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Report

Nek6 is involved in G_2/M phase cell cycle arrest through DNA damage-induced phosphorylation

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Nek6 is a recently identified NIMA-related kinase that is required for mitotic cell cycle progression. In the present study, we examined the role of Nek6 in the DNA damage response. We found that Nek6 is phosphorylated upon IR and UV irradiation through the DNA damage checkpoint in vivo. Nek6 is also directly phosphorylated by the checkpoint kinases Chk1 and Chk2 in vitro. Notably, Nek6 activation during mitosis is completely abolished by IR and UV irradiation. Moreover, the ectopic expression of Nek6 overrides DNA damage-induced G_2/M arrest. These results suggest that Nek6 is a novel target of the DNA damage checkpoint and that the inhibition of Nek6 activity is required for proper cell cycle arrest in the G_2/M phase upon DNA damage.

Introduction

Eukaryotic cells respond to DNA damage by triggering complex signaling pathways that activate various cellular responses, including DNA repair and arrest of cell cycle progression.[1] Cell cycle arrests at the G_1, S and G_2/M phases are essential steps in the proper DNA damage response because they allow cells sufficient time to repair the damaged DNA and thereby prevent the accumulation of mutations. The ATM/ATR-Chk1/Chk2 DNA damage checkpoint pathway plays a central role in the induction of cell cycle arrest in the G_1, S or G_2/M phases by regulating important cell cycle regulators, such as cdc25A, cdc25C, p53, Smc1 and NBS1, through phosphorylation.[2] Aberrant cell cycle regulation upon DNA damage is a hallmark of various cancer cells and frequently causes the failure of repair of DNA damage, thereby resulting in genomic instability. Thus, the identification of physiological targets and the exploration of underlying regulatory mechanisms for DNA damage-induced cell cycle arrest are critical issues.

Nek6 is a recently identified serine/threonine kinase that belongs to the Neks (NIMA-related kinases) family, which has been implicated in mitosis control.[3] Previous studies revealed that Nek6 plays an important role in mitotic cell cycle progression. It has been shown that the protein level and the activity of Nek6 are increased during mitosis.[4,5] Moreover, the overexpression of a dominant negative mutant form of Nek6 or of an RNAi designed to knock down Nek6 induced spindle defects, abnormal chromosome segregation, mitotic arrest and eventually apoptosis,[5] thus indicating that Nek6 is an essential mitotic kinase.

Interestingly, several lines of evidence suggest that the function of Nek6 is related to tumorigenesis. For example, it was recently shown that mRNA levels of Nek6 were upregulated in 70% of hepatic cell carcinomas.[6] Yin et al., showed that the overexpression of inactive Nek6 decreases the growth rate and induces apoptosis in human breast cancer cells.[4] Moreover, the Nek6 gene is localized on chromosome 9q33-34, a locus associated with several human cancers, such as bladder cancer, neuroblastoma, and renal cell carcinoma.[7,8] However, it is not known whether Nek6 is involved in cell cycle arrest upon DNA damage and whether Nek6 is regulated by the DNA damage-induced signal pathway. In this study, we describe our findings showing that Nek6 is a novel target of the DNA damage checkpoint and that the inhibition of Nek6 function is required for G_2/M arrest upon DNA damage.

Results

Nek6 is phosphorylated upon DNA damage in vivo. Previous studies have shown that DNA damage signals are frequently transmitted through phosphorylation.[1] In order to explore the role Nek6 in the DNA damage response, we first examined whether Nek6 is phosphorylated upon DNA damage by using an in vivo [^{32}P] orthophosphate labeling assay, a method that can detect the phosphorylation of a protein under physiological conditions. Since the immunological purification of endogenous Nek6 using currently available antibodies was not successful in our setting, Flag-tagged Nek6 protein was transiently expressed in Hela cells. After transfection, cells were treated with IR (10 Gy) or UV (50 J/m^2) and labeled with [^{32}P]orthophosphate for 2 h. Flag-Nek6 was immunoprecipitated with anti-Flag antibody and analyzed by autoradiography. Interestingly, a phosphorylated form of Flag-Nek6 was detected in lysates from IR- and UV-treated cells (Fig. 1A), indicating that Nek6 is phosphorylated upon DNA damage in vivo.

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We next examined whether IR- and UV-induced phosphorylation of Nek6 are mediated by the DNA damage signaling pathway by using caffeine, a potent chemical inhibitor of the ATM/ATR-Chk2/Chk1 signal pathway.[11] As shown in Figure 1B, caffeine treatment completely inhibited both IR- and UV-induced phosphorylation of Nek6 (Fig. 1B), suggesting that Nek6 is phosphorylated through the DNA damage checkpoint pathway.

Nek6 is a substrate for Chk1 and Chk2 kinase in vitro. Chk1 and Chk2 act as effector kinases in the DNA damage signaling pathway by regulating a wide range of target protein functions through phosphorylation.[2] Based on the findings described above, we examined whether Nek6 might be a direct target of Chk1 and Chk2. Nek6 was immunoprecipitated with anti-Flag antibody from HeLa cells expressing Flag-Nek6 and used as a substrate for the in vitro Chk1 and Chk2 kinase assay. We found that Nek6 was phosphorylated by both Chk1 and Chk2 at similar levels, while Flag-immunoprecipitates from vector-transfected Hela cell lysates showed no detectable phosphorylation product and auto-phosphorylation of Nek6 without Chk1 and Chk2 was very weak (Fig. 2A); these result suggests that Nek6 was specifically phosphorylated by Chk1 and Chk2, at least in vitro. This finding strengthens the notion that Nek6 is phosphorylated by the DNA damage checkpoint pathway.

The Chk kinase-mediated phosphorylation site is located in the N-terminal region of Nek6. To further demonstrate that Nek6 is phosphorylated by Chk1 and Chk2 and to locate the target site for phosphorylation, a series of Nek6 fragments (F1–F4) were generated as GST-fusion proteins (Fig. 2B). The GST-fusion proteins were purified from *E. coli* and used as substrates in a Chk1 and Chk2 in vitro kinase assay. As shown in Figure 2C, the F1 fragment was the only Nek6 fragment phosphorylated by Chk1 or Chk2. The in vitro kinase assay was performed several times, and the results were essentially same each time. Based on these findings, we conclude that the Chk1 and Chk2 phosphorylation target site is located in the 80-amino acid N-terminal region.

DNA damage inhibits Nek6 activation during mitosis. To determine the functional role of DNA damage-induced Nek6 phosphorylation, we next examined whether DNA damage affects the kinase activity of Nek6. Previously it has been shown that Nek6 is activated mainly in mitotic phase and play important role in mitotic cell cycle progression while the kinase activity of Nek6 is low in non-mitotic phase.[4,5] Thus, we examined the effect of IR and UV irradiation on the kinase activity of Nek6 after synchronizing cells in mitotic phase by nocodazole treatment. The results of cell cycle analysis indicated that more than 98% of the cells were in M phase after nocodazole treatment (Fig. 3A). The in vivo [^{32}P]orthophosphate labeling assay showed that Nek6 phosphorylation by IR and UV-irradiation after nocodazole treatment was comparable to that in asynchronous condition (Fig. 3B) and cell cycle distribution was not changed by IR and UV in this time point (Fig. 3A). Consistent with a previous report,[4] Nek6 activity was significantly upregulated in nocodazole-treated cells compared to asynchronous cells (Fig. 3B). However, this nocodazole-induced activation of Nek6 was completely abolished by IR and by UV irradiation. These results suggest that the mitotic activation of Nek6 is immediately inhibited upon DNA damage.

Overexpression of Nek6 overrides DNA damage-induced G_2/M arrest. Since Nek6 is necessary for mitotic cell cycle progression and because we observed that DNA damage inhibits Nek6 activation during M phase, it is likely that DNA damage-induced Nek6 suppression is required for proper G_2/M phase arrest. To test this possibility, we examined whether the ectopic overexpression of Nek6 overrides DNA damage-induced G_2/M arrest. As shown in Figure 4A, Hela cells that were transfected with the control vector were predominantly arrested at the G_2/M phase 12 h after IR irradiation, and this arrest was maintained for at least 24 h. However, the IR-induced G_2/M arrest at 12 h was significantly diminished in Hela cells overexpressing Nek6 (from 62% to 31%) (Fig. 4B). Instead, a G_1 population was observed at 12 h after IR, and a sub-G_1 fraction, which is indicative of cell death, emerged after 24 h while, consistent with previous report,[4] Nek6 overexpression itself showed no significant effect on cell cycle distribution. These results suggest that cells expressing a high level of Nek6 overcame the G_2/M arrest after DNA damage and were eventually subjected to cell death.

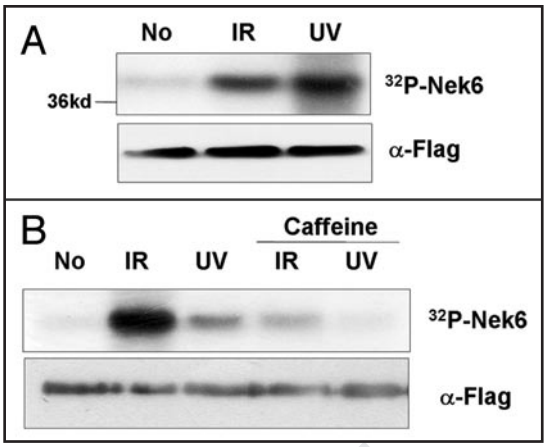

Figure 1. DNA damage-induced phosphorylation of Nek6 in vivo. (A) Hela cells were transfected with the Flag-Nek6 expression plasmid and labeled with [^{32}P]orthophosphate after treatment with or without IR (10 Gy) or UV (50 J/m^2). The phosphorylated Nek6 was analyzed as described in Materials and Methods. Parallel unlabelled samples were immunoprecipitated and analyzed by Western blotting using anti-Flag antibody (lower panel). (B) Hela cells transiently expressing Flag-Nek6 were pretreated for 30 min with or without caffeine (5 mM). Cells were then labeled with [^{32}P] orthophosphate for 2 h in the presence or absence of caffeine (5 mM) and in vivo phosphorylation of Nek6 was examined as described above.

Discussion

In this study, we found that Nek6 is phosphorylated upon IR and UV irradiation in vivo, and that this phosphorylation is mediated by the caffeine-sensitive ATM/ATR-Chk2/Chk1 pathway (Fig. 1). We also showed that Nek6 was directly phosphorylated by Chk1 and Chk2, at least in vitro (Fig. 2). These results revealed for the first time that Nek6 is a direct target of the DNA damage checkpoint.

Several recent studies have suggested that the checkpoint kinases Chk1 and Chk2 regulate important mitotic kinases, such as polo-like kinase (Plk) and Aurora. For example, it has been reported that Chk2 is physically associated with Plk1 and Plk3.[12,13] Recently, it was also revealed that Chk1 regulates the function of Aurora A and Aurora B.[14,15] Nonetheless, the underlying mechanisms of mitotic cell cycle regulation following DNA damage are not completely understood. In the present study, we show that mitotic activation of Nek6 is completely abolished by DNA damage (Fig. 3). Furthermore, the

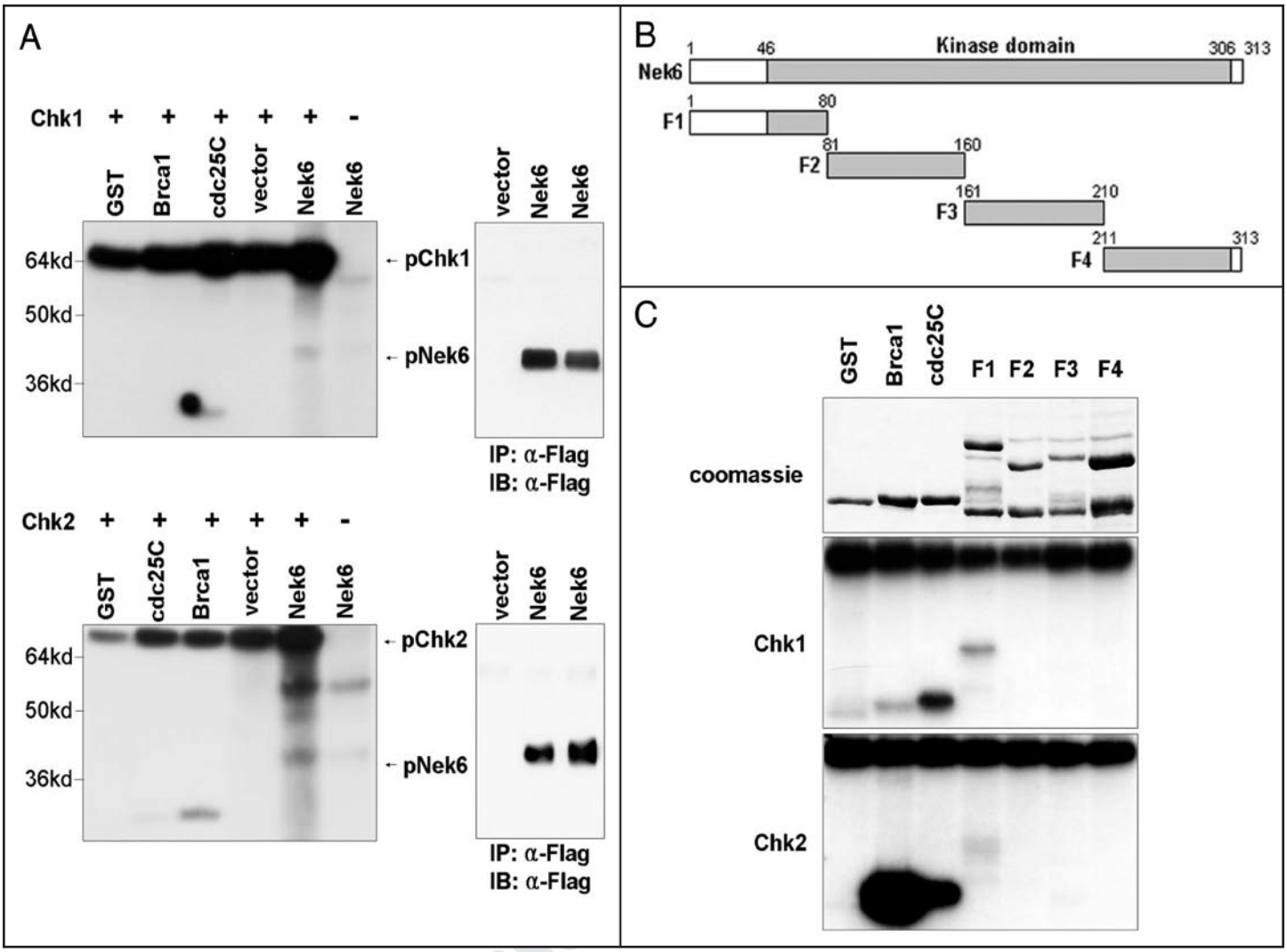

Figure 2. Direct phosphorylation of Nek6 by Chk1 and Chk2 in vitro. (A) Lysates from Hela cells transfected with vector (vec) or Flag-Nek6 (Nek6) were immunoprecipitated with anti-Flag antibody (M2) and used as substrates for the Chk1 and Chk2 in vitro kinase assay as described in Materials and Methods. The Cdc25C Ser216 and Brca1 Ser988 motifs[10] were used as positive controls. The immunoprecipitation of Flag-Nek6 was confirmed by Western blotting with anti-Flag antibody. (B) Schematic diagram of Nek6 and four GST-Nek6 fragments (F1–F4). (C) The phosphorylation of purified GST-Nek6 fragments (F1–F4) by Chk1 and Chk2 was examined in an in vitro kinase assay as described above.

overexpression of Nek6 overrides DNA damage-induced G_2/M arrest (Fig. 4). Nek6 activation is an essential step for mitotic cell cycle progression. Inactivation of Nek6 by either RNA interference or the expression of a dominant negative mutant form of Nek6 resulted in cell cycle arrest at mitosis.[4] These results suggest that one of the mechanisms involved in DNA damage-induced cell cycle arrest at the G_2/M phase, is the inhibition of Nek6 activation through Chk kinase-mediated phosphorylation.

Of the Nek family members, Nek6 and Nek7 are closely related in amino acid sequence and structure. These kinases share 87% amino acid homology in the kinase domain and show a significant difference only in their N-terminal regions.[16] Thus, it has been suggested that Nek6 and Nek7 play a redundant role in mitotic cell cycle progression. However, recent reports have demonstrated that their patterns of activation and subcellular localization are quite different.[16-18] In addition, Nek6 and Nek7 respond differently to serum deprivation conditions.[19] These studies suggest that Nek6 and Nek7 play distinct physiological roles. Consistent with this notion, Nek6 showed strong phosphorylation upon DNA damage, but Nek7 phosphorylation was not detected in the [^{32}P] in vivo labelling assay after IR and UV (data not shown). The location of the Chk kinase-dependent phosphorylation site in the N-terminal region of Nek6, which is the only variable domain between Nek6 and Nek7, also suggests that these two proteins are differently regulated upon DNA damage.

In conclusion, our study revealed that Nek6 is a novel target of the DNA damage checkpoint and that the inhibition of Nek6 is required for proper cell cycle arrest upon DNA damage. Further investigation of the detailed mechanism by which DNA damage checkpoint mediates Nek6 regulation will provide important insights into cell cycle regulation following DNA damage, as well as novel targets for cancer treatment.

Materials and Methods

Cell culture and treatments. HEK293 and Hela cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). For genotoxic

treatments, cells were treated with gamma irradiation (IR) by using a 131 Cs source, or with UV by using a germicidal UV lamp and UV radiometer (UVX-25, Ultra-Violet Products Ltd) with the indicated dose. To enrich the mitotic cell fraction, Hela cells were treated with 400 ng/ml of nocodazole (Sigma) for 24 h.

Nek6 In vivo phosphorylation assay. In vivo [^{32}P]orthophosphate labelling assays were performed as described previously.[9] Briefly, Hela cells were transfected with Flag-Nek6 expression plasmids and treated with 10 Gy of IR or 50 J/m^2 of UV after 48 h incubation. Cells were subsequently labeled with 0.5 mCi of [^{32}P]orthophosphate for 2 h. Phosphorylated Nek6 was immunoprecipitated with anti-Flag antibody and analyzed by autoradiography. To confirm the equal loading of Flag-Nek6, unlabelled samples that had been treated in parallel were immunoprecipitated and analyzed by Western blotting with anti-Flag antibody, as described previously.[10]

Cell cycle analysis. To determine cell cycle distribution, cells were fixed with 70% ethanol and stained with propidium iodide (PI). Flow cytometric analysis was performed using FACSCalibur and Cell Quest software (Becton Dickinson, Mountain View, CA).

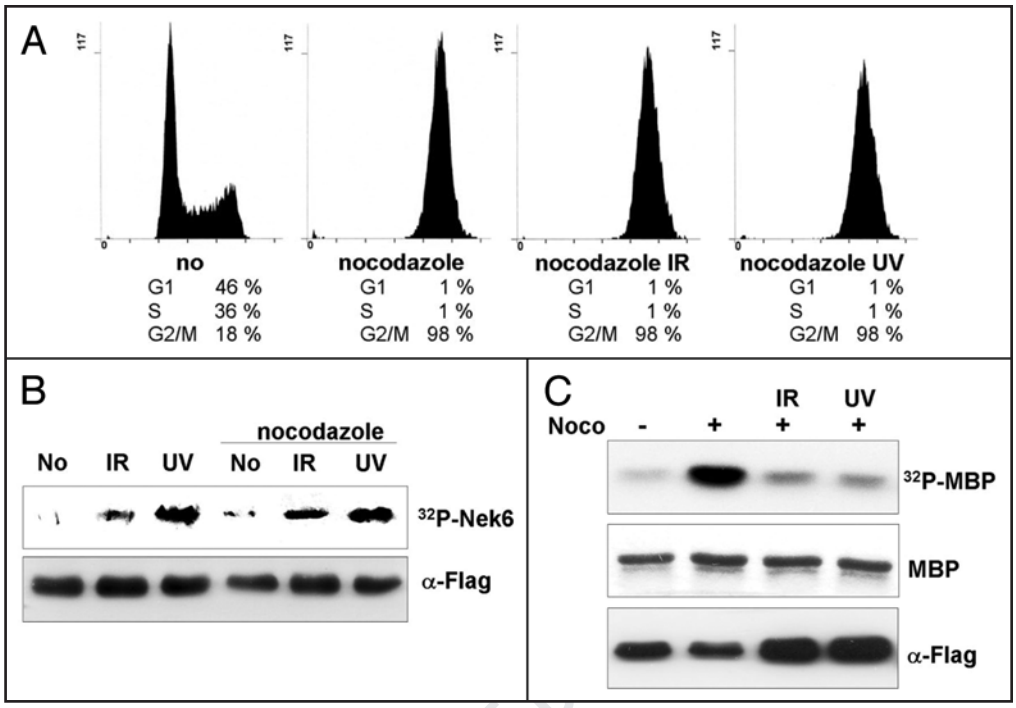

Figure 3. Mitotic activation of Nek6 is inhibited by DNA damage. (A) Hela cells were treated with or without (no) 0.4 μg/ml of nocodazole for 24 h. The nocodazole-treated cells were then irradiated with 10 Gy of IR or 50 J/m^2 of UV and harvested 2 h later. The cell cycle distribution was determined by flow cytometric analysis. (B) Hela cells transfected with Flag-Nek6 (Nek6) were treated with or without 0.4 μg/ml of nocodazole for 24 h. Cells were then treated with 10 Gy of IR or 50 J/m^2 of UV and in vivo phosphorylation of Nek6 was examined as described Figure 1. (C) Hela cells were transfected with Flag-Nek6 and incubated with nocodazole for 24 h. Cells were then treated with or without 10 Gy of IR or 50 J/m^2 of UV and harvested 2 h later. Nek6 kinase activity was measured by an in vitro kinase assay using MBP as substrate, as described in Materials and Methods.

Generation of GST-Nek6 fragments and purification. To generate GST-Nek6 fragments (F1–F4), PCR amplification was carried out using forward primers carrying an EcoRI site and reverse primers containing an XhoI site (the sequence of the oligonucleotides is available upon request). The amplified fragments were digested with EcoRI-XhoI and subcloned into the pGEX-5X-1 vector. The GST-fusion proteins were expressed in Escherichia coli BL21 (DE3) and purified as described previously.[10]

In vitro kinase assay. In vitro Chk1 and Chk2 kinase assays were performed essentially as described previously.[10] To examine the phosphorylation of full length Nek6, Flag-Nek6 purified by immunoprecipitation with anti-Flag antibody from HeLa cells transiently expressing the fusion protein or 2 μg of purified GST-Nek6 fragments were incubated in a kinase reaction buffer containing 0.1 μg of purified Chk1 or Chk2,[10] and 5 μCi [γ-^{32}P]ATP. The level of immunoprecipitated Flag-Nek6 was determined by Western blotting with an anti-Flag antibody. The reaction mixture was separated by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography.

To measure Nek6 kinase activity, an in vitro Nek6 kinase assay was performed as described previously.[4] Flag-Nek6 was immunoprecipitated from Hela cells transiently expressing the fusion protein and incubated with 2 μg of myelin basic protein (MBP) as substrate in a reaction buffer containing 5 μCi of [γ-^{32}P]ATP.

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References

1. Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. Nature 2000; 408:433-9.
2. Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 2003; 3:421-9.
3. O'Regan L, Blot J, Fry AM. Mitotic regulation by NIMA-related kinases. Cell Div 2007; 2:25.
4. Yin MJ, Shao L, Voehringer D, Smeal T, Jallal B. The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis. J Biol Chem 2003; 278:52454-60.
5. Belham C, Roig J, Caldwell JA, Aoyama Y, Kemp BE, Comb M, Avruch J. A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. J Biol Chem 2003; 278:34897-909.
6. Chen J, Li L, Zhang Y, Yang H, Wei Y, Zhang L, Liu X, Yu L. Interaction of Pin1 with Nek6 and characterization of their expression correlation in Chinese hepatocellular carcinoma patients. Biochem Biophys Res Commun 2006; 341:1059-65.
7. Li MZ, Yu L, Liu Q, Chu JY, Zhao SY. Assignment of NEK6, a NIMA-related gene, to human chromosome 9q33. 3→q34.11 by radiation hybrid mapping. Cytogenet Cell Genet 1999; 87:271-2.
8. Hashimoto Y, Akita H, Hibino M, Kohri K, Nakanishi M. Identification and characterization of Nek6 protein kinase, a potential human homolog of NIMA histone H3 kinase. Biochem Biophys Res Commun 2002; 293:753-8.
9. Yun J, Chae HD, Choi TS, Kim EH, Bang YJ, Chung J, Choi KS, Mantovani R, Shin DY. Cdk2-dependent phosphorylation of the NF-Y transcription factor and its involvement in the p53-p21 signaling pathway. J Biol Chem 2003; 278:36966-72.

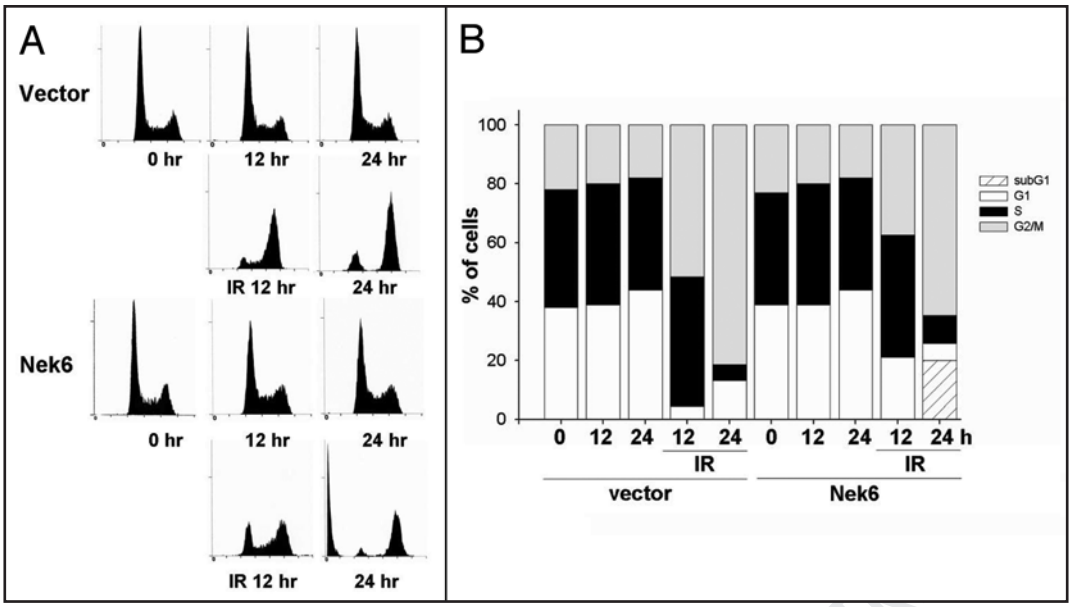

Figure 4. The overexpression of Nek6 overrides DNA damage-induced G_2/M arrest. (A) Hela cells were transfected with either 6 μg of vector or Flag-Nek6. Cells were then treated with or without 10 Gy of IR and harvested at the indicated time points. The cell cycle distribution was analyzed by flow cytometry. The experiment was repeated three times with similar results. (B) Quantification of the cell cycle distributions obtained in (A).

10. Kim MA, Kim HJ, Brown AL, Lee MY, Bae YS, Park JI, Kwak JY, Chung JH, Yun J. Identification of novel substrates for human checkpoint kinase Chk1 and Chk2 through genome-wide screening using a consensus Chk phosphorylation motif. Exp Mol Med 2007; 39:205-12.
11. Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, Abraham RT. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 1999; 59:4375-82.
12. Tsvetkov L, Xu X, Li J, Stern DF. Polo-like kinase 1 and Chk2 interact and co-localize to centrosomes and the midbody. J Biol Chem 2003; 278:8468-75.
13. Xie S, Wu H, Wang Q, Kunicki J, Thomas RO, Hollingsworth RE, Cogswell J, Dai W. Genotoxic stress-induced activation of Plk3 is partly mediated by Chk2. Cell Cycle 2002; 1:424-9.
14. Krystyniak A, Garcia-Echeverria C, Prigent C, Ferrari S. Inhibition of Aurora A in response to DNA damage. Oncogene 2006; 25:338-48.
15. Zachos G, Black EJ, Walker M, Scott MT, Vagnarelli P, Earnshaw WC, Gillespie DA. Chk1 is required for spindle checkpoint function. Dev Cell 2007; 12:247-60.
16. Kandli M, Feige E, Chen A, Kilfin G, Motro B. Isolation and characterization of two evolutionarily conserved murine kinases (Nek6 and nek7) related to the fungal mitotic regulator, NIMA. Genomics 2000; 68:187-96.
17. Belham C, Comb MJ, Avruch J. Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase. Curr Biol 2001; 11:1155-67.
18. Feige E, Motro B. The related murine kinases, Nek6 and Nek7, display distinct patterns of expression. Mech Dev 2002; 110:219-23.
19. Minoguchi S, Minoguchi M, Yoshimura A. Differential control of the NIMA-related kinases, Nek6 and Nek7, by serum stimulation. Biochem Biophys Res Commun 2003; 301:899-906.