modifications of ATF3.

**DISCUSSION**

In this study, functional screening of a protein kinase siRNA library indicates that ATR-Chk1 signaling plays a key role in conferring anticancer drug CPT-induced S-phase checkpoint in colorectal cancer cells. By contrast, ATM-Chk2 does not seem to be important for such a checkpoint in this experimental condition. This is consistent with the previous finding that ATR is more activated by agents that cause double strand DNA breaks (40,41). Our screening results also indicate that other kinases, including members of PLK family (Plk1) and MAPK family (ERK8, MAP2K2 and MAP3K3) may also be involved in modulating the S-phase checkpoint response. Indeed, the roles of Polo-like kinases and MAP kinases in regulating cell cycle checkpoint have been shown recently (26,28,44). Although knockdown of these kinases only had a moderate effect in S-phase checkpoint in the presence of Chk1, their roles may become more essential in cells lacking Chk1. Thus, functional investigation of these kinases in Chk1-null background should be warranted.

Given that Chk1 is the main kinase transducing signals from stalled replication forks and that pharmacological inactivation of Chk1 sensitizes p53-deficient cells to DNA damaging anticancer drugs (15,45), we set to investigate the mechanisms of such a sensitization. In the present study, we provided evidences that switching off key members of two stress signal pathways, p53 and Chk1, resulted in defects in G1, intra-S-phase and G2/M checkpoints. We found that change in Cdc25A was at least in part responsible for such a phenotype. In addition to the established roles of Chk1 and Chk2 in Cdc25A degradation (27,46), p53-dependent transcriptional repression of Cdc25C has also been shown (47,48). Unlike Cdc25C, Cdc25A promoter does not possess any p53-binding site and p53 does not bind directly to the Cdc25A promoter (49).

Here we show that p53 activation represses the Cdc25A expression through ATF3. We found that ectopic expression of ATF3 alone was insufficient to down-regulate Cdc25A in cycling cells but it occurred in cells treated with CPT. This suggests that a DNA damage signal is required for ATF3 to be fully functional in repressing its target genes. This might require the engagement of other cofactors or posttranslational modifications of ATF3 that were necessary for its activity. In view of
these findings, we propose that p53 and Chk1 cooperate to repress of Cdc25A activity on different levels – by proteasome degradation, caused by Chk1-mediated phosphorylation and by inhibition of Cdc25A transcription by ATF3 (Figure 5F). These mechanisms might occur in concert to have a broad effect in cell cycle checkpoints control.

At the mechanistic level, we provide evidences that one mechanism conferring the high sensitivity of p53/Chk1-deficient cells to DNA damage is the continuous and high expression of Cdc25A during stress response. Indeed, disruption of Chk1-pathway abolishes Cdc25A protein degradation occurred within first few hours of the drug treatment, while inactivation of p53-pathway resulted in constant expression of Cdc25A mRNA. Together, the high level of Cdc25A phosphatase speeds up the G2 transit of damaged cells, and eventually leads to apoptosis.

Cytotoxic agents activate p53 for apoptosis induction (i.e. in intestinal stem cells) that leads to severe complications and undesired side effects (50). It has been shown that p53 induction during DNA damage response does not protect mice from tumors and only induces cytotoxicity in normal tissues (51). Thus, it would be beneficial to transiently inhibit p53 both in normal and tumor tissue (52). Indeed, p53-silencing in slow proliferating or quiescent cells will protect them from apoptosis during short-term drug exposure. At the same time, simultaneous disruption of two check-point pathways (i.e. the combination of UCN-01 and PFT-α to block Chk1/2 and p53, respectively) in tumor cells with a highly proliferative phenotype together with drug exposure will result in cell death. Thereby, transiently modulating selected checkpoints pathways even during short time cytostatic therapy, we can fulfill two equally important aims - protect normal tissue from apoptosis and sensitize malignant cell to anti-cancer drugs.

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Figure Legends

**Figure 1.** ATR and Chk1 are the main kinases in CPT-induced S-phase arrest. (a) Cell cycle analysis of HCT116 cells transfected with negative control (NC) or Chk1 siRNA and treated with 0.2 μM CPT for 24 h. (b) The results of the screening of siRNA library of 780 human kinases from Dharmacon. Kinases with (G2/S) siRNA(kinase) to (G2/S) siRNA(NC) ratio more than 1.8 are shown.

**Figure 2.** Switching off both p53- and Chk1-dependent stress signaling pathways in cells treated with CPT abrogates the G1/S and intra-S-phase checkpoints and completely abolishes the degradation of Cdc25A phosphatase. (a) Cell cycle analysis of HCT116 and HCT116 p53-/- cells and their Chk1-depleted derivates treated or not treated with 0.2 μM or 1 μM CPT for 24 or 48 hours. (b) Western blot showing indicated proteins levels in cell lines described in panel (a) treated or not treated with 0.2 μM or 1 μM CPT for 6 or 24 hours. An anti-β-actin blot was using as a loading control.

**Figure 3.** The ability of HCT116 shChk1/p53-/- cells to by-pass the G2/M checkpoint and their subsequent death are partially dependent upon Cdc25A. (a) Flow cytometry analysis of phospho-histone H3 (Ser28)-positive HCT116 wild type and HCT116 p53-/- cells and their Chk1-depleted derivates treated or not treated with 0.2 μM or 1 μM CPT for 24 or 48 hours The values shown are the means ± standard deviations from three separate experiments. (b) Flow cytometry analysis of phospho-histone H3 (Ser28)-positive HCT116 shChk1/p53-/- cells transfected with siRNA targeting Cdc25A or with negative control (NC) siRNA and then treated or not treated with 0.2 μM or 1 μM CPT for 24 hours. The values shown are the means ± standard deviations from three separate experiments. To validate Cdc25A knock-down, cells were transfected siRNA targeting Cdc25A or with negative control (NC) siRNA and analyzed by immunoblotting with anti-Cdc25A antibodies. An anti-β-actin blot was using as a loading control. (c) Flow cytometry analysis of caspase-3 activation in cell lines described in (a) treated or not treated with 0.2 μM CPT for 24 or 48 hours. The values shown are the means ± standard deviations from three separate experiments. (d) Flow cytometry analysis of caspase-3 activation in HCT116 shChk1/p53-/- cells transfected for 24 hours with siRNA targeting Cdc25A or with negative control (NC) siRNA and then treated or not treated with 0.2 μM CPT for 48 hours. The values shown are the means ± standard deviations from three separate experiments. (e) Restoration of surviving colony numbers of NC siRNA transfected HCT116 p53-/- shChk1 cells exposed to 0.2 μM CPT compare to Cdc25A siRNA transfected cells. Cells were split 24 h after CPT treatment. Plates were stained with crystal violet.

**Figure 4** p53 down-regulates Cdc25A expression through induction of transcriptional repressor ATF3. (a) RT-PCR analysis of Cdc25A mRNA in HCT116 and HCT116 p53-/- cells treated or not treated with 1 μM CPT for 6 or 24 hours. RNA was normalized with GAPDH mRNA. (b) RT-PCR analysis of ATF3 mRNA in HCT116 and HCT116 p53-/- cells treated or not treated with 1 μM CPT for 6 or 24 hours. (c) RT-PCR analysis of ATF3 mRNA in HCT116 cells transfected for 24 hours with siRNA targeting ATF3 or with negative control (NC) siRNA and then treated or not treated with 1 μM CPT for indicated time points. (d) RT-PCR analysis of Cdc25A mRNA in HCT116 cells transfected for 24 hours with siRNA targeting ATF3 or with negative control (NC) siRNA and then treated or not treated with 1 μM CPT for indicated time points. (e) Scheme of the human Cdc25A promoter region. The arrow shows the transcription start site. The boxes indicate the putative binding sites for CREB/ATF transcriptional factors. The bars below the diagram represent the amplicons used for ChIP experiments. (f) ChIP assays were...
performed in HCT116 cells treated or not treated either with 1 μM CPT for 12 hours using antibodies to ATF3 and non-specific IgG. The Cdc25A promoter fragments were amplified by PCR using primers flanking the putative CREB/ATF binding regions. Amplification of the fragment from the first exon of the Cdc25A gene is shown as a negative control. Amplification of input the DNA is shown as a positive control. (g) ChIP assays were performed in HCT116 wt and p53-/- cells treated or not treated with 1 μM CPT 12 hours. Crosslinked chromatin was immunoprecipitated using anti-ATF3 antibodies.

Figure 5. *p53 and ATF3 down-regulate Cdc25A only under stress conditions.* (a) Western blot showing Cdc25A, p53 and ATF3 levels in HCT116 cells transfected with pcDNA3, pcp53 or pcATF3 vector for 24 hours. An anti-β-actin blot was using as a loading control. (b) ChIP assays were performed in HCT116 wt cells transfected with pcATF3 or an empty vector and then treated or not treated with 1 μM CPT for 12 hours. Crosslinked chromatin was immunoprecipitated using anti-ATF3 antibodies. (c) Western blot showing Cdc25A, p53 and ATF3 levels in HCT116 p53-/- cells transfected with pcDNA3, pcp53 or pcATF3 vector for 24 hours and then treated with 1 μM CPT for 24 hours. An anti-β-actin blot was using as a loading control. (d) Western blot showing Cdc25A and ATF3 levels in HCT116 shChk1 cells transfected with ATF3 (or negative control) siRNA and then treated with CPT for 24h. An anti-β-actin blot was using as a loading control. (e) Cell cycle analysis of HCT116 wild type and Chk1-depleted cells transfected with negative control (NC) or ATF3 siRNA and treated with 0.2 μM CPT for 24 h. (f) p53-ATF3 and ATR-Chk1 pathways cooperate in Cdc25A down-regulation after DNA damage.
Figure 1

A

200nM CPT, 24h

NC siRNA

Chk1 siRNA

B

| 1  | ABI1/NC      | 2.1 |
| 2  | ANKRD3/NC   | 2   |
| 3  | ATR/NC      | 8.1 |
| 4  | ATM/NC      | 2.2 |
| 5  | CD4/NC      | 2.1 |
| 6  | CHK1/NC     | 4.9 |
| 7  | CHK2/NC     | 2.5 |
| 8  | CSNK2B/NC   | 2.1 |
| 9  | DYRK3/NC    | 2   |
| 10 | EphB6/NC    | 1.8 |
| 11 | ERK8/NC     | 2.4 |
| 12 | FGFR4/NC    | 2.9 |
| 13 | LIM/NC      | 2   |
| 14 | MAP2K2/NC   | 1.8 |
| 15 | MAP3K3/NC   | 1.8 |
| 16 | PFTK1/NC    | 1.9 |
| 17 | PLK1/NC     | 3.3 |
| 18 | RPS6KA5/NC  | 1.8 |
| 19 | RPS6KC1/NC  | 1.8 |
| 20 | SGK1/NC     | 2   |
| 21 | SRPK1/NC    | 2.2 |
Figure 2

A

|        | CPT, 0.2 µM |        | CPT, 1 µM |
|--------|-------------|--------|-----------|
|        | c 24 h 48 h | 24 h   |           |
| wt     |             |        |           |
| shChk1 |             |        |           |
| p53 -/-|             |        |           |
| p53 -/- shChk1 | |        |           |

2N 4N 2N 4N 2N 4N 2N 4N

B

CPT, 1 µM:

| h  | wt  | shChk1 | p53 -/- | shChk1 p53 -/- |
|----|-----|--------|---------|----------------|
| 0  | 0   | 0      | 0       | 0              |
| 6  | 6   | 6      | 6       | 6              |
| 24 | 24  | 24     | 24      | 24             |

1 0.7 1.4 1 0.7 1.2 0.8 1.4 1.6 1.5

Cdc25A

Chk1

p53

actin
Figure 3

A

H3-phosphorylation (%)

C

Caspase-3 positive cells, %

E

siRNA: NC Cdc25A

0.2 µM CPT, 24 h

HCT116 p53-/- shChk1

Number of caspase-3 positive cells, folds of induction

B

H3-phosphorylation (%)

D

siRNA: NC Cdc25A

β-actin

Cdc25A

C

CPT, 0.2 µM, 48 h

wt shChk1 p53 -/- p53 -/- shChk1

Caspase-3 positive cells, %

Number of caspase-3 positive cells, folds of induction

NC siRNA Cdc25A siRNA

CPT, 0.2 µM, 24 h

CPT, 0.2 µM, 48 h
Figure 4

A. Relative cdc25a mRNA levels

B. Relative ATF3 mRNA levels

C. Relative ATF3 mRNA levels

D. Relative cdc25a mRNA levels

E. Schematic representation of ATF/CREB-1 and ATF/CREB-2 regions

F. Western blot analysis of ATF/CREB-1 and ATF/CREB-2

G. Fold enrichment

HCT116 wt  HCT116 p53-/-
Figure 5

A

B

C

D

E

F

double-strand breaks

ATR

Chk1

ATF3

cdc25a

Cdc25a

ATF3

actin

HCT116 wt

pcDNA3

pcATF3

pcp53

CPT, 0.2 µM:

0
24
0
24
h

Cdc25A

p53

ATF3

actin

HCT116 p53-/-

CPT, 1 µM:

0
24
0
24
h

Cdc25A

p53

ATF3

actin

HCT116 wt

NC siRNA

ATF3 siRNA

CPT, 0.2 µM:

0
24
0
24
h

Cdc25A

ATF3

actin

HCT116 shChk1

C

0.2 µM CPT, 24h

subG1=3±0.5%

subG1=11±1%

subG1=5±1%

subG1=26±2%

HCT116 wt

C

0.2 µM CPT, 24h

subG1=5±1%

subG1=12±2%

subG1=8±1%

subG1=35±2%

HCT116 shChk1

NC siRNA

ATF3 siRNA

p53

Cdc25A

ATF3

cell cycle arrest

DNA repair
Dual regulation of Cdc25A by CHK1 and p53-ATF3 in DNA replication checkpoint control
Anastasia R. Demidova, Mei Yee Aau, Li Zhuang and Qiang Yu

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