

C-terminal domain of Chk1 regulates its subcellular location and kinase activity for DNA repair

NING LiFeng^{1,2}, WANG HuiPing², SAN Dan¹ & SANG JianLi^{1*}¹ Key Laboratory for Cell Proliferation and Regulation Biology of Ministry of Education, College of Life Science, Beijing Normal University, Beijing 100875, China;² Department of Reproduction Immunology, National Research Institute for Family Planning, Beijing 100081, China

Received December 27, 2010; accepted April 19, 2011

Effector kinase Chk1 is an evolutionarily conserved protein kinase. It is a key mediator linking the mechanisms that monitor DNA integrity to components of the cell cycle engine. In this study, recombinant vectors pEGFP-C1-Chk1/CA288/CA334/CA368 were constructed and transfected into HeLa cells to study the effect of the Chk1 regulatory domain on the regulation of subcellular Chk1 location in response to DNA damage. We found that DNA damage-induced nuclear accumulation is regulated by 34 amino acids (334–368) in the C-terminal regulatory domain. Recombinant vectors pXJ41-Chk1/CA288/CA334/CA368 were co-transfected with reporter plasmid pEGFP-N2 into HeLa cells to study the repair abilities of the different human Chk1 truncation mutants. In addition, recombinant vectors were transfected into HeLa cells to study the effects of the different truncation mutants on the cell cycle. Furthermore, to study the kinase activity of the different truncation mutants, Ser216 phosphorylation of Cdc25C was studied by Western blot analysis. We found that the enzymatic activity of CA368, missing the 108 C-terminal amino acids (368–476), was higher than that of full-length Chk1, and CA368 delayed the cell cycle progression. The enzymatic activity of CA334, missing the 142 C-terminal amino acids (334–476), was equivalent to that of full-length Chk1. CA288, missing the 188 C-terminal amino acids (288–476), had almost no enzymatic activity, suggesting that the regulatory domain contains both inhibitory and regulatory elements. This study provides useful information for further research on Chk1 function.

Chk1, nuclear accumulation, domain, DNA damage, repair, cell cycle

Citation: Ning L F, Wang H P, San D, et al. C-terminal domain of Chk1 regulates its subcellular location and kinase activity for DNA repair. *Chinese Sci Bull*, 2011, 56: 3138–3147, doi: 10.1007/s11434-011-4538-4

Living organisms face tremendous challenges in accurately transmitting genetic material to subsequent generations. To maintain genomic integrity, cells have evolved mechanisms known as checkpoints that detect genomic perturbations and initiate a complex network of signal transduction pathways consisting of sensors, transducers, and effectors to mitigate deleterious effects [1]. The sensors detect DNA damage and aberrant DNA structures and initiate the signal response pathway. The transducers amplify the signal and relay it to effectors, which carry out a variety of activities, such as stopping cell cycle transitions, repairing damaged DNA and inducing cell death.

Checkpoints allow the cell cycle to be arrested and enable the induction of genes that facilitate DNA repair. These checkpoints ensure that damaged DNA is not replicated in the S phase or passed on to daughter cells in mitosis. These responses ensure that DNA replication and chromosome segregation are completed with high accuracy. Checkpoint defects result in genomic instability, cell death, and a predisposition to cancer.

Checkpoint kinase 1 (Chk1) was first identified in fission yeast. Chk1 homologs have since been identified in mammals, flies, budding yeast, worms, frogs and chickens. Checkpoint functions are largely conserved in these organisms [2]. Chk1 is an evolutionarily conserved protein kinase, which plays an important role in DNA damage-induced

*Corresponding author (email: jlsang@bnu.edu.cn)

checkpoint function [3,4]. Chk1 acts downstream of Atm-like proteins in yeasts and higher eukaryotes and regulates the checkpoint response that prevents chromosome segregation in the presence of DNA damage. Chk1 is a key mediator linking the mechanisms that monitor DNA integrity to components of the cell cycle engine [5]. Chk1 participates in the regulation of the cell cycle process, monitoring the integrity of genomic DNA and driving the repair of damaged genomic DNA. Over-expression of Chk1 contributes to the self-repair of cells. However, under-expression or knockdown of Chk1 impairs the ability of cells to arrest the cell cycle at the G2/M phase and to trigger apoptosis [6–8].

The cell cycle checkpoint consists of ataxia telangiectasia mutated (ATM) and ATM Rad3 Related (ATR) protein kinases, Chk1/2 and Cdc25A/B/C [9]. ATR and ATM phosphorylate and activate Chk1, which is the key event in the Chk1 response to DNA damage and the arrest of DNA replication. Activated Chk1 triggers the transduction and augmentation of the signal, leading to expression of target proteins and arrest of the cell cycle [10]. Inactivation of the Chk1 gene blocks the signaling pathway of the cell cycle checkpoint, as Chk1 is the target of ATM and ATR, which both control the signaling pathways that arrest the cell cycle after DNA damage [11].

One of the key events in the initiation of mitosis is the activation of CyclinB-Cdk1 [12]. Wee1 and Myt1 phosphorylate and inactivate CyclinB-Cdk1, while Cdc25C, a phosphatase, activates CyclinB-Cdk1 [13]. Chk1 has a pivotal role in the processes triggered by the checkpoint at the G2/M phase. When DNA damage occurs, Chk1 phosphorylates and inactivates Cdc25C, preventing the transition from G2 to M phase to allow for DNA repair [14]. Thus, the inhibitory phosphorylation of Cdc25C blocks mitosis and promotes the repair of damaged DNA. If DNA repair is completed, the cell cycle process continues [15].

Chk1 contains two structural domains: a kinase domain in the N-terminal half and a regulatory domain in the C-terminal half of the protein. An SQ/TQ domain (317–383) is located in the regulatory domain. Both ATR and ATM kinases phosphorylate serine and threonine residues that are followed by glutamine (SQ/TQ). ATM phosphorylates Ser317 and Ser345 of Chk1 in response to DNA damage. There is a nuclear location sequence (NLS) in motif 260–271 of Chk1 (Figure 1) [16–21]. In yeast and mammalian cells Chk1 is located both in the cytoplasm and nuclei before DNA damage, but it accumulates in nuclei after DNA damage, indicating a distinct change in subcellular location [22]. Recombinant Chk1, expressed in cultured cells, displayed only basal enzymatic activity [5]. Unlike the full-length protein, C-terminus-truncated Chk1 leads to autophosphorylation and phosphorylation of Cdc25C at Ser216. The subcellular location of Chk1 and its ability to repair DNA damage, however, requires further exploration.

In this study, a series of regulatory domain-truncated Chk1 mutations fused with green fluorescent protein (GFP)

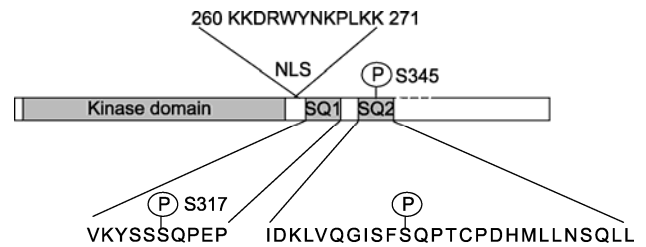


Figure 1 Structure of the Chk1 protein. A kinase domain is located in the N-terminal half and a regulatory domain is located in the C-terminal half of the protein. The SQ/TQ domain has several conserved Ser-Gln (SQ) or Thr-Gln (TQ) motifs, in which the serine or threonine residues are preferred phosphorylation sites of ATR or ATM. P represents phosphorylation sites. An SQ/TQ domain (317–383) is located in the regulatory domain. Both ATR and ATM kinases phosphorylate serine and threonine residues that are followed by glutamine (SQ/TQ). ATM phosphorylates Chk1 Ser317 and Ser345 in response to DNA damage. There is a nuclear location sequence (NLS) in motif 260–271.

was used to investigate the subcellular location of Chk1. Chk1 and its truncation mutants were constructed into a pXJ41 vector. Cells were co-transfected with the Chk1 mutant vectors and pEGFP-N2 to investigate the ability of the mutants to repair DNA damage. In addition, for analysis of the kinase activity of the different Chk1 truncation mutants, Ser216 phosphorylation of the Cdc25C protein, which is a downstream target of Chk1, was studied by Western blot analysis.

1 Materials and methods

1.1 Materials

HeLa cells, pXJ41-neo, and eukaryotic expression vector, pEGFP-C1, were maintained in our lab. The transfection reagent VigoFect was purchased from Vigorous (Vigorous, Beijing). Restriction enzymes were purchased from Promega (Promega, Madison, WI). Oligo(dT)₅ and Trizol RNA were purchased from Invitrogen (Invitrogen, Carlsbad, California). DMEM was purchased from GIBCO (GIBCO BRL, Invitrogen, USA). Phospho-cdc25C (Ser216)(63F9) rabbit mAb and cdc25C (5H9) rabbit mAb were purchased from Cell signaling Technology (Cell signaling Technology, Danvers, MA) and anti-rabbit IRDye800-linked IgG was obtained from Rockland Immunochemicals (Rockland Immunochemicals, Inc., Gilbertsville, PA). Nucleoid occlusion protein (NOC), hydroxyurea (HU) and propidium iodide (PI) were purchased from Sigma (Sigma, St. Louis, MO).

1.2 Construction of recombinant vectors

Full-length human Chk1 cDNA was obtained by RT-PCR and cloned into pMD18-T. The target sequences of truncation mutations were amplified by PCR from pMD18-T-Chk1 and ligated into the pXJ41/pEGFP-C1 vector after both had been digested with *EcoR* I/*Xho* I (Promega). All PCR primers

were synthesized by Saibaisheng, China. All inserts in recombinant constructs were verified by DNA sequencing.

1.3 Cell culture and transfection

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (GIBCO BRL) and maintained in a humid incubator at 37°C with 5% CO₂. Recombinant expression vectors were transfected into HeLa cells grown on cover slips placed in 6-well plates with VigoFect (Vigorous) according to the manufacturer's instructions. The medium was replaced after 12 h.

1.4 Nuclear accumulation assay of the different Chk1 mutants after DNA damage

Forty hours after transfection with pEGFP-C1-Chk1, HeLa cells were damaged by UV irradiation (90 J/m²) for 0, 5, 10 or 15 min and observed under a microscope (Olympus Fluoview 300) to choose the optimal time to study the changes in subcellular location of Chk1 in response to DNA damage.

pEGFP-C1-Chk1, pEGFP-C1-CA288, pEGFP-C1-CA334 and pEGFP-C1-CA368 were transfected into HeLa cells. Forty hours later, cells were UV-irradiated (90 J/m²) for 0, 5, 10 or 15 min, respectively. The medium was discarded, and cells were fixed with methyl aldehyde for 10 min at -20°C. Cells were washed 3 times with PBS and once with distilled water. Distribution of GFP-Chk1 and the GFP-Chk1 mutants was studied using microscopy (Olympus Fluoview 300). To evaluate and quantify the nuclear location of GFP-Chk1 and the GFP-Chk1 mutants, positive cells were classified as follows: (1) N=C cells, the fluorescence intensity is approximately equal in the nucleus and cytoplasm; (2) N>C cells, the fluorescence intensity in the nucleus is stronger than that in the cytoplasm. The number of cells belonging to each category and the number of cells emitting fluorescence were determined in different microscope fields. The relative ratios of cells belonging to the two categories were compared before and after DNA damage to analyze the changes in Chk1 location. Values were obtained by averaging the results from at least 4 fields.

1.5 Analysis of the abilities of the different truncation mutants to repair DNA damage

The pEGFP-N2 plasmid was damaged by UV irradiation (90 J/m²) for 0, 10, 20, 30, 40 or 50 min and then transfected into HeLa cells. GFP expression of transfected cells was then studied under a microscope (Olympus Fluoview 300) to choose the optimal time to study the abilities of the different truncation mutants to repair DNA damage.

HeLa cells were co-transfected with the truncation mutants and pEGFP-N2 plasmids treated with UV, and were then studied under a fluorescence microscope (Olympus Fluoview 300). Cells emitting fluorescence were catego-

rized as positive cells. The fluorescence intensity and total cell number were analyzed in different microscope fields for cells transfected with each of the different truncation mutants. At least 4 microscope fields were counted for each cell type. The fluorescence intensity, representing overall GFP expression, was calculated by Image Pro Plus (Media Cybernetics Inc.).

1.6 Analysis of the effects of the different C-terminal truncation mutants on the cell cycle

The cell cycle was halted at the G2/M phase, 24 h after transfection with the truncation mutants, by treatment with 0.1 µg/mL NOC (Sigma) for 16 h, after which the cells were harvested. For RT-PCR identification of transfection efficiency, total RNA was used as a template for amplification of the neo gene. Cells were also fixed overnight in 70% ethanol, then washed twice with PBS, and finally incubated with 50 µg/mL RNase at 37°C for 30 min. After incubation on ice for 1 min, PI (Sigma) was added and the cells were incubated at 4°C in the dark. This was followed by flow cytometry analysis (Becton Dickinson, San Jose, CA).

1.7 Analysis of the kinase activity of the different C-terminal truncation mutants

Cells were treated with 5 mmol/L HU (Sigma) for 18 and 48 h after transfection with the different C-terminal truncation mutants and then harvested. Protein concentration was determined by the BCA protein assay. To investigate the kinase activity of the different C-terminal truncation mutants, Ser216 phosphorylation of the Cdc25C protein was analyzed by Western blot. Proteins were probed with a Phospho-cdc25C(Ser216)(63F9) rabbit mAb (Cell signaling) or a cdc25C (5H9) rabbit mAb (Cell signaling), followed by incubation with an anti-rabbit IRDye800-linked IgG (Rockland Immunochemicals). Imaging films were scanned using the Odyssey infrared imaging system and read in gray values. Cdc25C was used as an internal reference to adjust the gray values of the target protein. The ratio of Ser216 phosphorylation of the Cdc25C protein in the cells without treatment by HU was set as 1. Data from 3 repeated experiments with stable results were used for statistical analysis. For all experiments, statistical analyses were performed using Prism 4.0 software and $P < 0.05$ was considered statistically significant.

2 Results

2.1 Vector construction of Chk1 and Chk1 truncation mutants

Full-length human Chk1 was cloned from the cDNA of HeLa cells. To investigate the functions of different Chk1 domains, several truncation mutations were inserted into

pXJ41 and pEGFP-C1, including CA288, missing the 188 C-terminal amino acids (288–476), CA334, missing the 142 C-terminal amino acids (334–476) and CA368, missing the 108 C-terminal amino acids (368–476) (Figure 2). All of the plasmids were confirmed by sequencing.

2.2 Nuclear accumulation of the different Chk1 truncation mutants in response to DNA damage

HeLa cells were transfected with pEGFP-C1-Chk1 and 40 h later cells were treated with UV irradiation for different durations. The subcellular localization of GFP-Chk1 was analyzed under a fluorescence microscope. Nuclear accumulation of GFP-Chk1 increased with increasing duration of UV treatment (Figure 3). The percentage of cells with nuclear accumulation of GFP-Chk1 increased to a maximum after UV treatment for 10 min (Figure 4). However, the percentage of cells with nuclear accumulation of GFP-Chk1 declined (Figure 4) and the number of cells emitting fluorescence (Figure 3) was reduced after UV treatment for 15 min. Cells were seriously damaged after UV treatment for 15 min. We, therefore, chose 10 min as the optimal time to study the subcellular localization of Chk1 in response to DNA damage.

Figures 5 and 6 show that the percentage of cells with nuclear accumulation of GFP-CA368 and GFP-Chk1 increased in response to DNA damage caused by UV treatment for

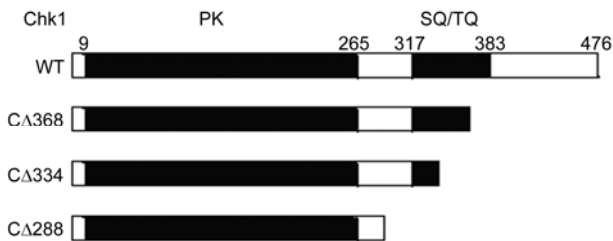


Figure 2 Structure of Chk1 and Chk1 truncation mutants.

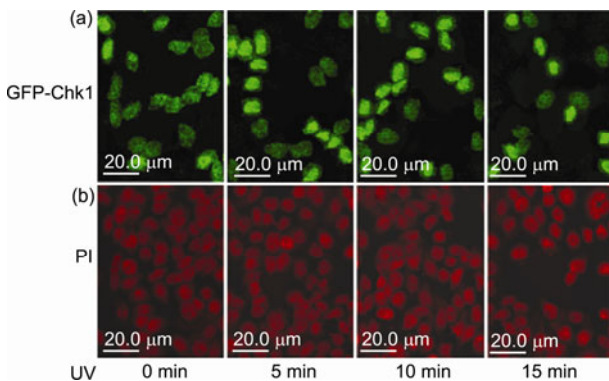


Figure 3 Nuclear accumulation of full-length Chk1 after UV treatment (90 J/m^2) for 0, 5, 10 and 15 min, 24 h after transfection with pEGFP-C1-Chk1. (a) Expression of GFP-Chk1. (b) Propidium iodide (PI) staining.

(a)

Cell	Type	Rate (N/C)
	N=C	0.95–1.14
	N>C	>1.15

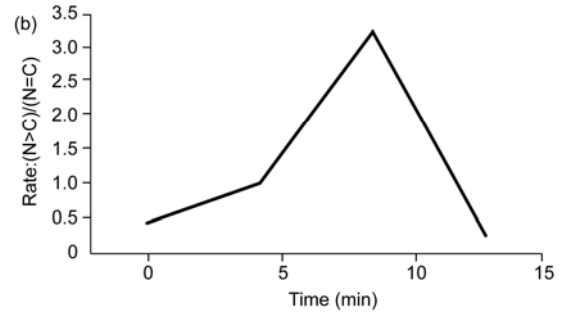


Figure 4 Relative ratios of cells with different subcellular locations of GFP-Chk1. (a) Cell categories. N=C cells, the GFP-Chk1 fluorescence intensity is approximately equal in the nucleus and cytoplasm; N>C cells, the GFP-Chk1 fluorescence intensity in the nucleus is stronger than that in the cytoplasm. (b) The relative ratio of cells with different subcellular locations of GFP-Chk1 0, 5, 10 and 15 min after UV treatment.

10 min. The percentage of cells with nuclear accumulation of GFP-CA368 is similar to the percentage of cells with nuclear accumulation of GFP-Chk1 after UV treatment. However, the percentages of cells with nuclear accumulation of GFP-CA334 and GFP-CA288 did not change after UV

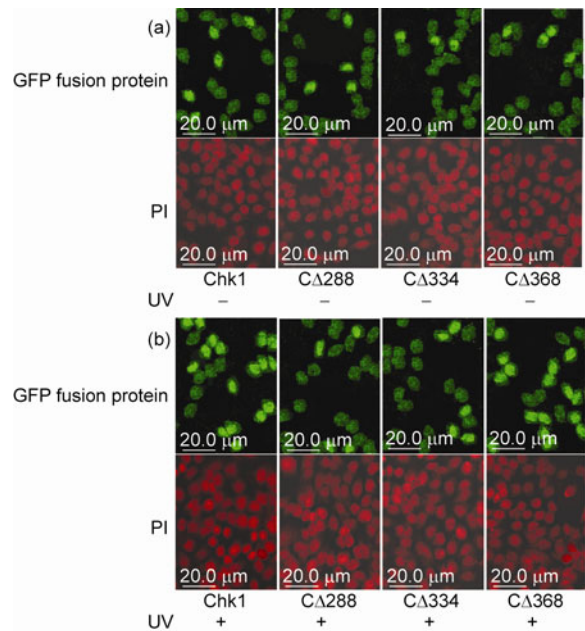


Figure 5 Nuclear accumulation of the different Chk1 truncation mutants after UV treatment. (a): Without UV treatment (-). (b): With UV treatment (+).

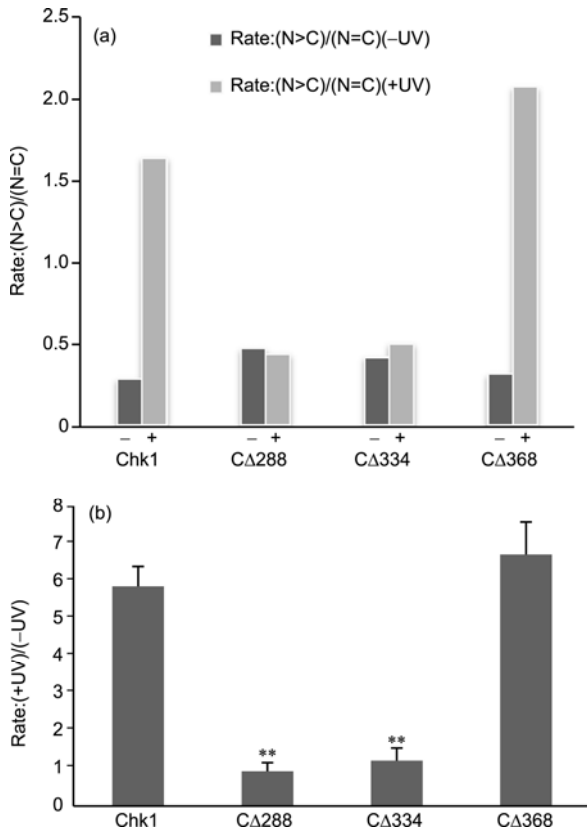


Figure 6 Nuclear accumulation of the different Chk1 truncation mutants after UV treatment. (a) Relative ratios of cells with different subcellular locations of the GFP-Chk1 mutants before (+) and after (-) UV treatment for 10 min. (b) Changes in localizations of the GFP-Chk1 mutants before and after UV treatment for 10 min expressed as the ratio between the (+) column and the (-) column in (a). Values are mean \pm SD for at least 4 microscope fields per cell type. **, compare with Chk1, $P < 0.01$.

treatment. This demonstrates that the sequence of 34 amino acids between GFP-CΔ334 and GFP-CΔ368 is very important for the regulation of nuclear accumulation of Chk1 induced by DNA damage.

2.3 Abilities of the different Chk1 truncation mutants to repair DNA damage

pEGFP-N2 plasmids were treated with UV irradiation for different durations. HeLa cells were transfected with the treated pEGFP-N2 plasmids. Figures 7 and 8 show that 48 h after transfection the percentage of cells emitting fluorescence decreased with increasing duration of UV treatment. When UV treatment time exceeded 40 min, the pEGFP-N2 plasmid was damaged too severely to express the GFP protein. We chose 40 min as the optimal treatment time to study the abilities of the different truncation mutants to repair DNA damage.

To study the abilities of the different Chk1 truncation mutations to repair DNA damage, pEGFP-N2 plasmids were treated with UV for 40 min. Undamaged or damaged pEGFP-N2 plasmids and different Chk1 truncation mutants were then co-transfected into HeLa cells. Undamaged pEGFP-N2 co-transfected with pXJ41 into HeLa cells is control 1, damaged pEGFP-N2 co-transfected with pXJ41 into HeLa cells is control 2, and damaged pEGFP-N2 co-transfected with pXJ41-Chk1 into HeLa cells is control 3. Figures 9 and 10 show that the fluorescence intensity of control 2 was markedly decreased compared with control 1. The fluorescence intensity of control 3 was stronger than the fluorescence intensity of control 2. The fluorescence intensity of cells co-transfected with damaged pEGFP-N2 and pXJ41-Δ368 was stronger than that of control 3, the fluorescence intensity of cells co-transfected with damaged pEGFP-N2 and pXJ41-Δ334 was equivalent to that of control 3, and the fluorescence intensity of cells co-transfected with damaged pEGFP-N2 and pXJ41-Δ228 was markedly weaker than that of control 3. These results indicate that the repair ability of CΔ368 was higher than that of full-length Chk1, the repair ability of CΔ334 was equivalent to that of full-length Chk1, and CΔ288 had almost no repair ability. These data indicate that deletion of part of the Chk1 regulatory domain activated

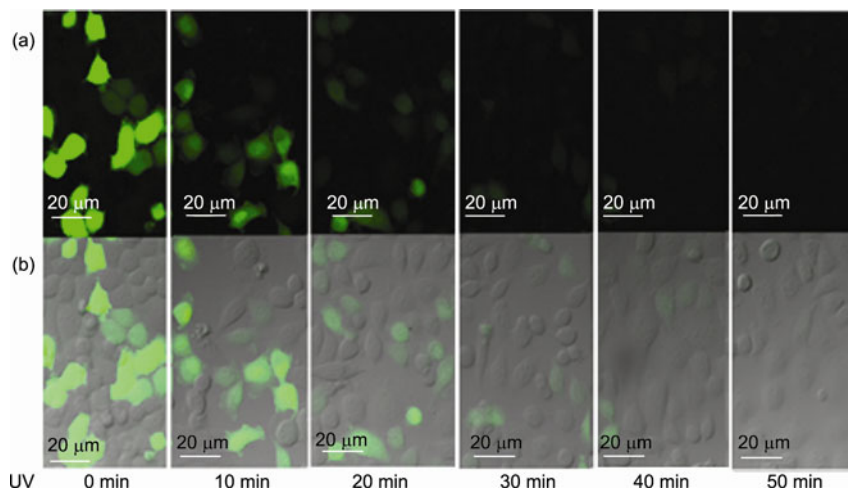


Figure 7 Fluorescence intensity of GFP after UV treatment of pEGFP-N2 for different durations. (a): GFP expression. (b): Overlay of bright field and green fluorescence images.

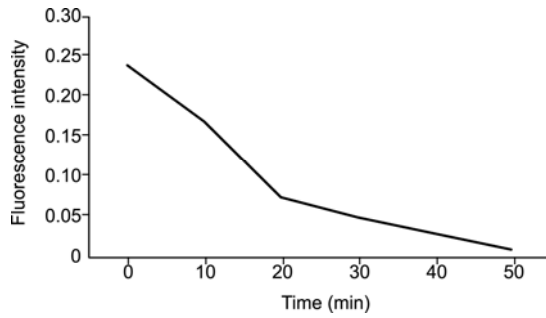


Figure 8 Fluorescence intensity of GFP after UV irradiation of pEGFP-N2 for different durations.

the kinase. When the entire SQ/TQ domain was deleted from the regulatory domain, Chk1 had no enzymatic activity.

2.4 Effects of over-expression of the different Chk1 truncation mutants on the cell cycle

To study the effects of the different Chk1 mutants on the

cell cycle, cells transfected with Chk1 or the Chk1 truncation mutants were treated with NOC, and cell cycles were analyzed by flow cytometry 16 h after transfection. RT-PCR for the *neo* gene indicated that transfection efficiencies of the different plasmids were approximately equal (Figure 11). Untransfected cells are referred to as control 1, cells transfected with pXJ41 are referred to as control 2 and cells treated with NOC after transfection with pXJ41 are referred to as control 3. Figure 12 shows that there were no clear differences between the cell cycle progression of control 2 and control 1. Cells of control 3 were trapped in the G2/M phase. Compared with control 3, cells treated with NOC after transfection with pXJ41-Chk1, pXJ41-CA288 or pXJ41-CA334 were trapped in the G2/M phase. Cells treated with NOC after transfection with pXJ41-CA368 were not trapped in G2/M but had a delayed G1/S phase. Over-expression of Chk1, CA334 or CA288 did not disrupt cell cycle progression, but expression of the active CA368 delayed the G1 or S phase. These results demonstrate that the enzymatic activities of the different Chk1 truncation mutants correlated

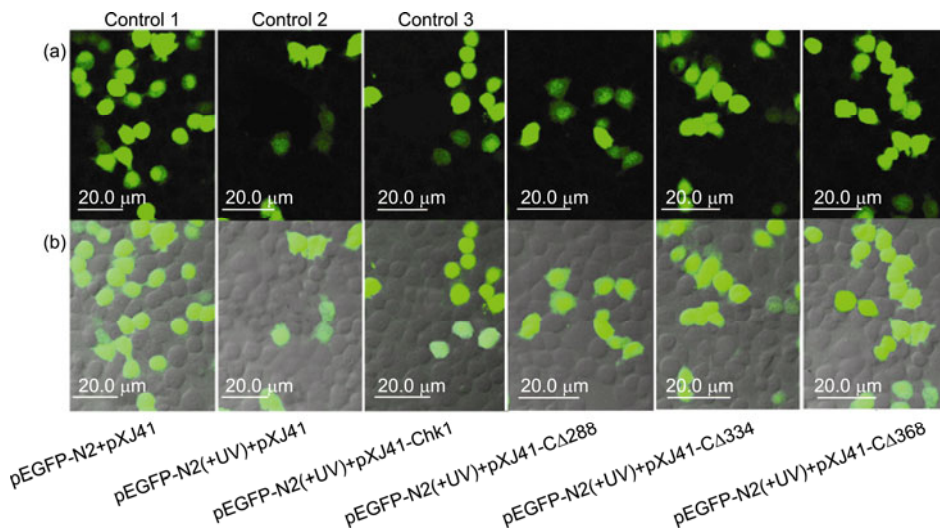


Figure 9 Abilities of the different Chk1 truncation mutants to repair DNA damage. (a) GFP expression. (b) Overlay of bright field and green fluorescence images.

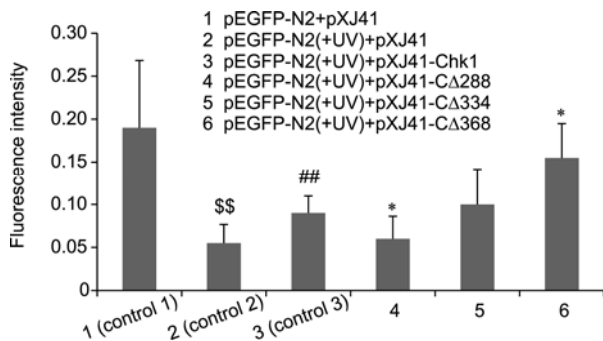


Figure 10 Abilities of the different Chk1 truncation mutants to repair DNA damage, expressed as the fluorescence intensity. Values are mean \pm SD for at least 4 microscope fields per cell type. \$\$, compare with control 1, $P < 0.01$; ##, compare with control 2, $P < 0.01$; *, compare with control 3, $P < 0.05$.

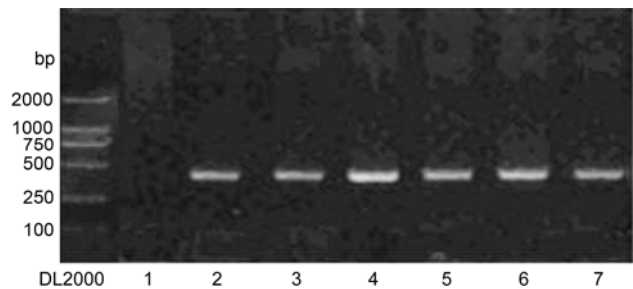


Figure 11 Identification of the *neo* gene in cells transfected with the different Chk1 truncation mutants. 1, normal; 2, pXJ41; 3, pXJ41; 4, pXJ41-Chk1; 5, pXJ41-CA288; 6, pXJ41-CA334; 7, pXJ41-CA368.

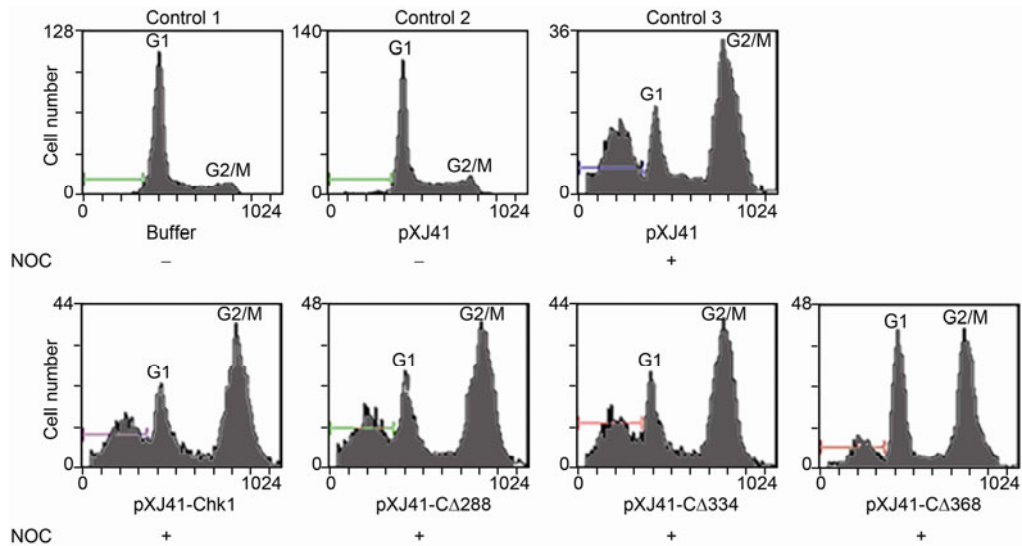


Figure 12 Effect of Chk1 and the different Chk1 mutations on the cell cycle with (+) and without (-) NOC treatment.

with their effects on the cell cycle. Steric hindrance may exist in the regulatory domain of Chk1, and CΔ368, which is missing the 108 C-terminal amino acids (368–476), may have an exposed latent active site because of disruption of this steric hindrance. This results in an enzymatic activity of CΔ368 that is higher than that of full-length Chk1. These results demonstrate that the spatial structure of the C-terminal regulatory domain of Chk1 plays an important role in Chk1 function.

2.5 Kinase activity of the different Chk1 truncation mutants

To study the kinase activity of the different Chk1 truncation mutants, cells transfected with Chk1 or different Chk1 truncation mutants were treated with HU, and Ser216 phosphorylation of the Cdc25C protein was analyzed by Western blot 18 h after HU treatment. HeLa cells transfected with pXJ41 are referred to as control 1, cells treated with HU after transfection with pXJ41 are referred to as control 2, and cells treated with HU after transfection with pXJ41-Chk1 are referred to as control 3. Figures 13 and 14 show that there was clearly greater Cdc25C Ser216 phosphorylation in control 2 compared with control 1. This may be related to the kinase activity of endogenous Chk1. Ser216 phosphorylation of Cdc25C was stronger in control 3 than in control 2. This may be because of the combined kinase activity of endogenous and exogenous Chk1. Phosphorylation activity of CΔ368 was stronger than that of full-length Chk1, phosphorylation activity of CΔ334 was equivalent to that of full-length Chk1, and phosphorylation activity of CΔ288 was equivalent to control 2. These results show that the kinase activity of CΔ368 was higher than that of full-length Chk1, the kinase activity of CΔ334 was equivalent to that of full-length Chk1, and that CΔ288 almost had

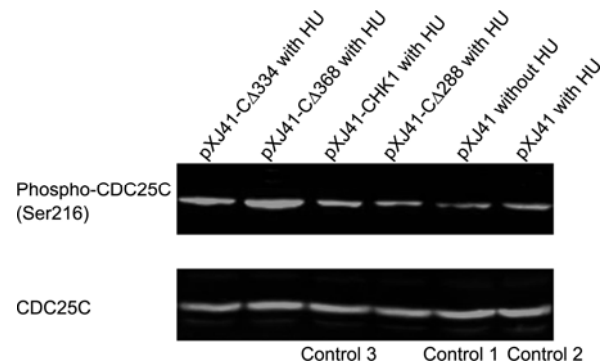


Figure 13 Analysis of the kinase activity of the different Chk1 truncation mutants.

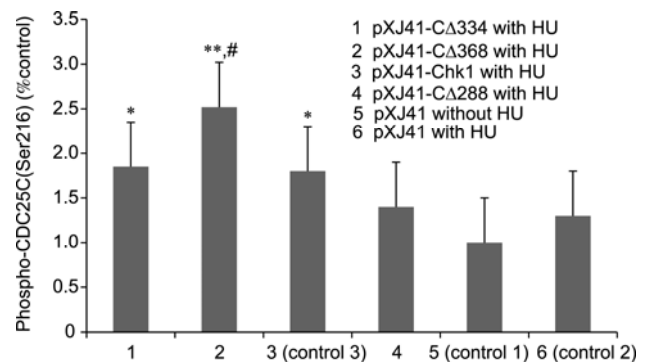


Figure 14 Relative kinase activities of the different C-terminal truncation mutants. Cdc25C was used as an internal reference to adjust the gray values of the target protein. The ratio of Cdc25C Ser216 phosphorylation in cells without HU treatment was set as 1. Values are mean \pm SD for 3 repeated experiments with stable results per cell type. HU = hydroxyurea. Compare with control 2, *, $P < 0.05$; **, $P < 0.01$; #, compare with control 3, $P < 0.05$.

no kinase activity. It further proves that deletion of part of the Chk1 regulatory domain activated the kinase. When the

entire SQ/TQ domain was deleted from the regulatory domain, Chk1 had no enzymatic activity.

3 Discussion

Chk1 accumulates in the nucleus of fission yeast cells that have been treated with the drug camptothecin, a DNA replication inhibitor. In a previous study, fusion proteins were generated by attaching enhanced yellow fluorescent protein (EYFP) in-frame to the amino terminus of full-length Chk1. After HeLa cells transfected with the EYFP vector were treated with the DNA-damaging agents, HU or methyl methane sulfonate (MMS) [22,23], accumulation of EYFP-Chk1 was observed in the nucleus of the cells [21]. These studies demonstrated a change in the subcellular localization of EYFP-Chk1 in response to HU and MMS-induced DNA damage. It is, therefore, necessary to study the nuclear accumulation sequence of Chk1 that regulates DNA damage-induced Chk1 localization and function. Subcellular localization of a Chk1 deletion mutant missing the 115 C-terminal amino acids (361–476) did not alter the checkpoint-induced localization/retention of Chk1 [21]. These results suggest that the first 460 amino acids of Chk1 may be required for DNA damage-induced nuclear localization and retention of Chk1. An SQ/TQ domain (residues 317–383) contains putative phosphorylation sites for ATM/ATR and lies within the regulatory domain. Chk1 is phosphorylated on Ser317 and Ser345 in response to replication stress or other types of DNA damage in an ATM/ATR-dependent fashion [16–20]. To determine which part of the Chk1 protein is required for DNA damage-induced Chk1 nuclear localization and retention, we generated fusion proteins by attaching GFP in-frame at the C-terminus of Chk1 mutants missing different parts of the regulatory domain. HeLa cells were transfected with the different GFP fusion constructs and treated with UV irradiation to induce DNA damage, after which the nuclear localization of the different Chk1 mutants was observed under a fluorescence microscope. Our results showed that the Chk1 deletion mutant CA368, which lacks the 108 C-terminal amino acids (368–476), accumulated in the nucleus after UV treatment, while the subcellular localizations of the Chk1 deletion mutants CA334, missing the 142 C-terminal amino acids (334–476), and CA288, missing the 188 C-terminal amino acids (288–476), were not significantly different from wild-type Chk1. This suggests that a specific sequence of 34 amino acids (334–368) in the C-terminal regulatory domain is involved in regulating the DNA damage-induced nuclear retention of Chk1. In *S. pombe*, the interaction of Chk1 with 14-3-3 proteins, which preferentially associate with active phosphorylated Chk1, is stimulated tenfold in response to DNA damage [24]. Human Chk1 also interacts with 14-3-3 protein, and the association between these proteins is stimulated by DNA damage [21]. It has recently been shown that

14-3-3 proteins have a functional role in controlling the localization of certain binding partners [25]. In the case of Cdc25C, human Chk1 can phosphorylate the Ser216 residue of Cdc25 [26], creating a site for 14-3-3 protein binding [27]. Given the observations that Chk1 interacts with 14-3-3 proteins and that 14-3-3 proteins are involved in the subcellular localization of many of their binding partners, furthermore, when cells incur DNA damage, Chk1 becomes phosphorylated and its interaction with 14-3-3 protein is stimulated approximately tenfold. We speculate that 14-3-3 proteins regulate the subcellular localization of Chk1 by binding with Chk1, and the association of Chk1 with 14-3-3 proteins plays a critical role in regulating Chk1 accumulation in the nucleus in response to DNA damage. If the sequence that regulates the nuclear accumulation of Chk1 in response to DNA damage is deleted, the subcellular localization of Chk1 does not change after DNA damage.

Ng et al. [5] incubated full-length Chk1 and a truncated mutant, CA390, which lacked the 86 C-terminal amino acids (390–476), with radioactive ATP and found that full length Chk1 was only weakly radiolabeled, while CA390 was robustly autophosphorylated. To measure the kinase activity of Chk1 against external substrates, a GST-Cdc25C₁₉₅₋₂₅₉ fusion protein containing the Chk1 phosphorylation site (Ser216) was used. Consistent with the autophosphorylation activity, Cdc25C was phosphorylated more strongly by CA390 than by full-length Chk1. Further truncations of the C-terminus were made to characterize the regulatory regions. CA309, missing the 167 C-terminal amino acids (309–476), still displayed some autophosphorylation activity, while CA284, which lacks the 192 C-terminal amino acids (284–476) containing the entire SQ/TQ domain, displayed no autophosphorylation [5]. We, therefore, hypothesize that the C-terminal regulatory domain plays an important role in modulating the kinase domain.

To investigate which part of the regulatory domain is involved in regulating the kinase domain we first studied the abilities of the different Chk1 truncation mutants to repair DNA damage. Previous studies have used pEGFP-N2 as the reporter plasmid to investigate the ability of cells to repair DNA [28]. Therefore, we co-transfected HeLa cells with pEGFP-N2 and the Chk1 truncation mutants. We determined the fluorescence intensity as a measure of the repair capabilities of the different truncation mutants. The repair mediated by CA368 was higher than that mediated by full-length Chk1, the repair mediated by CA334 was equivalent to that mediated by full-length Chk1, while CA288 had almost no ability to repair DNA. This suggests that the regulatory domain inhibits the enzymatic activity of Chk1, and deletion of part of the Chk1 regulatory domain activates the kinase. When the entire SQ/TQ domain was deleted from the regulatory domain, Chk1 had no enzymatic activity.

We then studied the effects of the Chk1 truncation mutants on the cell cycle to determine how the kinase domain of Chk1 is regulated by its regulatory domain. HeLa cells

were transfected with the different Chk1 truncation mutants. Flow cytometry analysis was performed after treatment with NOC. We found that whereas over-expression of Chk1, CA334 and CA288 did not perturb the cell cycle progression, expression of CA368 severely compromised the NOC-induced G2/M block. These results demonstrate that expression of the active CA368 delays the G1 or S phase. We found that the G1/S delay induced by the various truncation mutants of Chk1 correlated with their kinase activity. Among the studied truncation mutants, the enzymatic activity of CA368 was the highest.

Our results showed that CA334, in which the 34 amino acids (334–368) involved in regulating the DNA damage-induced nuclear accumulation of Chk1 were deleted, did not completely accumulate in the nucleus after DNA damage, while its repair competence was equivalent to full length Chk1. This suggests that nuclear accumulation of Chk1 is not the only pathway responding to DNA damage. Chk1 phosphorylates and inactivates Cdc25C by other signaling pathways to prevent the cell cycle from progressing past the G2/M checkpoint to allow DNA repair. The enzymatic activity of CA368 was higher than that of full-length Chk1, while the enzymatic activity of CA334 was equivalent to that of full-length Chk1. We speculate that the 142 C-terminal amino acids (334–476) form a special spatial structure that blocks a latent active site lying within those 142 amino acids from external contact. This may be the inhibitory element of the regulatory domain. Once the latent active site is exposed by destruction of the spatial structure, the enzymatic activity of CA368 is higher than that of full-length Chk1. While CA334 lacks the integral spatial structure formed by these 142 amino acids, it still has the regulatory elements of the regulatory domain and, thus, its enzymatic activity is equivalent to that of full-length Chk1. CA288 lacks all regulatory elements of the regulatory domain, and it has almost no enzymatic activity. To verify this hypothesis, phosphorylation of Cdc25C was studied by Western blot to analyze the kinase activities of the different Chk1 truncation mutants. We found that the kinase activity of CA368 was higher than that of full-length Chk1, the kinase activity of CA334 was equivalent to that of full-length Chk1, and CA288 had almost no kinase activity. These results suggest that differences in phosphorylation of Cdc25C of the different deletion mutants relate to their differences in kinase activity.

The results show that the kinase domain has enzymatic activity, and the regulatory domain controls the kinase domain by molecular interaction. The spatial structure of the C-terminal regulatory domain of Chk1 plays an important role in Chk1 function. Thus, removing parts of the regulatory domain could undermine these interactions. Deleting parts of the regulatory domain of Chk1 increased the kinase activity, and removal of the entire SQ/TQ domain deleted the kinase activity almost completely. These results suggest

that the regulatory domain contains both inhibitory and regulatory elements: the SQ/TQ domain stimulates the kinase domain, but this is counteracted by suppression from the C-terminal region. The C-terminal region may act directly on the kinase domain or it may inhibit the activating potential of the SQ/TQ domain. The regulatory domain affects the kinase domain simply through steric hindrance, blocking the access of the kinase to Mg^{2+} -ATP or substrates. Stress-induced phosphorylation of the SQ/TQ domain may remove the constraints of the C-terminal region on the kinase domain.

In conclusion, a specific 34 amino acid sequence in the C-terminal regulatory domain of Chk1 is involved in the regulation of nuclear accumulation of Chk1 in response to DNA damage. When parts of the regulatory domain were truncated, Chk1 kinase activity was enhanced. However, when the entire SQ/TQ domain was removed the kinase activity of Chk1 disappeared almost entirely. This suggests that the regulatory domain is involved in regulating the kinase domain. The mechanism of how the regulatory domain is involved in the regulation of DNA damage-induced nuclear accumulation and the kinase domain of Chk1 still requires further exploration.

This work was supported by the Major Research Program of China (CB914401) and the National High Technology Research and Development Program of China (2006AA02Z4A6).

- 1 Zhou B B, Elledge S J. The DNA damage response: Putting checkpoints in perspective. *Nature*, 2000, 408: 433–439
- 2 Zachos G, Rainey M D, Gillespie D A. Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects. *EMBO J*, 2003, 22: 713–723
- 3 Merry C, Fu K, Wang J, et al. Targeting the checkpoint kinase Chk1 in cancer therapy. *Cell Cycle*, 2010, 9: 279–283
- 4 Li X F, Ward T, Yao X B, et al. Chk1 prevents abnormal mitosis of S-phase HeLa cells containing DNA damage. *Chinese Sci Bull*, 2009, 54: 4205–4213
- 5 Ng C P, Lee H C, Ho C W, et al. Differential mode of regulation of the checkpoint kinases CHK1 and CHK2 by their regulatory domains. *J Biol Chem*, 2004, 279: 8808–8819
- 6 Sweeney G. Leptin signalling. *Cell Signal*, 2002, 14: 655–663
- 7 Bolderson E, Richard D J, Zhou B B, et al. Recent advances in cancer therapy targeting proteins involved in DNA double-strand break repair. *Clin Cancer Res*, 2009, 15: 6314–6320
- 8 Carrassa L, Sanchez Y, Erba E, et al. U2OS cells lacking Chk1 undergo aberrant mitosis and fail to activate the spindle check-point. *J Cell Mol Med*, 2009, 13: 1565–1576
- 9 Karlsson-Rosenthal C, Millar J B. Cdc25: Mechanisms of checkpoint inhibition and recovery. *Trends Cell Biol*, 2006, 16: 285–292
- 10 Pan Y, Ren K H, He H W, et al. Knockdown of Chk1 sensitizes human colon carcinoma HCT116 cells in a p53-dependent manner to lidamycin through abrogation of a G2/M checkpoint and induction of apoptosis. *Cancer Biol Ther*, 2009, 8: 1559–1566
- 11 McNeely S, Conti C, Sheikh T, et al. Chk1 inhibition after replicative stress activates a double strand break response mediated by ATM and DNA-dependent protein kinase. *Cell Cycle*, 2010, 9: 995–1004
- 12 Xiao Z, Xue J, Gu W Z, et al. Cyclin B1 is an efficacy-predicting biomarker for Chk1 inhibitors. *Biomarkers*, 2008, 13: 579–596
- 13 Nigg E A. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol*, 2001, 2: 21–32

- 14 Ling H, Wen L, Ji X X, et al. Growth inhibitory effect and Chk1-dependent signaling involved in G2/M arrest on human gastric cancer cells induced by diallyl disulfide. *Braz J Med Biol Res*, 2010, 43: 271–278
- 15 Wilsker D, Petermann E, Helleday T, et al. Essential function of Chk1 can be uncoupled from DNA damage checkpoint and replication control. *Proc Natl Acad Sci USA*, 2008, 105: 20752–20757
- 16 Liu Q, Guntuku S, Cui X S, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev*, 2000, 14: 1448–1459
- 17 Zhao H, Watkins J L, Piwnica-Worms H. Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. *Proc Natl Acad Sci USA*, 2002, 99: 14795–14800
- 18 Lopez-Girona A, Tanaka K, Chen X B, et al. Serine-345 is required for Rad3-dependent phosphorylation and function of checkpoint kinase Chk1 in fission yeast. *Proc Natl Acad Sci USA*, 2001, 98: 11289–11294
- 19 Guo Z, Kumagai A, Wang S X, et al. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in *Xenopus* egg extracts. *Genes Dev*, 2000, 14: 2745–2756
- 20 Gatei M, Sloper K, Sorensen C, et al. Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. *J Biol Chem*, 2003, 278: 14806–14811
- 21 Jiang K, Pereira E, Maxfield M, et al. Regulation of Chk1 includes chromatin association and 14-3-3 binding following phosphorylation on Ser-345. *J Biol Chem*, 2003, 278: 25207–25217
- 22 Dunaway S, Liu H Y, Walworth N C. Interaction of 14-3-3 protein with Chk1 affects localization and checkpoint function. *J Cell Sci*, 2004, 118: 39–50
- 23 Kim E M, Burke D J. DNA damage activates the SAC in an ATM/ATR-dependent manner, independently of the kinetochore. *PLoS Genet*, 2008, 4: e1000015
- 24 Chen L, Liu T H, Walworth N C. Association of Chk1 with 14-3-3 proteins is stimulated by DNA damage. *Genes Dev*, 1999, 13: 675–685
- 25 Seimiya H, Sawada H, Muramatsu Y, et al. Involvement of 14-3-3 proteins in nuclear localization of telomerase. *EMBO J*, 2000, 19: 2652–2661
- 26 Muslin A J, Tanner J W, Allen P M, et al. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, 1996, 84: 889–897
- 27 Zeng Y, Piwnica-Worms H. DNA damage and replication checkpoints in fission yeast require nuclear exclusion of the Cdc25 phosphatase via 14-3-3 binding. *Mol Cell Biol*, 1999, 19: 7410–7419
- 28 Atanassov B S, Ninova P D, Anachkova B B, et al. Relationship between DNA repair capacity and resistance to genotoxins in four human cell lines. *Cancer Detect Prev*, 2003, 27: 24–29

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.