Cancer Letters 449 (2019) 114–124

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

USP39 regulates DNA damage response and chemo-radiation resistance by deubiquitinating and stabilizing CHK2

Jinhuan Wu[a,b,c,1], Yuping Chen[a,b,1], Guohe Geng[d], Lei Li[a,b], Ping Yin[e], Somaira Nowsheen[e], Yunhui Li[a,b], Chenming Wu[a,b], Jiaqi Liu[e], Fei Zhao[e], Wootae Kim[e], Qin Zhou[e], Jinzhou Huang[e], Guijie Guo[e], Chao Zhang[e], Xinyi Tu[e], Xiumei Gao[g], Zhenkun Lou[e,f,****], Kuntian Luo[e,f,***], Haixuan Qiao[c,**], Jian Yuan[a,b,*]

[a] Research Center for Translational Medicine, East Hospital, Tongji University School of Medicine, Shanghai, 200120, China
[b] Key Laboratory of Arrhythmias of the Ministry of Education of China, East Hospital, Tongji University School of Medicine, Shanghai, 200120, China
[c] School of Biomedical Engineering and Technology, Tianjin Medical University, 300070, Tianjin, China
[d] School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, 325035, China
[e] Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, MN, 55905, USA
[f] Department of Oncology, Mayo Clinic, Rochester, MN, 55905, USA
[g] Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, 300193, Tianjin, China

ARTICLE INFO

Keywords:
USP39
Deubiquitination
CHK2
Lung cancer
Chemo-radiation resistance

ABSTRACT

The serine/threonine kinase, CHK2 (checkpoint kinase 2), is a key mediator in DNA damage response and a tumor suppressor, which is implicated in promoting cell cycle arrest, apoptosis and DNA repair. Accumulating evidence suggests that these functions are primarily exerted through phosphorylation downstream factors such as p53 and BRCA1. Recent studies have shown that ubiquitination is an important mode of regulation of CHK2. However, it remains largely unclear whether deubiquitinases participate in regulation of CHK2. Here, we report that a deubiquitinase, USP39, is a new regulator of CHK2. Mechanistically, USP39 deubiquitinates and stabilizes CHK2, which in turn enhances CHK2 stability. Short hairpin RNA (shRNA) mediated knockdown of USP39 led to deregulate CHK2, which resulted in compromising the DNA damage-induced G2/M checkpoint, decreasing apoptosis, and conferring cancer cells resistance to chemotherapy drugs and radiation treatment. Collectively, we identify USP39 as a novel regulator of CHK2 in the DNA damage response.

1. Introduction

The serine/threonine kinase CHK2 is a key mediator of DDR (DNA damage response) and comprises of an N-terminal SQ/TQ cluster domain (SCD), central forkhead-associated (FHA) domain and C-terminal serine/threonine kinase domain (KD) [1,2]. CHK2 functions as a crucial module and participates in several cellular processes, including cell cycle regulation, apoptosis and DNA damage response [3–5]. CHK2 inactivation, mutation or depletion is associated with diverse pathophysiological states including tumorigenesis and chemo-resistance [6–8]. As a serine/threonine kinase, CHK2 regulates these cellular functions by phosphorylating multiple substrates. For instance, CHK2 phosphorylates p53 on Serine (Ser) 20, which leads to p53 stabilization and activation, resulting in cell cycle arrest and cell apoptosis [4,9,10]. Moreover, CHK2 phosphorylates E2F1 on Ser364 and promyelocytic leukemia protein (PML) on Ser117 to regulate apoptosis [11–13]. Additionally, CHK2 phosphorylates CDC25A on Ser123, 127, and 292 and CDC25C on Ser216 to promote cell cycle arrest [14–18]. Furthermore, CHK2 regulates DNA repair by mediating phosphorylation of BRCA1 [19–21].

CHK2 activity is majorly regulated through posttranslational modification [22]. Following DNA damage, CHK2 is phosphorylated by ATM at Thr68 site, which in turn induced CHK2 dimerization and autophosphorylation at multiple S/T sites in its kinase domain [23–28].

* Corresponding author. Research Center for Translational Medicine, East Hospital, Tongji University School of medicine, Shanghai, 200120, China.
** Corresponding author. School of Biomedical Engineering and Technology, Tianjin Medical University, 300070, Tianjin, China.
*** Corresponding author. Department of Oncology, Mayo Clinic, Rochester, MN, 55905, USA.
**** Corresponding author. Department of Oncology, Mayo Clinic, Rochester, MN, 55905, USA.
E-mail addresses: Lou.zhenkun@mayo.edu (Z. Lou), Luo.kuntian@mayo.edu (K. Luo), qiaohaixuan@aliyun.com (H. Qiao), yuanjian229@hotmail.com (J. Yuan).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.canlet.2019.02.015
Received 4 December 2018; Received in revised form 7 February 2019; Accepted 10 February 2019

CHK2 autophosphorylation induces further conformational change and disassociates the CHK2 dimer to fully active monomers. In addition, DNA-PKcs phosphorylates and activates CHK2, and then prevents spindle disruption [29,30]. Moreover, PLK3 phosphorylates CHK2 at Ser62 and Ser73, which in turn promotes CHK2 fully activation by ATM [31]. Beside the phosphorylation mediated CHK2 activation, recent reports showed that ubiquitination also plays an important role in regulation of CHK2. For example, the E3 ubiquitin ligase RNF8 and PIRH2 have been demonstrated to regulate ubiquitination and degradation of CHK2 [34,35]. Previous reports showed that upregulated RNF8 correlated with EMT features, cancer chemo resistance and poor patient survival in breast cancer though activating Twist by triggering K63-linked ubiquitination of Twist [32]. PIRH2 is found to be upregulated in multiple cancers, including lung, prostate, head and neck cancers, and hepatocellular carcinoma [33]. In addition, hypoxia induced SIAH2 mediated ubiquitination of CHK2 and promotes its degradation and impacts its function in regulation of apoptosis and cell cycle [37]. Previous studies showed that SIAH2 is markedly upregulated in castration-resistant prostate cancer (CRPC) and promoting expression of select androgen receptor (AR) target genes implicated in lipid metabolism, cell motility, and proliferation [36]. Taken together, the ubiquitination modifications of CHK2 are well studied. However, it remains largely unknown how deubiquitination process regulates CHK2 stabilization.

Here, We report that a deubiquitinase, ubiquitin Specific Peptidase 39 (USP39), regulates apopotosis and cell cycle checkpoint through deubiquitinating and stablizing CHK2. USP39 is a deubiquitinase containing the ubiquitin specific protease domain and the UBP-type zinc finger domain [38]. We found that USP39 directly deubiquitinate and stablize CHK2 both *in vitro* and *in vivo*. Furthermore, we demonstrate that USP39 regulates G2/M checkpoint and cell apoptosis in response to chemotherapy drugs treatment through CHK2. Knockdown of USP39 leads to a significant decrease in CHK2 protein level resulting in compromises G2/M checkpoint and apoptosis, thereby promoting cancer cell resistance to chemotherapy drugs and radiation treatment. Moreover, low level of USP39 is observed in lung cancers, which is correlated with the low level of CHK2 in these cancer samples, suggesting that USP39-CHK2 may play a role in treatment of lung cancers.

2. Materials and methods

2.1. Reagents, plasmids, antibodies

Cisplatin, Cycloheximide (CHX), puromycin, IgG agarose, streptavidin-linked agarose, anti-FLAG M2 agarose and anti-HA M2 agarose were purchased from Sigma Aldrich. HA-FLAG-USP39 was purchased from Addgene (Plasmid #22581) and subcloned into PLVX3 lentiviral or pGEX-4T-2 vector (Clontech). USP39 C306A mutant was generated by site-directed mutagenesis.

The anti-GAPDH was purchased from proteintech (60004-1-lg). Anti-USP39 (ab131244) and anti-P53 (PAb 240) antibody were purchased form Abcam. Anti-phospho-p53 (Ser20) (9287) was purchased from CST (Cell signaling Technology). Anti-Chk2 (05–649) antibody was purchased form Millipore. The Anti-β-actin (A1978), Anti-FLAG (F1804) and anti-HA (F9658) antibodies were purchased from Sigma.

2.2. Cell culture and transfection

The human lung cancer cell lines A549, H460, H1299, H1650 and lung normal cell lines IMR90 and HEK293T were purchased from ATCC. Without specially mentioned, the above cells were cultured in DMEM supplemented with 10% FBS. IMR90 was cultured in ATCC-formulated EMEM with 10% FBS. Cells were transfected with plasmids by using Mirus (Catalog No. MIR600PM). The medical genome facility (MGF) of Mayo Clinical in Rochester, Minnesota confirmed the identities of all the cell lines.

2.3. GST-pull down assay

Containing Flag-CHK2 protein whole cell lysate was prepared in HEK239T cells. GST, GST fusion USP39 wild type, 1-219aa truncation and 220-565aa truncation proteins were prepared in bacteria. GST fusion proteins were bound to glutathione sepharose for 3 h at 4 °C. Containing Flag-CHK2 protein whole cell lysate was incubated with indicated GST fusion protein for 2 h at 4 °C. After washing with NETN for 5 times, the bound proteins were eluted and separated by SDS-PAGE and immunoblotting with indicated antibodies.

2.4. Protein stability assay

To assess Chk2 protein turnover, Cells were treated with cycloheximide (CHX) (0.1 mg/ml) and harvested at different time points. Harvested cells were then lysed in NETN buffer and the lysate samples were separated by SDS-PAGE and blotted with indicated antibodies. Finally, we quantified of the proteins levels by ImageJ.

2.5. Colony formation assay

Cells (500–2000) were seeded in triplicate in each well of six-well plates. For drug or radiation sensitivity assay, after 1 day, cells were exposed to ionizing radiation or treated with cisplatin as indicated dose, and left for 10–14 days at incubator to allow colony formation.

2.6. Examining apoptotic cells

Cells overexpressed constructs or stably transfected with lentiviruses as indicated were untreated or treated with cisplatin for 48 h before harvested, washed twice using cold phosphate-buffered saline (PBS), fixed at −20 °C with cold 70% ethanol overnight and resuspended in PBS. The suspension was filtrated through a 400-mesh membrane. The cells were stained with propidium iodide (BD, NO: 51-66211E) or FITC Annexin V (BD, NO: 51–65874X) and analyzed using a BD FACSCalibur™ Flow Cytometer with Kaluza Analysis software (version 1.3; BD Biosciences). Experiments were repeated three times.

2.7. Tissue microarray

The tissue arrays of Lung cancer samples and adjacent normal tissue samples were purchased from Alenabio (www.Alenabio.com) (LC10013A and LC1503). Immunohistochemistry staining of USP39 (dilution 1:300), Chk2 (dilution 1:300) were carried out using IHC Select HRP/DAB kit (Cat. DAB50, Millipore). The immunostaining was blindly scored by pathologists. The statistical analysis of the IHC results were performed as previously [39].

2.8. Survival analysis of cancer patients

Lung cancer patients' data from TCGA (TCGA Research Network: http://cancergenome.nih.gov/) was analyzed by Statistical Program for Social Sciences 19.0 software (SPSS) software and generated Survival curve.

2.9. Statistics

For apoptotic cell analysis, data is represented as the mean ± S.E.M of three independent experiments. Statistical analyses were performed with the Student's t-test or X^2 test. Statistical significance is represented in figures by: *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

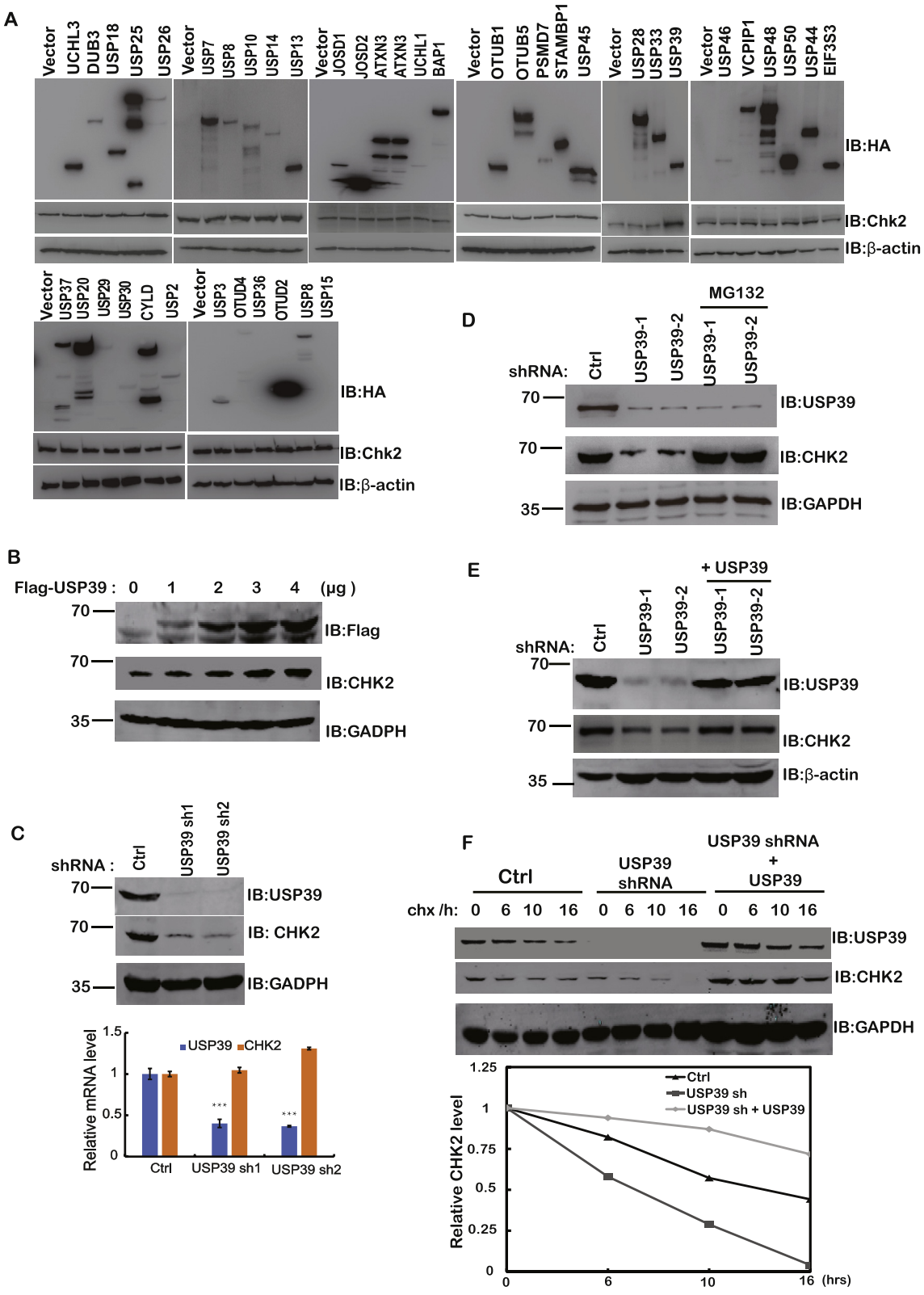
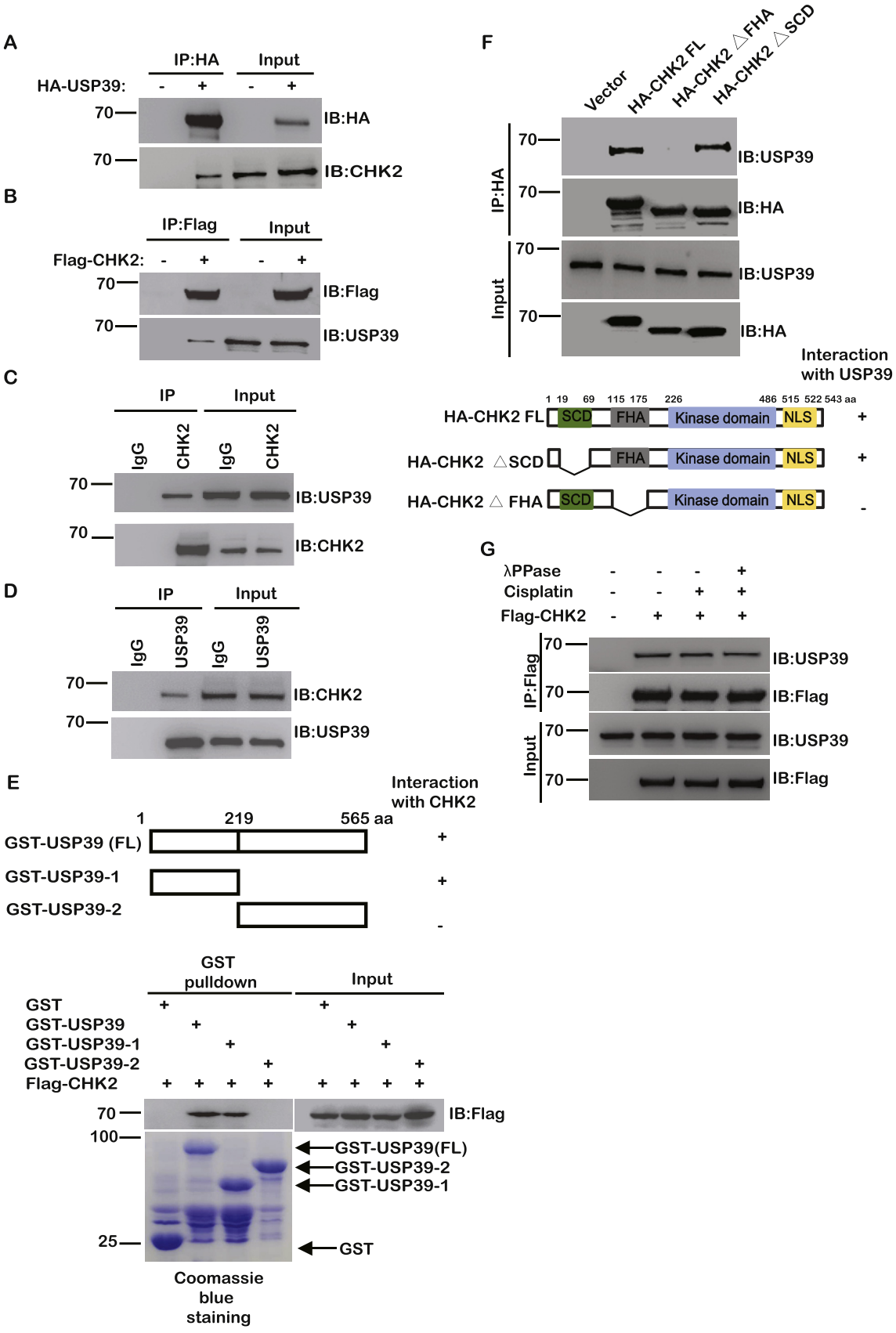


Fig. 1. USP39 regulates CHK2 protein level. (A) HEK293T Cells were transfected indicated deubiquitinases (DUBs). After 48 h, cells were lysed, and Western blot was performed with the indicated antibodies. **(B)** A549 cells were transfected indicated dose of vector-expressing Flag-USP39. After 48 h, cells were lysed, and Western blot was performed with the indicated antibodies. **(C)** Cells stably expressing control or USP39 shRNAs were harvested. Half of the cells were lysed, and Western blot was performed with the indicated antibodies. Lower panel: The mRNAs were extracted from the rest of the cells and subjected to qPCR. Error bars represent ± S.E.M. from three independent experiments. ***$p < 0.001$. Statistic analyses were performed with the Student's t-test. **(D)** Cells stably expressing USP39 shRNAs were treated with vehicle or MG132 for 6 h, and then lysed. Western blot was performed with the indicated antibodies. **(E)** Cells stably expressing control or two different USP39 shRNAs transfected with indicated constructs, after 48 h, were lysed and cell lysates were then blotted with the indicated antibodies. **(F)** Cells stably expressing control shRNA, USP39 shRNA and USP39 shRNA together with shRNA-resistant USP39 were treated with cycloheximide (1.0 mg/ml), and harvested at the indicated times. The upper panels showed immunoblots of CHK2 and USP39. Lower panels: quantification of the CHK2 level relative to GAPDH.



(caption on next page)

Fig. 2. USP39 interacts with CHK2. (A) USP39 interacts with CHK2 in cells. Cells were transfected with HA-USP39. HA-USP39 was purified on anti-HA-agarose, and then coprecipitating endogenous CHK2 was detected by anti-CHK2 antibody. **(B)** Cells were transfected with Flag-CHK2. Flag-CHK2 was purified on anti-Flag-agarose, and then coprecipitating endogenous USP39 was detected by anti-USP39 antibody. **(C)(D)** Endogenous USP39 coprecipitates with endogenous CHK2. Cells lysates were subjected to immunoprecipitation with control IgG, **(C)** anti-CHK2, or anti-USP39 **(D)** antibody. The immunoprecipitates were then blotted with the indicated antibodies. **(E)** HEK293T cells were transfected with Flag-CHK2, and then cell lysates were incubated with sepharose coupled with GST, GST-USP39, GST-USP39-1, GST-USP39-2, after washing, proteins bound on sepharose were blotted with indicated antibodies. **(F)** HEK293T cells transfected with HA tagged WT, deletion of FHA mutant or deletion of SCD mutant of CHK2 were lysed, and then cell lysates were subjected to immuoprecipitation with anti-HA-agarose. The immunoprecipitates were then blotted with the indicated antibodies. **(G)**. HEK293T cells transfected with Flag tagged CHK2 were untreated or treated with cisplatin for 24 h, and then cell lysates were subjected to immuoprecipitation with anti-Flag-agarose. As indicated, one of samples was additionally treated with lambda protein phosphatase (λPP). The immunoprecipitates were then blotted with the indicated antibodies.

3. Results

3.1. USP39 upregulates CHK2 protein level

CHK2 is a key kinase and tumor suppressor that plays an important role in multiple cellular processes such as cell cycle arrest, apoptosis and DNA repair [1,40]. In human cells, following DNA damage, CHK2 is activated by phosphorylation via ATM on Thr68, which in turn activates CHK2 and triggers CHK2 to phosphorylate multiple downstream targets to induce different cellular responses including cell cycle arrest, apoptosis or DNA damage repair [22]. Ubiquitination has been reported to exert a key function in DNA damage signaling pathway [41–43]. Several studies have reported that ubiquitination mediates CHK2 turnover via ubiquitin-proteasome system, and regulates CHK2 function [34,35,37,44,45]. However, how deubiquitinating process regulates CHK2 is largely unknown. To identify potential DUBs that can deubiquitinate and stabilize CHK2, we overexpressed a panel of deubiquitinases in HEK293T cells individually and examined the CHK2 protein level. As shown in Fig. 1A, overexpression of ubiquitin-specific protease USP39 could dramatically increase CHK2 protein level. To further confirm the result, we expressed USP39 in a lung cancer cell line, A549, in increasing dosage. As shown in Fig. 1B, overexpression of USP39 increased CHK2 protein level in a dose dependent manner. Furthermore, USP39 wild type (WT) and USP39 C306A (enzyme inactive mutant) mutant were overexpressed in another lung cancer cell line H1299. Only USP39 WT but not USP39 CA mutant dramatically increased CHK2 level (Supplementary Fig. 1A). On the other hand, we depleted USP39 using two different USP39-specific short hairpin RNAs (shRNAs) in A549 cells and then detected CHK2 protein level. We found that downregulation of USP39 decreased CHK2 protein level but not CHK2 mRNA level (Fig. 1C). Moreover, we also examined the proteins level of other upstream critical components of DDR, including ATM, ATR and CHK1. However, loss of USP39 didn't affect the expression of these proteins (Supplementary Figs. 1B and C). In addition, the decrease in CHK2 protein level was rescued by treated with proteasome inhibitor MG132 in A549 and H1299 cells (Fig. 1D and Supplementary Fig. 1D). Moreover, CHK2 protein level could also be rescued by supplementing shRNA-resistant USP39 WT but not the USP39 CA mutant in USP39 depleted cells (Figs. 1E and 6E and Supplementary Fig. 2I). To further establish whether USP39 regulates CHK2 protein stability, we treated cells with cycloheximide (CHX) and determined the half-life of CHK2. As shown in Fig. 1F, CHK2 stability was dramatically decreased in USP39 depleted cells. While, reconstitution of USP39 could rescue CHK2 protein stability in USP39 depleted cells. Taken together, these results suggest that USP39 regulates CHK2 protein level and stability in a proteasome-dependent manner and USP39 is a potential DUB for CHK2.

3.2. USP39 interacts with CHK2

To further demonstrate the mechanism by which USP39 regulates CHK2 protein level, we tested whether the USP39 binds to CHK2. As shown in Fig. 2A, immunoprecipitating HA-tagged USP39 in 293T cells was able to pulldown CHK2. Reciprocally, we could also detect USP39 in Flag-CHK2 immunoprecipitating complex (Fig. 2B). Additionally, endogenous immunoprecipitation further confirmed that USP39 binds to CHK2 (Fig. 2C and D). To further investigate the mechanism of binding between USP39 and CHK2, purified GST or various GST USP39 fusion proteins were performed the GST-pull down assay. We found that the N terminal of USP39 (1-219aa) was responsible for binding with CHK2 (Fig. 2E). Additionally, CHK2 truncations constructs were employed to perform semi-endogenous immunoprecipitation. We found the FHA domain of CHK2 was essential for it binding to USP39 (Fig. 2F). It has been well characterized that the FHA domain of CHK2 is a phosphopeptide binding motif [46–48]. Hence, we next examined whether the interaction of USP39 and CHK2 was mediated by DNA damage. However, as shown in Fig. 2G, the binding between USP39 and CHK2 did not increase following cisplatin treatment. In addition, the binding of USP39 and CHK2 didn't change upon Lambda protein phosphatase (λPP) treatment (Fig. 2G), suggesting that the interaction between USP39 and CHK2 is independence of DNA damage. Taken together, our findings suggest that USP39 interacts with CHK2.

3.3. USP39 deubiquitinates and stabilizes CHK2

To further clarify the mechanism of USP39 mediated CHK2 stabilization, we assessed whether USP39 deubiquitinated CHK2 in cells. As shown in Fig. 3A and supplementary Fig. 1E, knockdown of USP39 resulted in a dramatic increase in CHK2 polyubiquitination in A549 and H1299 cells. Conversely, overexpressing USP39 WT in A549 and HEK293T cells resulted in a significant decrease in polyubiquitination of CHK2, while overexpressing USP39 CA mutant failed to alter the level of CHK2 polyubiquitination (Fig. 3B and Supplementary Fig. 1F). To further investigate whether USP39 directly deubiquitinated CHK2, bacterially purified GST-USP39 and ubiquitinated CHK2 proteins were incubated together in a cell-free system. As shown in Fig. 3C, USP39 WT, but not the USP39 CA mutant, deubiquitinated CHK2 *in vitro*. Taken together, these results indicated that USP39 could deubiquitinate CHK2 both *in vitro* and *in vivo*.

3.4. Effect of USP39 on CHK2-mediated G2/M checkpoint and apoptosis

CHK2 is a key mediator for the regulation of cell apoptosis and DNA repair in response to DNA damage [22]. Hence, it is possible that USP39 regulates these functions via deubiquitinating and stabilizing CHK2. To test this hypothesis, cells with CHK2 knock down, USP39 knock down or combined USP39 and CHK2 knock down were employed to detect cell apoptosis and cell cycle checkpoint. As shown in Fig. 4A, depletion of USP39 resulted in sharp reduction in the protein level of CHK2 and phosphor-p53 (p53 phosphorylated at Ser20), which is phosphorylated by CHK2 upon DNA damage. This suggests that depletion of USP39 blunts DNA damage response. Furthermore, depletion of USP39 led to reduction in DNA damage induced PARP1 cleavage and BAX levels, suggesting that USP39 might compromise DNA damage induced apoptosis. To further confirm this result, we performed the flow cytometry to detect cisplatin-induced apoptosis in cells. As showed in Fig. 4B and C, overexpression of USP39 resulted in an increase in cisplatin induced apoptosis. On the other hand, depletion of USP39 dramatically decreased cisplatin induced apoptosis. However, in CHK2 knockdown cells, depletion of USP39 didn't further decrease cisplatin

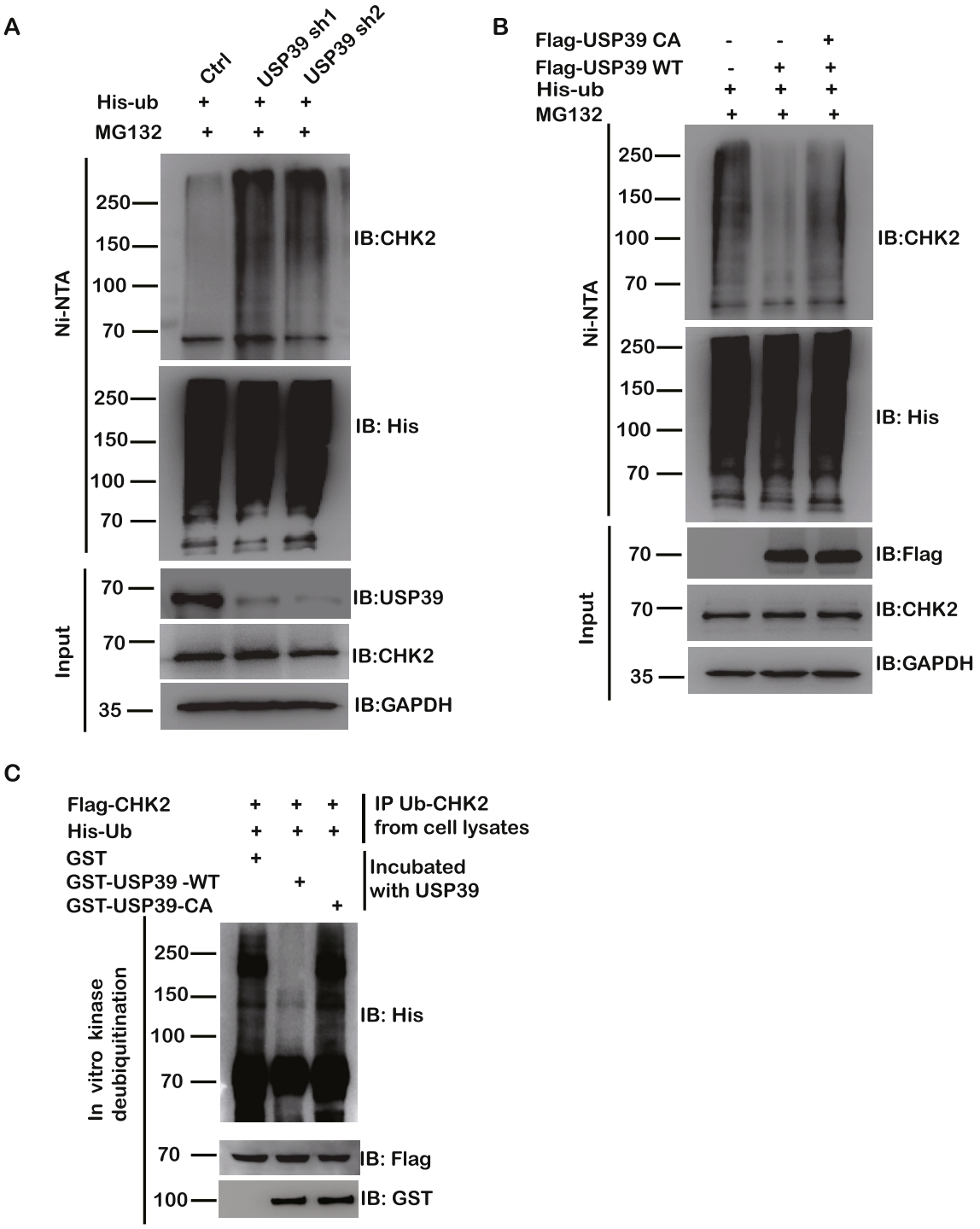


Fig. 3. USP39 deubiquitinates CHK2. (A) USP39 deubiquitinates CHK2 in cells. Cells stably expressing control or USP39 shRNAs were transfected with His-Ub, and then were treated with MG132 for 4 h before harvested. Covalently modified proteins purified on NiNTI-agarose under denatured conditions. Ubiquitinated CHK2 was detected by anti-CHK2 antibody. **(B)** Cells transfected with indicated constructs were treated with MG132 for 4 h before harvested. Covalently modified proteins were purified on NiNTI-agarose under denatured conditions and then blotted with indicated antibodies. **(C)** Deubiquitination of CHK2 *in vitro* by USP39. Ubiquitinated CHK2 was incubated with purified GST fusion USP39WT or USP39CA *in vitro* and then blotted with indicated antibodies.

induced apoptosis (Figs. 4D and 6A). These results suggested USP39 regulates DNA damage mediated cell apoptosis through CHK2. We next examined whether USP39 regulated G2/M checkpoint, which are regulated by CHK2. As shown in Figs. 4E and 6E, depletion of USP39 in A549 compromised G2/M checkpoint upon cisplatin treatment. Re-constituting USP39 knockdown cells with USP39 WT but not the USP39 CA mutant rescued these phenotypes (Figs. 4E and 6E). In addition, knockdown USP39 in depletion of CHK2 cells had no further effect on DNA damage-induced G2/M checkpoint (Figs. 4F and 6A). Taken together, USP39 regulates apoptosis and cell cycle checkpoint upon irradiation or cisplatin induced DNA damage via CHK2.

3.5. *USP39 is positively correlated to CHK2 in clinical lung cancer samples*

CHK2 has been identified as a tumor suppressor, which is mutated or depleted in various cancers, including breast, colon, bladder, ovary

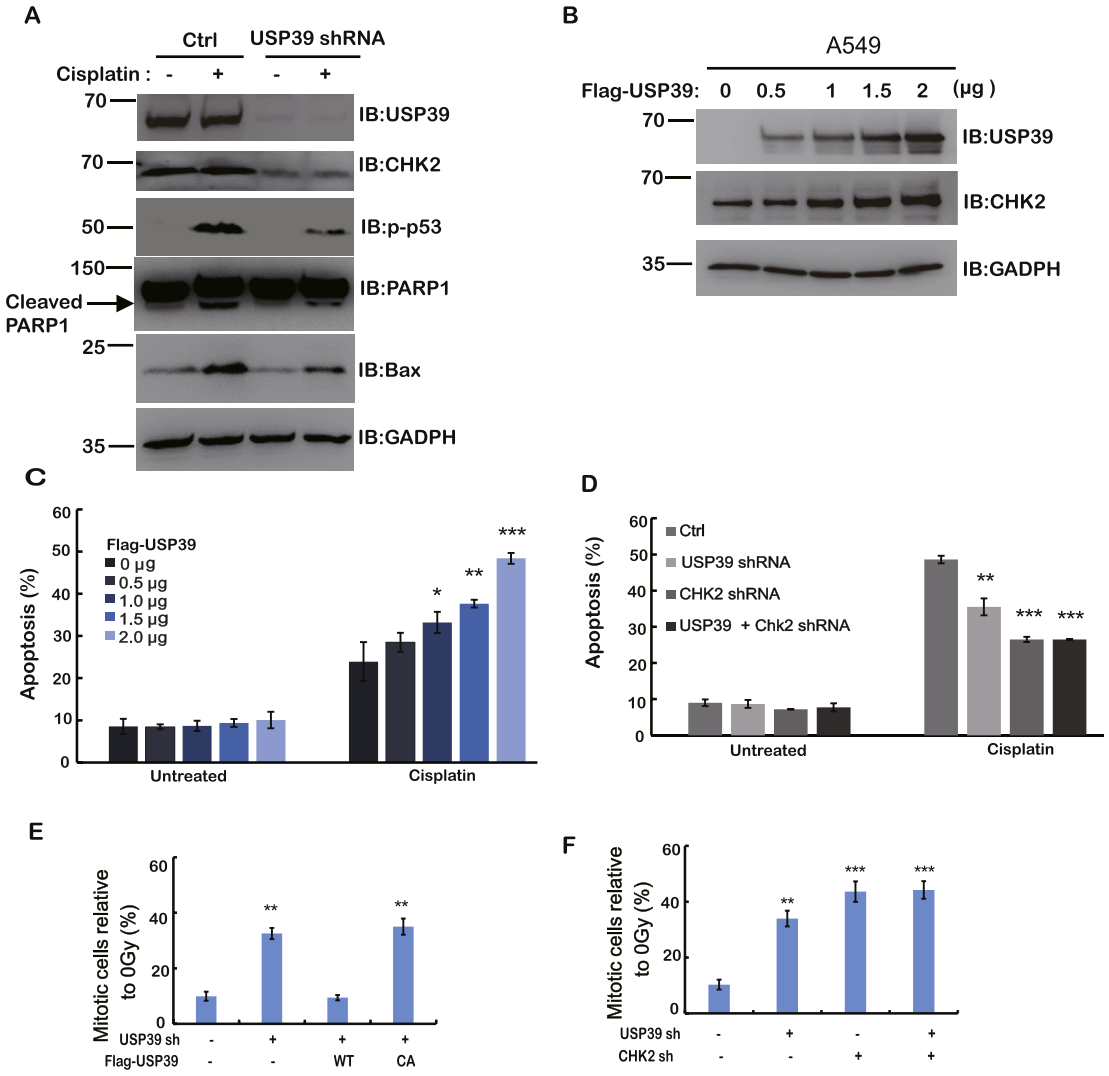


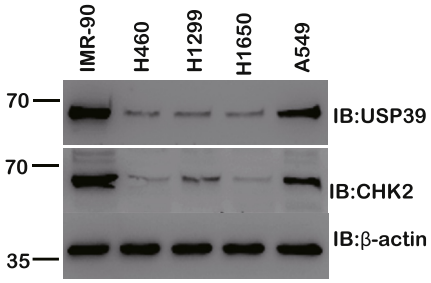
Fig. 4. Effect of USP39 on CHK2-mediated apoptosis and cell cycle checkpoint. (A) Cells stably expressing control or USP39 shRNAs were treated with vehicle or 20 μm cisplatin, and then were lysed. Western blot was performed with the indicated antibodies. **(B)** Cells were transfected as indicated doses of USP39. The expression of CHK2 was analyzed by western blot. **(C)** Cells from **(B)** were treatment with vehicle or 20 μm cisplatin for 48 h before harvested. Apoptotic cells were determined by flow cytometry. **(D)** Cells stably expressing control or as indicated shRNAs were treatment with vehicle or 20 μm cisplatin for 48 h before harvested. Apoptotic cells were determined by flow cytometry. **(E)** Control, depleted USP39, and depleted USP39 cells stably expressing the indicated constructs were left untreated or treated with IR (2 Gy). After a further 1 h, cells were collected, fixed and stained with anti-phospho-H3 antibodies to determine the mitotic population (mitotic index). **(F)** Control, and depleted USP39, depleted CHK2 or both depletion of USP39 and CHK2 cells were subjected to mitotic population determination as in **(E)**. **(C–F)** Error bars represent ± S.E.M. from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. Statistic analyses were performed with the Student's t-test.

and prostate carcinomas, albeit at low frequencies [49–52]. Besides mutated in cancer, low level of CHK2 was also observed in lung cancers, which contributes to chemo-radiation resistance [53–55]. However, the mechanism of downregulation of CHK2 in lung cancer is still unclear. We are interested in investigate whether USP39 regulates CHK2 level in lung cancers. We firstly examined the expression of USP39 and CHK2 in multiple lung cancer cell lines. As shown in Fig. 5A, compared to normal lung cell line, the expression of USP39 and CHK2 is lower in lung cancer cell lines, especially in H460 and H1650 cells. Furthermore, we tested USP39 and CHK2 expression in lung cancer samples. As shown in Fig. 5B and C, USP39 and CHK2 expression were lower in lung cancer tissue compared to normal tissue. Furthermore, the expression of USP39 and CHK2 in Lung cancer tissue were positively correlative (Fig. 5D). Since USP39-CHK2 axis regulates cell cycle checkpoint and apoptosis, we next examined the expression of Ki67 and cleaved caspase 3 in these cancer samples. As shown in Fig. 5E–F, the expression of USP39 and Ki-67 were negatively correlative in Lung cancer samples, while the expression of USP39 and cleaved caspase 3 were positively correlative in Lung cancer tissue (Fig. 5E and G). In addition, TCGA data showed that patients with low expression of USP39 have poor clinical outcomes (Fig. 5H).

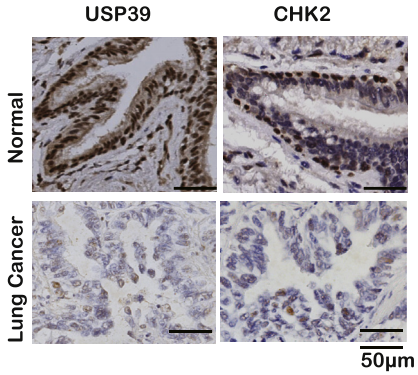
3.6. *USP39 regulates chemo-radiation resistance via CHK2*

Previous studies have shown that loss of CHK2 resulted in radio-chemo resistance [4,7,8,52,56–59]. Furthermore, our data shown USP39-CHK2 axis playing an important role in regulation of apoptosis and cell cycle checkpoint induced by DNA damage. Hence, we next examined whether USP39-CHK2 axis affects lung cancer cells response to chemotherapy and radiation. We depleted USP39 with shRNA in A549 and H1299 cells and tested the chemo-radiation response with cisplatin, etoposide and radiation. As shown in Fig. 6A–D and Supplementary Figs. 2E–H, loss of USP39 resulted in conferring cells resistance to cisplatin (Fig. 6B and Supplementary Fig. 2F), etoposide

A



B



C

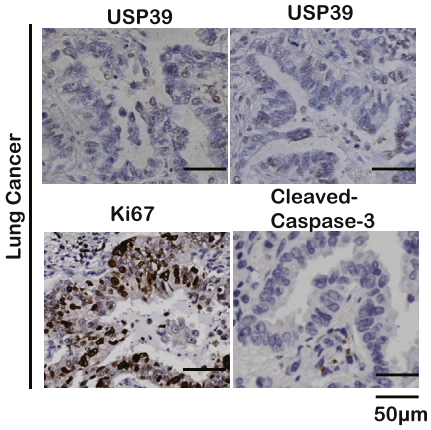
CHK2	High (%)	Low (%)	Total
Normal	38 (67%)	19(23%)	57
Carcinoma	30 (25%)	88 (75%)	118
P<0.001			

USP39	High (%)	Low (%)	Total
Normal	39 (68%)	18 (22%)	57
Carcinoma	22 (19%)	96 (81%)	118
P<0.001			

D

	USP39 Low	USP39 High	Total
CHK2 Low	79	9	88
CHK2 High	17	13	30
Total	96	22	118
P<0.001, R=0.37			

E



F

	USP39 Low	USP39 High	Total
Ki-67 Low	29	18	47
Ki-67 High	67	4	71
Total	96	22	118
P<0.001, R= -0.411			

G

		USP39 Low	USP39 High	Total
Cleaved caspase-3	Low	80	10	90
Cleaved caspase-3	High	16	12	28
Total		96	22	118
P<0.001, R=0.347				

H

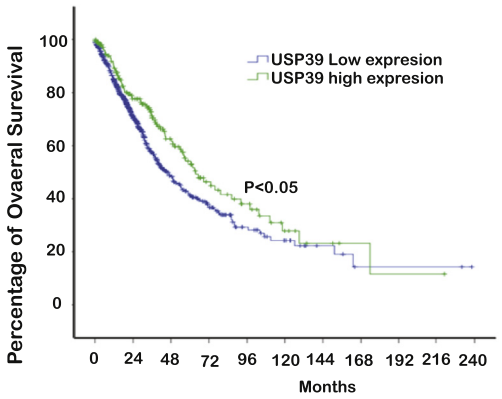


Fig. 5. USP39 expression is correlated with CHK2 in clinical lung cancer sample. (A) Cell lysates from lung cancer cell lines were blotted with USP39 and CHK2 antibodies. Lysates from normal lung cell line IMR-90 were used as a control. **(B)** Representative images of immunohistochemistry staining of USP39 and CHK2 in normal and lung carcinoma. Scar bars, 50 μm **(C)** Quantification of USP39 and CHK2 protein levels in normal and lung carcinoma. Statistical analyses were performed with Student's t-test. **(D)** Correlation study of USP39 and CHK2 in lung carcinoma. Statistical analyses were performed with X^2 test. R, pearson correlation coefficient. **(E)** Representative images of immunohistochemistry staining of USP39, Ki-67 and cleaved caspase 3 in lung carcinoma. Scar bars, 50 μm. **(F)** Correlation study of USP39 and Ki-67 in lung carcinoma. Statistical analyses were performed with X^2 test. R, pearson correlation coefficient. **(G)** Correlation study of USP39 and cleaved caspase 3 in lung carcinoma. Statistical analyses were performed with X^2 test. R, pearson correlation coefficient. **(H)** Survival analysis of lung cancer patients was performed by SPSS. Data from TCGA, patients were categorized into high and low expression of USP39.

(Fig. 6C and Supplementary Fig. 2G) and radiation (Fig. 6D and Supplementary Fig. 2H). Reconstituting USP39 knockdown cells with USP39-WT, but not the USP39-CA mutant, rescued these phenotypes (Fig. 6E–H and Supplementary Figs. 2I–K). Furthermore, we overexpressed USP39 in H460 cells (a cell line with lower expression of USP39 (Fig. 5A)). The cells were subsequently treated with chemotherapy drugs (cisplatin and etoposide) and radiation. As shown in Supplementary Figs. 2A–D and Fig. 6I–L, overexpression of USP39

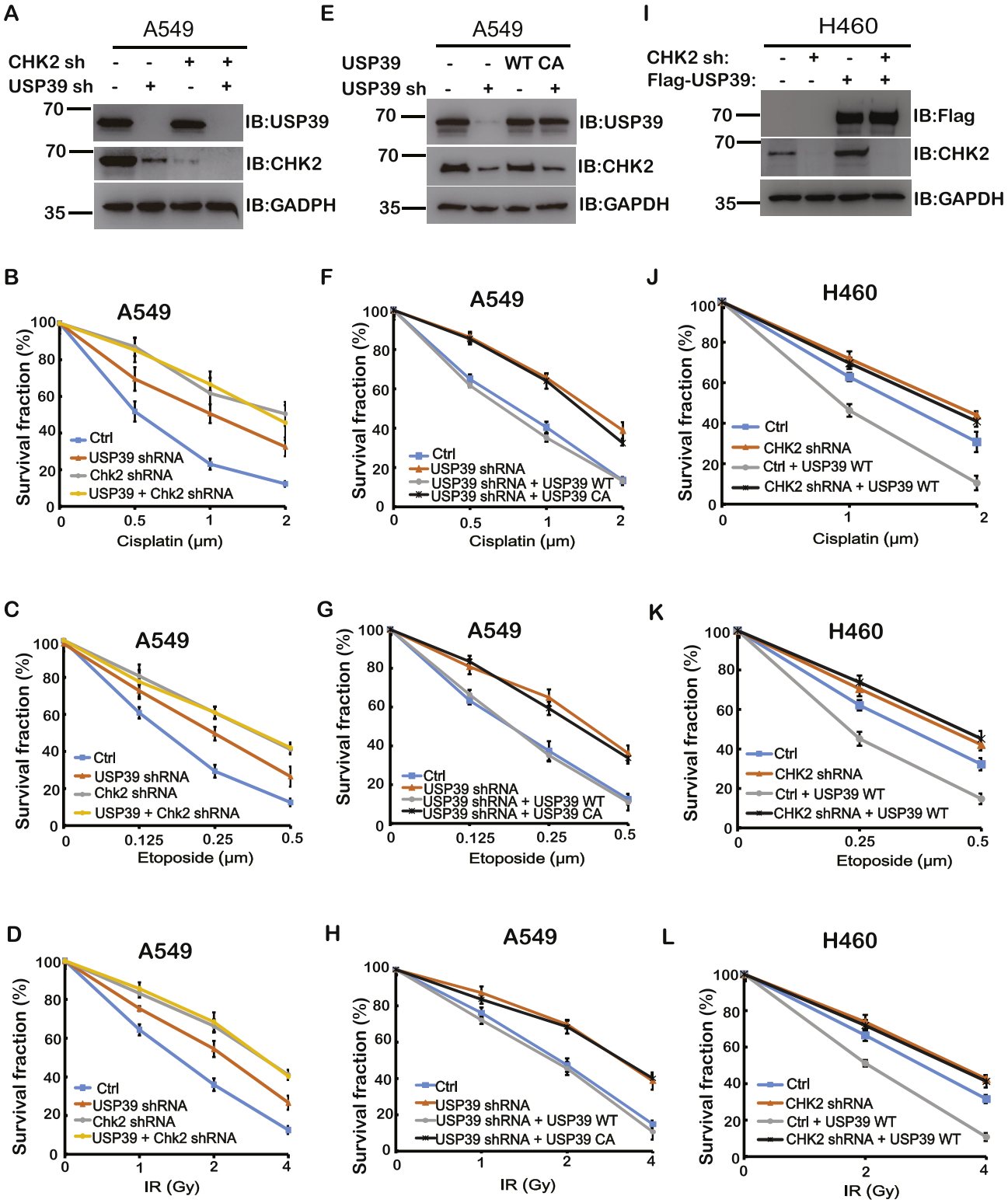


Fig. 6. USP39 regulates chemo-radiation resistance via CHK2. **(A)** A549 Cells stably expressing indicated constructs were lysed. The expression of CHK2 was analyzed by western blot. **(B–D)** cells from **(A)** were treated with **(B)** cisplatin, **(C)** etoposide or **(D)** radiation (IR). Cell survival was performed by colony formation. **(E)** A549 Cells stably expressing control or USP39 shRNAs were transfected with indicated constructs. The expression of CHK2 was analyzed by western blot. **(F–H)** cells from **(E)** were treated with **(F)** cisplatin, **(G)** etoposide or **(H)** radiation (IR). Cell survival was performed by colony formation. **(I)** H460 cells stably expressing control or CHK2 shRNAs were transfected with indicated constructs. The expression of CHK2 was analyzed by western blot. **(J–L)** cells from **(I)** were treated with **(J)** cisplatin, **(K)** etoposide or **(L)** radiation (IR). Cell survival was performed by colony formation. The data presented are mean ± SD (n = 3).

sensitized cells to cisplatin (Supplementary Fig. 2B and Fig. 6J), etoposide (Supplementary Fig. 2C and Fig. 6K) and radiation (Supplementary Fig. 2D and Fig. 6L). However, the USP39 overexpression mediated chemo-radiation sensitivity was abolished in depleted CHK2 cells (Fig. 6I–L). Taken together, our findings support an important role of USP39 in regulating the response of lung cancer cells

to chemotherapy and radiation through CHK2.

In summary, we demonstrated that USP39 deubiquitinates and stabilizes CHK2, which in turn regulates cell cycle checkpoint, cell apoptosis and chemo-radiation response. Furthermore, USP39 and CHK2 are correlatively downregulated in lung cancer, suggesting a potential prognostic role of USP39 in lung cancer.

4. Discussion

CHK2 is a core player in DNA damage response involved in regulation of cell cycle checkpoint, apoptosis and cell proliferation [22]. It is well studied that phosphorylation-dephosphorylation balance are important for maintaining CHK2 activity in cells [2]. Following DNA damage, CHK2 is phosphorylated, which in turn induces its conformation change and fully activation. On the other hand, CHK2 is maintained in a dephosphorylation and inactive status by phosphatases PP1, PP2A and WIP1 in the absence of DNA damage. Recent studies suggested that ubiquitination also play an important role in regulation of CHK2 protein level and functions [34,35,37,44,45]. Multiple E3 ligases were reported to regulate CHK2 protein turnover and activation. For example, PIRH2 and SIAH2 ubiquitinate CHK2 and promote its degradation and impact its function in the regulation of apoptosis and cell cycle [34,37]. In addition, MDM2 and PCAF mediate CHK2 ubiquitination and degradation independently of their E3 ligase activities [44]. Furthermore, Culin1 promotes CHK2 ubiquitination in response to DNA damage, which does not regulate CHK2 turnover, but instead activates CHK2 [45]. All these studies demonstrate important roles of ubiquitination in multiple layers regulation of CHK2. Previous studies showed that knockdown of USP28 decreased CHK2 level in IR treated cells [60]. However, it is difficult to clarify USP28 as a bona fide DUB of CHK2, since no direct evidence showed that USP28 deubiquitinated CHK2 in the previous publication [60]. Taken together, the process of deubiquitination of CHK2 remains unclear. Here we identified USP39 function as a bona fide DUB of CHK2, which in turn stabilized CHK2 and regulated CHK2 mediated apoptosis and cell cycle checkpoint. Knockdown of USP39 compromised G2/M checkpoint, thereby decreasing apoptosis in response to chemotherapy.

Several papers indicated that CHK2 plays a critical role in maintenance G2/M arrest and facilitates apoptosis in response to radiation or chemotherapy drugs treatment [4,7,57]. Loss of CHK2 compromised G2/M checkpoint and apoptosis, which in turn rendered chemo-radiation resistance. Thus, we also examined the roles of USP39-CHK2 axis in chemo-radiation response. In line with previous reports, we found depletion of USP39 led to CHK2 degradation, in turn rendered lung cancer cells resistance to chemotherapy and radiation treatment. However, knockdown of USP39 in depleted CHK2 cells had no further effects on radio-chemo response. In addition, overexpression of USP39 WT but not that of USP39 CA mutant sensitized cells to chemo-radiation treatment. These findings indicated that USP39 regulated chemo-radiation response through deubiquitinating CHK2.

Previous studies show that CHK2 is downregulated in lung cancers which led to chemoresistance [53,54]. However, the mechanism is still unclear. We propose that downregulation of USP39 might be a mechanism for CHK2 downregulation in cancer. In this study, we demonstrated that USP39 and CHK2 levels are correlatively downregulated in lung cancer cell lines and cancer samples. In addition, downregulation of USP39 facilitates lung cancer cell resistance to chemotherapy and radiation treatment through CHK2. Hence, our findings suggest that USP39-CHK2 axis may function as the potential biomarker for predicting chemo-radiation response in lung cancer.

Conflicts of interest

No author has conflict of interest with the contents of this article.

Acknowledgments

This work was supported by National Natural Science Foundation of China (91749115, 81872298, 81802754), China Scholarship Council (201706260203).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.015.

References

[1] J. Ahn, M. Urist, C. Prives, The Chk2 protein kinase, DNA Repair 3 (2004) 1039–1047.
[2] J. Bartek, J. Falck, J. Lukas, CHK2 kinase–a busy messenger, Nat. Rev. Mol. Cell Biol. 2 (2001) 877–886.
[3] H.C. Reinhardt, M.B. Yaffe, Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2, Curr. Opin. Cell Biol. 21 (2009) 245–255.
[4] A. Hirao, Y.-Y. Kong, S. Matsuoka, A. Wakeham, J. Ruland, H. Yoshida, D. Liu, S.J. Elledge, T.W. Mak, DNA damage-induced activation of p53 by the checkpoint kinase Chk2, Science 287 (2000) 1824–1827.
[5] B.B. Zhou, S.J. Elledge, The DNA damage response: putting checkpoints in perspective, Nature 408 (2000) 433–439.
[6] M.B. Kastan, J. Bartek, Cell-cycle checkpoints and cancer, Nature 432 (2004) 316.
[7] J. Bartek, J. Lukas, Chk1 and Chk2 kinases in checkpoint control and cancer, Cancer Cell 3 (2003) 421–429.
[8] V.R. Gogineni, A.K. Nalla, R. Gupta, D.H. Dinh, J.D. Klopfenstein, J.S. Rao, Chk2-mediated G2/M cell cycle arrest maintains radiation resistance in malignant meningioma cells, Cancer Lett. 313 (2011) 64–75.
[9] S.-Y. Shieh, J. Ahn, K. Tamai, Y. Taya, C. Prives, The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites, Genes Dev. 14 (2000) 289–300.
[10] N.H. Chehab, A. Malikzay, M. Appel, T.D. Halazonetis, Chk2/hCds1 functions as a DNA damage checkpoint in G1 by stabilizing p53, Genes Dev. 14 (2000) 278–288.
[11] L. Schmitz, I. Grishina, Regulation of the tumor suppressor PML by sequential post-translational modifications, Front. Oncol. 2 (2012) 204.
[12] S. Yang, C. Kuo, J.E. Bisi, M.K. Kim, PML-dependent apoptosis after DNA damage is regulated by the checkpoint kinase hCds1/Chk2, Nat. Cell Biol. 4 (2002) 865.
[13] C. Stevens, L. Smith, N.B. La Thangue, Chk2 activates E2F-1 in response to DNA damage, Nat. Cell Biol. 5 (2003) 401.
[14] J. Falck, N. Mailand, R.G. Syljuåsen, J. Bartek, J. Lukas, The ATM–Chk2–Cdc25A checkpoint pathway guards against radioresistant DNA synthesis, Nature 410 (2001) 842.
[15] S. Matsuoka, M. Huang, S.J. Elledge, Linkage of ATM to cell cycle regulation by the Chk2 protein kinase, Science 282 (1998) 1893–1897.
[16] H. Ashra, K. Rao, Elevated phosphorylation of Chk1 and decreased phosphorylation of Chk2 are associated with abrogation of G2/M checkpoint control during transformation of Syrian hamster embryo (SHE) cells by Malachite green, Cancer Lett. 237 (2006) 188–198.
[17] I. Park, K.-K. Park, J.H.Y. Park, W.-Y. Chung, Isoliquiritigenin induces G2 and M phase arrest by inducing DNA damage and by inhibiting the metaphase/anaphase transition, Cancer Lett. 277 (2009) 174–181.
[18] S.Y. Shin, Y. Yong, C.G. Kim, Y.H. Lee, Y. Lim, Deoxypodophyllotoxin induces G2/M cell cycle arrest and apoptosis in HeLa cells, Cancer Lett. 287 (2010) 231–239.
[19] A. Stolz, N. Ertych, A. Kienitz, C. Vogel, V. Schneider, B. Fritz, R. Jacob, G. Dittmar, W. Weichert, I. Petersen, The CHK2–BRCA1 tumour suppressor pathway ensures chromosomal stability in human somatic cells, Nat. Cell Biol. 12 (2010) 492.
[20] J. Zhang, H. Willers, Z. Feng, J.C. Ghosh, S. Kim, D.T. Weaver, J.H. Chung, S.N. Powell, F. Xia, Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair, Mol. Cell Biol. 24 (2004) 708–718.
[21] J.S. Lee, K.M. Collins, A.L. Brown, C.H. Lee, J.H. Chung, hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response, Nature 404 (2000) 201–204.
[22] L. Zannini, D. Delia, G. Buscemi, CHK2 kinase in the DNA damage response and beyond, J. Mol. Cell Biol. 6 (2014) 442–457.
[23] J.Y. Ahn, J.K. Schwarz, H. Piwnica-Worms, C.E. Canman, Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation, Cancer Res. 60 (2000) 5934–5936.
[24] J.Y. Ahn, X. Li, H.L. Davis, C.E. Canman, Phosphorylation of threonine 68 promotes oligomerization and autophosphorylation of the Chk2 protein kinase via the forkhead-associated domain, J. Biol. Chem. 277 (2002) 19389–19395.
[25] X. Xu, L.M. Tsvetkov, D.F. Stern, Chk2 activation and phosphorylation-dependent oligomerization, Mol. Cell Biol. 22 (2002) 4419–4432.
[26] C.H. Lee, J.H. Chung, The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation, J. Biol. Chem. 276 (2001) 30537–30541.
[27] J.K. Schwarz, C.M. Lovly, H. Piwnica-Worms, Regulation of the Chk2 protein kinase by oligomerization-mediated cis- and trans-phosphorylation, Mol. Canc. Res. 1 (2003) 598–609.
[28] X. Wu, J. Chen, Autophosphorylation of checkpoint kinase 2 at serine 516 is required for radiation-induced apoptosis, J. Biol. Chem. 278 (2003) 36163–36168.

[29] J. Li, D.F. Stern, Regulation of CHK2 by DNA-dependent protein kinase, J. Biol. Chem. 280 (2005) 12041–12050.
[30] Z.F. Shang, B. Huang, Q.Z. Xu, S.M. Zhang, R. Fan, X.D. Liu, Y. Wang, P.K. Zhou, Inactivation of DNA-dependent protein kinase leads to spindle disruption and mitotic catastrophe with attenuated checkpoint protein 2 Phosphorylation in response to DNA damage, Cancer Res. 70 (2010) 3657–3666.
[31] M. Bahassi el, D.L. Myer, R.J. McKenney, R.F. Hennigan, P.J. Stambrook, Priming phosphorylation of Chk2 by polo-like kinase 3 (Plk3) mediates its full activation by ATM and a downstream checkpoint in response to DNA damage, Mutat. Res. 596 (2006) 166–176.
[32] H.-J. Lee, C.-F. Li, D. Ruan, S. Powers, P.A. Thompson, M.A. Frohman, C.-H. Chan, The DNA damage transducer RNF8 facilitates cancer chemoresistance and progression through twist activation, Mol. Cell 63 (2016) 1021–1033.
[33] Y.-S. Jung, Y. Qian, X. Chen, Pirh2 RING-finger E3 ubiquitin ligase: its role in tumorigenesis and cancer therapy, FEBS Lett. 586 (2012) 1397–1402.
[34] M. Bohgaki, A. Hakem, M.J. Halaby, T. Bohgaki, Q. Li, P.A. Bissey, J. Shloush, T. Kislinger, O. Sanchez, Y. Sheng, R. Hakem, The E3 ligase PIRH2 polyubiquitylates CHK2 and regulates its turnover, Cell Death Differ. 20 (2013) 812–822.
[35] L. Feng, J. Chen, The E3 ligase RNF8 regulates KU80 removal and NHEJ repair, Nat. Struct. Mol. Biol. 19 (2012) 201–206.
[36] J. Qi, M. Tripathi, R. Mishra, N. Sahgal, L. Fazil, S. Ettinger, W.J. Placzek, G. Claps, L.W. Chung, D. Bowtell, The E3 ubiquitin ligase Siah2 contributes to castration-resistant prostate cancer by regulation of androgen receptor transcriptional activity, Cancer Cell 23 (2013) 332–346.
[37] C. Garcia-Limones, M. Lara-Chica, C. Jimenez-Jimenez, M. Perez, P. Moreno, E. Munoz, M.A. Calzado, CHK2 stability is regulated by the E3 ubiquitin ligase SIAH2, Oncogene 35 (2016) 4289–4301.
[38] D. Komander, M.J. Clague, S. Urbe, Breaking the chains: structure and function of the deubiquitinases, Nat. Rev. Mol. Cell Biol. 10 (2009) 550–563.
[39] K. Luo, Y. Li, Y. Yin, L. Li, C. Wu, Y. Chen, S. Nowsheen, Q. Hu, L. Zhang, Z. Lou, USP49 negatively regulates tumorigenesis and chemoresistance through FKBP51-AKT signaling, EMBO J. 36 (2017) 1434–1446.
[40] C.H. McGowan, Checking in on Cds1 (Chk2): a checkpoint kinase and tumor suppressor, Bioessays 24 (2002) 502–511.
[41] J.M. Dybas, C. Herrmann, M.D. Weitzman, Ubiquitination at the interface of tumor viruses and DNA damage responses, Curr. Opin. Virol. 32 (2018) 40–47.
[42] K. Ramadan, I. Dikic, Ubiquitin and ubiquitin-relative SUMO in DNA damage response, Front. Genet. 8 (2017) 188.
[43] A. Al-Hakim, C. Escribano-Diaz, M.-C. Landry, L. O'Donnell, S. Panier, R.K. Szilard, D. Durocher, The ubiquitous role of ubiquitin in the DNA damage response, DNA Repair 9 (2010) 1229–1240.
[44] E.M. Kass, M.V. Poyurovsky, Y. Zhu, C. Prives, Mdm2 and PCAF increase Chk2 ubiquitination and degradation independently of their intrinsic E3 ligase activities, Cell Cycle 8 (2009) 430–437.
[45] C.M. Lovly, L. Yan, C.E. Ryan, S. Takada, H. Piwnica-Worms, Regulation of Chk2 ubiquitination and signaling through autophosphorylation of serine 379, Mol. Cell Biol. 28 (2008) 5874–5885.
[46] D. Durocher, J. Henckel, A.R. Fersht, S.P. Jackson, The FHA domain is a modular phosphopeptide recognition motif, Mol. Cell 4 (1999) 387–394.
[47] J. Li, B.L. Williams, L.F. Haire, M. Goldberg, E. Wilker, D. Durocher, M.B. Yaffe, S.P. Jackson, S.J. Smerdon, Structural and functional versatility of the FHA domain in DNA-damage signaling by the tumor suppressor kinase Chk2, Mol. Cell 9 (2002) 1045–1054.
[48] D. Durocher, I.A. Taylor, D. Sarbassova, L.F. Haire, S.L. Westcott, S.P. Jackson, S.J. Smerdon, M.B. Yaffe, The molecular basis of FHA domain: phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms, Mol. Cell 6 (2000) 1169–1182.
[49] L. Antoni, N. Sodha, I. Collins, M.D. Garrett, CHK2 kinase: cancer susceptibility and cancer therapy - two sides of the same coin? Nat. Rev. Canc. 7 (2007) 925–936.
[50] R. Perona, V. Moncho-Amor, R. Machado-Pinilla, C. Belda-Iniesta, I. Sanchez Perez, Role of CHK2 in cancer development, Clin. Transl. Oncol. 10 (2008) 538–542.
[51] A. Stolz, N. Ertych, H. Bastians, Tumor suppressor CHK2: regulator of DNA damage response and mediator of chromosomal stability, Clin. Cancer Res. 17 (2011) 401–405.
[52] S.E. Meyer, B.E. Peace, E.M. Bahassi, G.M. Kavanaugh, P.K. Wagh, S.B. Robbins, M. Yin, S.I. Wells, G.M. Zinser, P.J. Stambrook, Chk2* 1100delC Acts in synergy with the Ron receptor tyrosine kinase to accelerate mammary tumorigenesis in mice, Cancer Lett. 296 (2010) 186–193.
[53] P. Zhang, J. Wang, W. Gao, B.Z. Yuan, J. Rogers, E. Reed, CHK2 kinase expression is down-regulated due to promoter methylation in non-small cell lung cancer, Mol. Canc. 3 (2004) 14.
[54] S. Matsuoka, T. Nakagawa, A. Masuda, N. Haruki, S.J. Elledge, T. Takahashi, Reduced expression and impaired kinase activity of a Chk2 mutant identified in human lung cancer, Cancer Res. 61 (2001) 5362–5365.
[55] P. Zhang, Lung cancer drug resistance and DNA damage signaling, Clin. Pract. 2 (2005) 381.
[56] H. Jiang, H.C. Reinhardt, J. Bartkova, J. Tommiska, C. Blomqvist, H. Nevanlinna, J. Bartek, M.B. Yaffe, M.T. Hemann, The combined status of ATM and p53 link tumor development with therapeutic response, Genes Dev. 23 (2009) 1895–1909.
[57] M. Squatrito, C.W. Brennan, K. Helmy, J.T. Huse, J.H. Petrini, E.C. Holland, Loss of ATM/Chk2/p53 pathway components accelerates tumor development and contributes to radiation resistance in gliomas, Cancer Cell 18 (2010) 619–629.
[58] H. Takai, K. Naka, Y. Okada, M. Watanabe, N. Harada, S.i. Saito, C.W. Anderson, E. Appella, M. Nakanishi, H. Suzuki, Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription, EMBO J. 21 (2002) 5195–5205.
[59] Q. Liu, S. Guntuku, X.-S. Cui, S. Matsuoka, D. Cortez, K. Tamai, G. Luo, S. Carattini-Rivera, F. DeMayo, A. Bradley, Chk1 is an essential kinase that is regulated by Atr and required for the G2/M DNA damage checkpoint, Genes Dev. 14 (2000) 1448–1459.
[60] D. Zhang, K. Zaugg, T.W. Mak, S.J. Elledge, A role for the deubiquitinating enzyme USP28 in control of the DNA-damage response, Cell 126 (2006) 529–542.