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# Regulation of Nek6 Functions by Its SUMOylation on the $K^{252}$ Residue

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**Abstract:** Nek6 belongs to NIMA1 (never in mitosis, gene A) related kinase, which was originally identified in *Aspergillus nidulans* as a serine/threonine kinase critical for cell cycle progression. We noticed that the putative SUMOylation site is localized on the $K^{252}$ residue in $^{251}$FKsD$^{254}$ of Nek6, based on the consensus sequence ΦKxE; where Φ represents L, I, V or F and x is any amino acid. We observed that the Nek6 SUMO mutant (K252R) has decreased protein kinase activity, nuclear speckle localization and protein stability, compared with that of the Nek6 wild type. However, the Nek6 SUMO mutant increased the cell survival rate of COS-1 cells as determined by FACS analysis. Therefore, our data suggest that SUMOylation on the $K^{252}$ residue of Nek6 is required for its normal functions, such as proper nuclear localization, kinase activity and protein stability, to control cell cycle.

**Key words:** Nek6, serine/threonine kinase, cell cycle, SUMOylation, Apoptosis

NIMA1 (never in mitosis, gene A) related kinase was originally identified in *Aspergillus nidulans* as a serine/threonine kinase critical for cell cycle progression (See the review by Quarmby and Mahjoub, 2005; Malumbres and Barbacid, 2007). The sequencing of the human genome has revealed a relatively divergent family of 11 mammalian Neks (Kandli et al., 2000; Belham et al., 2001). The Neks are most closely related to NIMA in their N-terminal catalytic domain sequences, but each diverges substantially from NIMA in its non-catalytic C-terminal tail (Kandli et al., 2000; Belham et al., 2001; Hashimoto et al., 2002). However, the functions of Neks are largely unknown (Quarmby and Mahjoub, 2005; Malumbres and Barbacid, 2007). Nek6 has been identified as one of the Neks. Nek6 and the closely related Nek7 were shown to phosphorylate not only the protein kinase p70 S6 on Thr-412, but also SGK1 on Ser-422 within a hydrophobic motif in the activation loop (Kandli et al., 2000; Belham et al., 2001; Hashimoto et al., 2002). Thus, Nek6 has been suggested as a PDK2, which is able to phosphorylate Ser/Thr residues in a hydrophobic motif in the AGC kinase activation loop (Roig et al., 2002). However, studies to determine the specificity of substrate, which is known to be LxxS/TΦ, where Φ is a hydrophobic amino acid, using peptides derived from annotated SUMOylation sites in human proteins have suggested that Nek6 does not phosphorylate p70 S6 kinase either *in vitro* or *in vivo* (Lizcano et al., 2002). Among the 11 Neks, Nek9, Nek6 and Nek7 are closely related to one another, and Nek9 was identified based on its association with Nek6 and Nek7 (Roig et al., 2002; Belham et al., 2003). Further, since it has been reported that Nek9 binds Ran GTPase and regulates mitotic progression, it was suggested that Nek9, Nek6 and Nek7 might play a role in mitotic regulation (Minoguchi et al., 2003; Yin et al., 2003). However, the function of Nek6 in mitotic regulation remains to be characterized.

SUMO (Small Ubiquitin-like Modifier) modification (SUMOylation) of proteins, especially of transcriptional regulators and nuclear pore proteins, has been previously described (reviewed by Gill, 2004; Dohmen, 2004). SUMOylation is a new covalent modification leading to attachment of SUMO to specific lysine residues of target proteins (Wilson and Rangasamy, 2001; Dorval and Fraser, 2006; Kikuchi et al., 2006). SUMO represents a class of ubiquitin-like proteins conjugated, like ubiquitin, by a set of enzymes to cellular proteins (Melchior and Hengst, 2002).

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However, SUMOylation does not promote protein degradation, and distinct enzymes are involved in SUMOylation. Thus, SUMOylation appears to regulate diverse cellular processes, including nuclear transport, signal transduction, apoptosis, autophagy, cell cycle control, ubiquitin-dependent degradation, and gene transcription. For SUMOylation of substrate proteins, SAE1/SAE2 heterodimer acts as an E1 enzyme in mammals (Aos1/Uba2 in yeast), and Ubc9 acts as an E2 SUMO-conjugating enzyme (Boggio et al., 2004). The mammalian PIAS (protein inhibitor of activated STAT) family, Ran Bp (Ran binding protein) 2 and the polycomb PC2 repressor, have recently been shown to function as E3-type SUMO ligases (Pichler et al., 2002; Kagey et al., 2003; Bischof et al., 2006; van Waardenburg et al., 2006). Analysis of many SUMOylation substrates indicates that it occurs at a particular sequence, thus the specificity of SUMO conjugation might be conferred by recognition of this sequence by the thioester-linked Ubc9-SUMO conjugate (Duprez et al., 1999; Sampson et al., 2001; Yang et al., 2006). Lysine residues targeted for SUMOylation are often found within specific sequences, such as its consensus sequence (Melchior and Hengst, 2002; Zhang et al., 2002). Even though lysine residues targeted for SUMOylation are not always consistent, they are often found within specific sequences (ΦKxE; where Φ represents L, I, V or F and x is any amino acid).

Using consensus sequence analysis, we noticed the putative SUMOylation site on the $K^{252}$ residue in $^{251}$F**K**s$D^{254}$ of Nek6. Here, we present evidence that the $K^{252}$ residue of Nek6 is the SUMOylation site, based on site directed mutagenesis analysis. Additionally, by analyzing the functional consequences of impairing Nek6 SUMOylation, we show that SUMOylation of Nek6 is required for its stability and nuclear speckle localization around the nuclear pore, which affects cell viability. These additional results represent a new mechanism for its functional regulation.

## MATERIALS AND METHODS

### Cell culture

COS-1 was purchased from ATCC (Manassas, VA, USA). Media and supplements were obtained from GIBCO (Grand Island, NY, USA). The cell line was maintained in Dulbecco's Modified Essential Medium (DMEM) containing 10% heat inactivated (for 30 min at 56°C) fetal bovine serum (FBS), 100 U potassium penicillin/ml, 100 μg streptomycin/ml, 2 mM glutamine and 20 mM sodium bicarbonate. Cells were incubated in a chamber containing 5% $CO_2$, maintained at 95 % humidity and 37°C. The growth medium was changed every 3 days. SUMO fusion protein was obtained from Calbiochem (Grand Island, NY, USA).

### Antibodies

Monoclonal antibody against the hexahistidine GFP epitope or GFP was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). Antibodies against Nek6, actin, phospho-threonine/serine-phenylalanine mouse mAb, or phospho-(Ser/Thr) PDK1 docking motif (18A2) mouse mAb were purchased from Cell Signaling (Boston MA, USA). Antibodies against ubiquitin or SUMO-1 were purchased from ABGENT (San Diego, CA, USA).

### Site-directed mutagenesis of Nek6

To generate Nek6 S252A or K252R mutations 5'-GAG AAC GGC TAC AAC TTC AGG TCC GAC ATC TGG TCC-3' and 5'-GCC CAA GGA CCA GAT GTC GGA CCT GAA GTT GTA GCC-3' from EGFP Human Nek6 or GST human Nek6 (purchased from GeneCopia, CA, USA) were used with a "Chameleon" double-stranded site-directed XL mutagenesis kit (Stratagene, CA, USA), according to the manufacturer's instructions. Every mutation was confirmed by DNA sequencing.

### Nek6 expression vector transfection and purification

For mammalian expression, EGFP-Nek6 or its SUMO mutant constructs were transfected to COS-1 cells using the lipofectin transfection method (Gibco-BRL Co). The transfected cells ($2 \times 10^7$) were lysed in RIPA lysis buffer. Anti-GFP polyclonal antibody was incubated with 1,000 μl of pre-cleaned cell lysate, and precipitated with protein A agarose beads. The beads were washed three times with excess cell lysis buffer, and the final pellet was used for immuno assay to detect SUMOyaltion. Western blots were performed to detect the presence of SUMO with an anti-SUMO-1 antibody (Dorval and Fraser, 2006). To detect $S^{231}$ residue phosphorylation of Nek6, phospho-serine/threonine-phenylalanine mouse mAb was used for western blot.

### Nek6 kinase assay

SGK1 protein was purchased from Cell Signaling. Nek6 kinase assays were performed for 30 min at 30°C in a 25-μl reaction volume containing 20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 0.2 mM EGTA, and 1 μg phosphatidylserine, and 1 μg human SGK1 protein as a Nek6 substrate protein. The phosphorylated SGK1 protein was detected by western blot analysis with the phospho-(Ser/Thr) PDK1 docking motif (18A2) mouse mAb.

### Double Immunofluorescence Microscopy

COS-1 cells were plated at low confluence (~30%) on two-well Lab-Tek Permanox slides (Nalgene Nunc International, Naperville, IL, USA) and transiently transfected with

EGFP-Nek6 wt or EGFP-Nek6 SUMO mutant (K252R) plasmid using the lipofectamine procedure. Cells were starved for 36 h and subsequently treated with 10% calf serum for 15 h. At no time did cell confluency exceed 60%. Cells were fixed, permeabilized, and processed for indirect double immunofluorescence microscopy as described previously, with minor modifications. Cells were blocked in normal goat and diluted (1 : 30) in PBS, for 15 min, then incubated with affinity-purified antibodies at a 1 : 150 dilution, in combination with a 1 : 1,000 dilution of murine anti- Nek6 or SUMO-1 monoclonal antibodies (ABGENT) for 1-2 h at room temperature on a rocking platform. Washed slides were incubated for 1 h at room temperature with 1 : 150 dilutions of both anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Molecular Probes Inc., Eugene, OR, USA) and Texas red-conjugated goat anti-mouse secondary antibody (Molecular Probes Inc.). Slides were washed and then mounted with Vectashield mounting medium (Vector Laboratories Inc) and examined using Zeiss Axiophot optics at The Core Facility of Chungbuk National University.

### Expression and purification of recombinant proteins

Wild type Nek6 or SUMO mutant (K252R) protein tagged with GST was expressed in *Escherichia coli* BL21 and purified with GST-agarose beads according to the manufacturer's instructions (Amersham Biosciences Co). Purified proteins were used for SUMOylation assay substrate protein.

### In vitro SUMO-1 conjugation assay

*In vitro* SUMO-1 conjugation assay was performed with a SUMO assay kit purchased from Corgen Inc. (Taipei, Taiwan), according to the manufacturer's recommended protocol. One μg of purified GST-tagged Nek6 mutants was mixed with 250 ng of Ubc9, 125 ng of Aos1/Uba2 with or without 2 μg of SUMO-1, then incubated for 2 h at 30°C in the presence of 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, with or without (for the negative control) 2 mM ATP in a 20 μl volume. Reactions were stopped with SDS-PAGE sample buffer and SUMO conjugates were separated by SDS-PAGE and analyzed by western blotting using mouse monoclonal antibody against SUMO-1 to detect Nek6 (Dorval and Fraser, 2006). The relative optical density (OD) was measured by image analysis of the dried SDS-PAGE gel with the Fuji Image Quant software (Fujifilm, Tokyo, Japan), according to the manufacturer's instructions.

### Protein Stability Experiments

COS-1 cells ($2.5 \times 10^5$ cells per well) in 10 cm plates were transfected with 1.0 μg of expression vector with EGFP-Nek6 wt or EGFP-Nek6 SUMO mutant plasmids. The medium was replaced with medium containing 200 μg/ml cycloheximide 36 h after the transfection (0-h time point). Cell lysates were harvested at 0, 8, 16, 24 h and analyzed by immunoprecipitation and western blotting using anti-GFP antibodies, and assayed five times. (Vega et al., 2004). The relative OD was measured as described above.

### FACS

EGFP Nek6 (wt), SUMO mutant (K252R), or EGFP vector was transfected and the rate of apoptosis was measured by FACS. The transfected cells were washed twice in cold PBS and then resuspended in binding buffer (0.01 M HEPES/NaOH (pH 7.4), 0.14 M NaCl and 2.5 mM $CaCl_2$). $1 \times 10^5$ cells in 100 μl were transferred to a 5 ml culture tube and 5 ml of EGFP antibody and 5 ml of 7-amino-actinomycin D were added. The cells were vortexed gently and incubated for 15 min at 25°C in the dark. 400 ml of binding buffer was added to each tube, and the samples were subsequently labeled with FITC-conjugated rabbit anti-mouse immunoglobulin G on ice for 30 min. The cells were washed twice and collected in Hanks' balanced salt solution and 5% human pooled serum. Within 1 h, FACS was performed on a Coulter Epics Elite equipped with a gated amplifier and upgraded with enhanced system performance at The Core Facility of Chungbuk National University.

## RESULTS

### Nek6 functional domain and SUMOylation

Based on the SUMOylation consensus sequence information (ΦKxE; where Φ represents L, I, V or F and x is any amino acid), the $K^{252}$ residue in $^{251}$F**K**s$D^{254}$ of Nek6 is found in its kinase domain, as shown in Fig. 1. Therefore, we predicted that Nek6 is a SUMO modified protein (Kandli et al., 2000; Belham et al., 2001; Hashimoto et al., 2002; Dorval and Fraser, 2006; Yang et al., 2006). To evaluate this, we used site directed mutagenesis to construct the Nek6 mutant, K252R, as indicated in Fig. 1. The Nek6 mutant (K252R) was then inserted into EGFP fusion expression vector or GST fusion expression vector.

To determine whether SUMOylation of Nek6 had occurred, we conducted an *in vitro* SUMOylation assay using GST-Nek6 fusion protein, which was purified from *E. coli*. As shown in Fig. 1B, SUMOylated Nek6 was detected with SUMO-1 antibody with an expected molecular weight of 70 kD (left lane). As a negative control, SUMO assay for Nek6 wt was performed without ATP (right lane). Western blot of each sample was performed with Nek6 antibody to monitor the amount of GST-Nek6 protein in the experiment (bottom). To confirm the requirement of the lysine residue in FKxE for the substrate protein, SUMO assay was also performed against integrin linked kinase 1 which has no

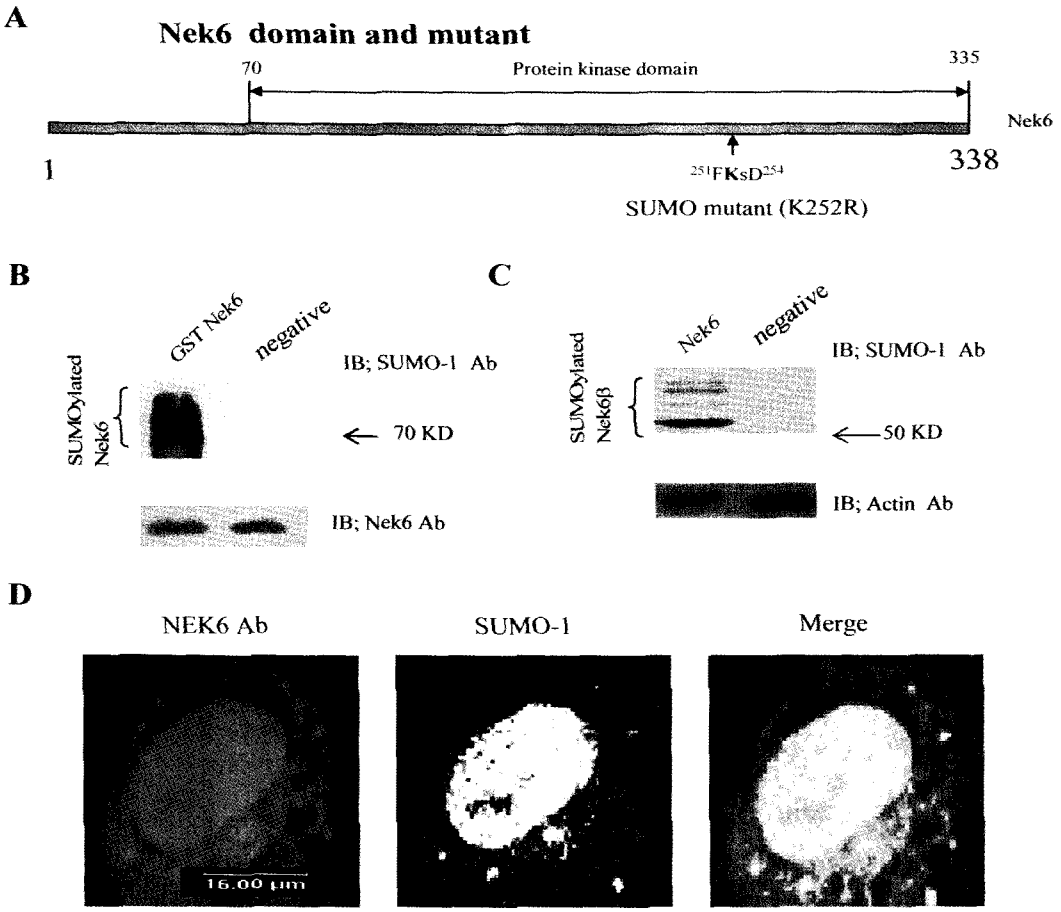


**Fig. 1.** Nek6 functional domain and SUMOylation site. Nek6 functional domain and its SUMOylation site ($K^{252}$ residue in $^{251}$F**K**s$D^{254}$) is indicated below (A). Nek6 mutant (K252R) was constructed by site directed mutagenesis. Nek6 SUMO mutant (K252R) was inserted into GST fusion (for bacteria) or EGFP fusion (for cell line) expression vectors. (B) Nek6 wild type (wt) protein purified from *E. coli* was assayed for SUMOylation. As a negative control, the same assay condition was used without ATP (left lane). Western blot of the same sample was performed with Nek6 monoclonal antibody to monitor the amount of protein (bottom). The arrows indicate the molecular weight of GST- Nek6 (66Kd). (C) Western blot of the immunopurified Nek6 from COS 1 was performed using SUMO-1 specific antibody. SUMOylation of Nek6 was detected as high molecular weight protein bands (right lane). As a negative control, an unrelated mouse antibody was used (left lane). To monitor the amount of total protein, western blot was performed with actin monoclonal antibody (bottom). The arrows indicate the molecular weight of endogeneous Nek6 (43Kd). (D) Confocal microscopic analysis of endogenous Nek6 wt (green) and SUMO-1 (in red). Nek6 was detected. The SUMO-1 modified Nek6 was mainly detected as nuclear speckles around the nuclear pore (in yellow). All figures represent results from three experiments repeated independently.

SUMOylation consensus sequence; We observed no SUMOylation signal, similar to the negative control (data not shown). Thus, this result suggested that Nek6 is a SUMOylated protein.

To further confirm SUMOylation of Nek6, we performed western blot of Nek6 immunopurified from COS1 cells using SUMO-1 specific antibody. As shown in Fig. 1C, SUMOylation of Nek6 was detected as high molecular weight protein bands (left lane), similar to the results shown in Fig. 1B. As a negative control, an unrelated mouse antibody was used (right lane). To monitor the total protein amount, western blot was performed using an actin monoclonal antibody (bottom) (Fig. 1C). These results also suggested that Nek6 was SUMOylated in COS 1 cells, consistent with the results in Fig. 1B.

To confirm further whether endogenous Nek6 undergoes SUMOylation in the cell, confocal microscopy observation of COS 1 cells using Nek6 or SUMO-1 specific antibodies was conducted. As shown in Fig. 1D, Nek6, which was detected in the nuclear speckles around nuclear pores, overlapped with SUMO-1 very strongly. Thus, consistent with both Fig. 1B and C, these results also suggested that the endogenous Nek6 in COS 1 was modified with SUMO-1. Interestingly, SUMOylation of Nek6 seemed to be required for its localization around nuclear pore regions as nuclear speckles (Fig. 1 D). Overall, these results (Fig. 1A, B, C, and D) suggest that Nek6 is a SUMOylated protein.

### SUMOylation sites in Nek6

To determine if SUMOylation occurred on the $K^{252}$ residue in $^{251}$F**K**s$D^{254}$ of NEK6, we purified the GST-fusion proteins (Nek6 wt and Nek6 K252R; SUMO mutant) from *E. coli*

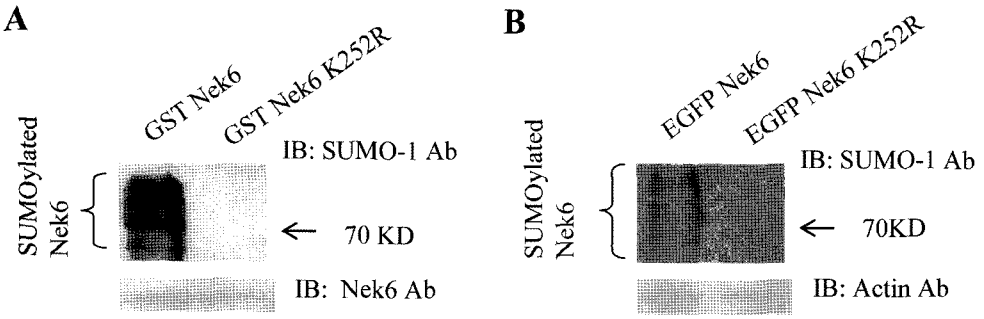


**Fig. 2.** SUMOylation Site in Nek6. The purified GST-Nek6 wt or GST-Nek6 SUMO mutant (K252R) fusion protein (A) was used as the substrate protein in the SUMOylation assay. SUMOylation of Nek6 wt was detected as a high molecular weight protein band (right lane), whereas that of SUMO mutant (K252R) was not detected (left lane). (B) EGFP-Nek6 wt or EGFP-Nek6 SUMO mutant was transfected to COS1 cells and immunoprecipitated with EGFP mouse monoclonal antibody. The immunoprecipitants were subjected to western blot against SUMO-1. The SUMOylation of Nek6 wt was shown as several high molecular weight protein bands (left lane), whereas that of SUMO mutant was not detected (right lane). To monitor the protein expression of Nek6, the immunoprecipitants were subjected to western blot with Nek6 polyclonal antibody (bottom). The arrows indicate molecular weight of GST- or EGFP-Nek6 (70Kd).

and used them as substrate proteins in the SUMOylation assay. As shown in Fig. 2A, SUMOylation of Nek6 wt was detected as several high molecular weight protein bands (right lane), whereas that of the Nek6 SUMO mutant was totally inhibited (left lane). To monitor the protein amount used in the experiment, western blot against Nek6 was performed (Fig. 2A bottom). Thus, these results suggested that the $K^{252}$ residue in $^{251}$FKSD$^{254}$ of Nek6 is the SUMOylation site.

To further confirm our observations, we performed western blot against SUMO-1 using the immunopurified EGFP-Nek6 wt or EGFP- Nek6 SUMO mutant (K252R) from COS1 cells (Fig. 2B). To monitor the total protein amount in the cell lysates, western blot against actin was performed (bottom) (Fig. 2B). As shown in Fig. 2B, SUMOylation of Nek6 wt was detected as high molecular weight protein bands (right lane), whereas Nek6 SUMO mutant (K252R) was not SUMOylated. Thus, Fig. 2 A and B suggest that SUMOylation of Nek6 occurs on the $K^{252}$ residue in $^{251}$FKSD$^{254}$.

## Confocal microscopic analysis with NEK6 SUMO mutant

To further determine whether SUMOylation occurs on the $K^{252}$ residue of Nek6, we analyzed COS-1 cells that were transiently transfected with constructs encoding the NEK6 mutants depicted Fig. 1. The transfected EGFP-Nek6 wt or its SUMO mutant (K252R) (all constructs shown in green) was detected by fluorescence microscopy and the SUMO-1 appeared as red (Fig. 3). We observed that the SUMO-1 modification proteins were primarily detected in the nuclear speckle (Fig. 3 middle lane). EGFP-Nek6 wt overlapped with SUMO-1 in the nuclear speckles around the nuclear pore (Fig. 3A). EGFP-Nek6 wt, which was observed as a fibrous form in the cytoplasm (similar to Fig. 1D), did not overlap with SUMO-1. Thus, these results also suggest that Nek6 is SUMOylated, consistent with the results presented in Fig. 2. Because both Nek6 with SUMO-1 (yellow color) were detected in the nuclear speckles (dots) around the nuclear pore but not the cytoplasm (Fig. 3A right lane), SUMOylation of Nek6 seems to be related to its nuclear subcellular localization.

Similar to the confocal results of Nek 6 wt, its SUMO mutant was also primarily detected in the nucleus (Fig. 3 B). However, the Nek6 SUMO mutant (which was diffusive, not dot form) did not overlap well with SUMO-1 at the nuclear pore (Fig. 3B right). Therefore, these results suggested that SUMOylation on $K^{252}$ of Nek6 is required for the nuclear speckle localization, consistent with the results shown in Fig. 1D and 3A. Further, consistent with the results shown in Fig. 2, these results (Fig. 3B) again confirmed that $K^{252}$ of Nek6 is the SUMOylation site based on confocal microscopic analysis.

## SUMOylation of Nek6 is required for its kinase activation

To further define biological significance of Nek6 SUMOylation, we compared the kinase activity of Nek6 with that of the Nek6 SUMO mutant. Each EGFP-Nek6 wt or its SUMO mutant (K252R) expression vector was transfected into COS-1 cells and immunoprecipitated with EGFP polyclonal antibody. To monitor the expression of EGFP-Nek6 wt or Nek6 SUMO mutant, western blot was performed using an anti-Nek6 antibody (Fig. 4A). We monitored the $S^{231}$ residue phosphorylation of Nek6 using its phosphorylation specific antibody, because this seemed to be required for its activation (Fig. 4B). The $S^{231}$ residue of Nek6 is located in its activation loop and the $T^{235}$ residue of Nek6 is found in the LxxT/S motif, a putative Nek6 autophosphorylation site (Belham et al., 2001; Lizcano et al., 2002; Roig et al., 2002; Belham et al., 2003; Yin et al., 2003). As shown in Fig. 4B, we observed that $S^{231}$

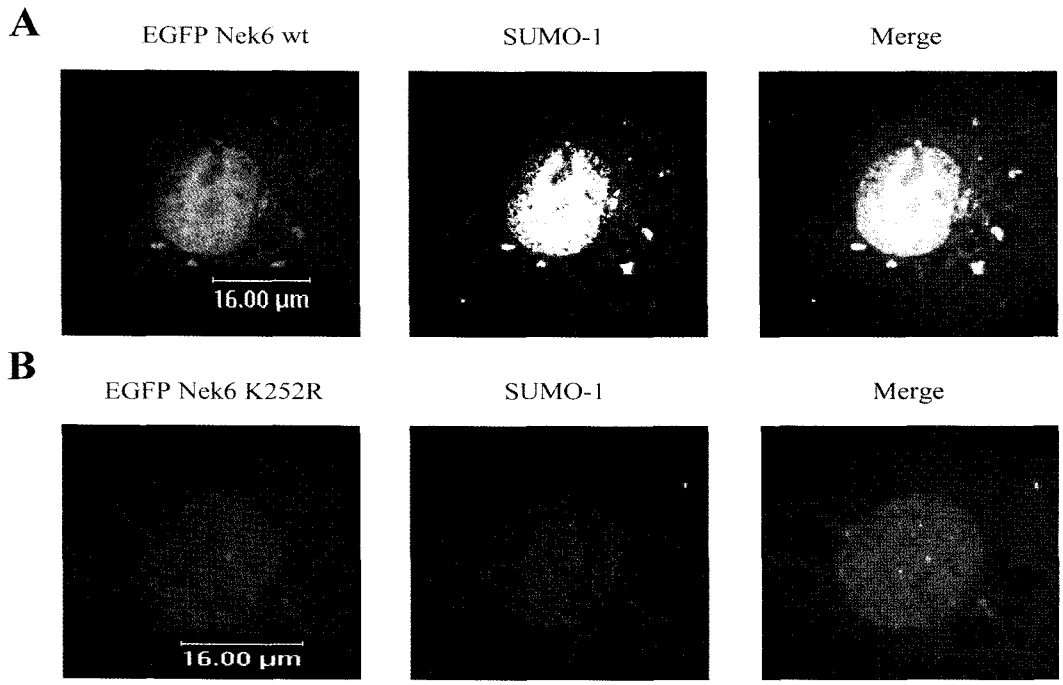


**Fig. 3.** Confocal microscopic analysis of Nek6 wt or its SUMO mutant. Confocal microscopic analysis of transfected EGFP-Nek6 wt (A), EGFP-Nek6 SUMO mutant (B) (green) was performed. The transfected EGFP-Nek6 wt (detected in both the cytoplasm and the nucleus) overlapped (yellow) with SUMO-1 (in red) nuclear speckles around nuclear pore (A). The transfected EGFP-Nek6 SUMO mutant (K252R) was not detected in nuclear speckles around the nuclear pores (B). The SUMO-1 modification proteins were detected as nuclear speckles around nuclear pore. Nek6 SUMO mutant (K252R) did not overlap with SUMO-1 around the nuclear pore (B right lane).

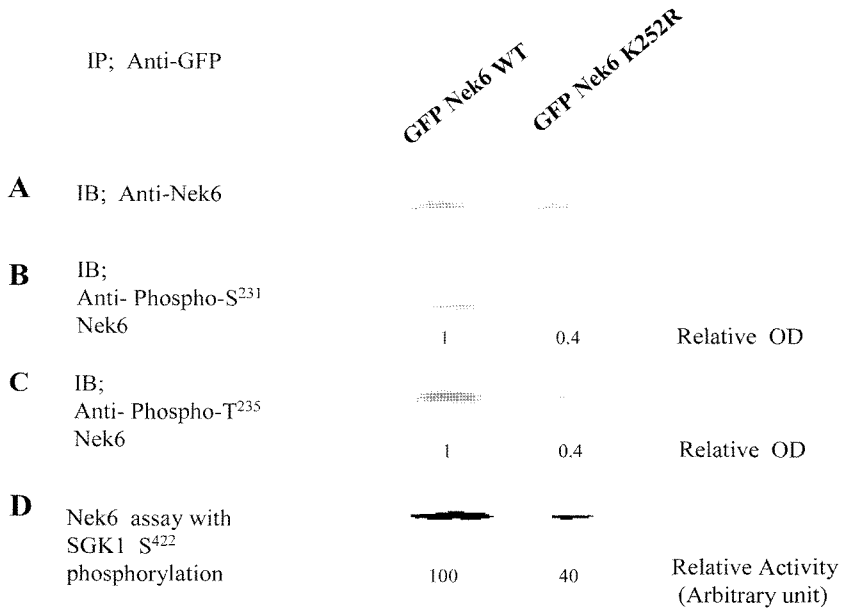


**Fig. 4.** Kinase activity of Nek6 wt or its SUMO mutant. The immunopurified EGFP-Nek6 wt or its SUMO mutant protein with EGFP antibody from COS 1 was immunoblotted with Nek6 (A), anti-Nek6 phospho $S^{231}$ monoclonal antibody (B), or anti- Nek6 phospho $T^{231}$ monoclonal antibody (C). The relative OD is indicated below. The Nek6 kinase activity was measured using human SGK1 protein as a substrate (C). The $S^{422}$ residue phosphorylation of human inactive SGK1 protein was detected with its specific antibody. The relative Nek6 activity by image analysis is indicated below. These results are from one of three repeated experiments.

phosphorylation of the EGFP-Nek6 SUMO mutant was reduced to 60%, compared to that of the EGFP-Nek6 wt (Fig. 4B). Even though the amounts of the expressed proteins were approximately the same (Fig. 4A), the phosphorylation of $T^{235}$ residue of Nek6 SUMO mutant was also reduced to 60% of the EGFP-Nek6 wt (Fig. 4C). In order to detect phosphorylation of the $S^{231}$ or $T^{235}$ residues, we used phospho-threonine/serine phenylalanine

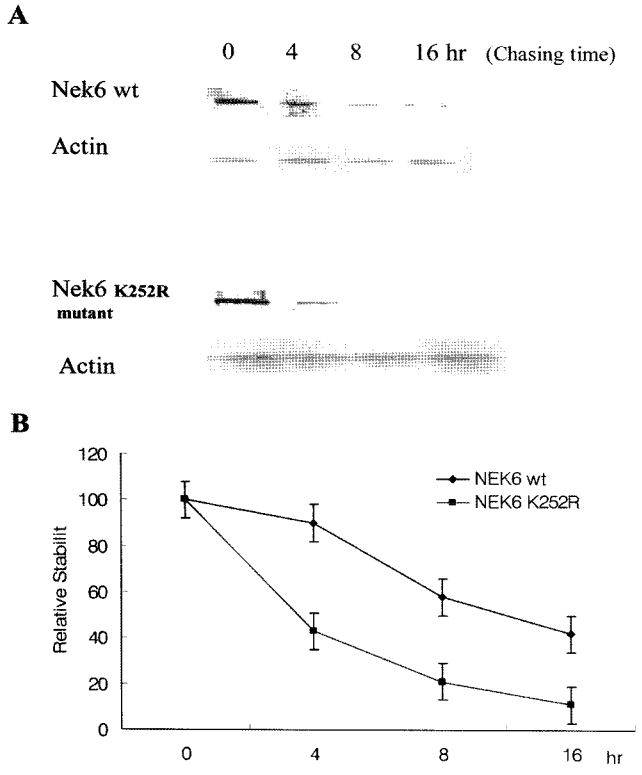


**Fig. 5.** Protein stability of Nek6 wt and its SUMO mutant. EGFP Nek6 and Nek6 SUMO mutant were transfected into COS-1 cells and the cells were treated with cyclohexamide. The Nek6 proteins were chased for the indicated time periods. EGFP-Nek6 proteins were immunoprecipitated with a polyclonal anti-GFP antibody and subjected to SDS-PAGE followed by western blot with a monoclonal Nek6 antibody (A). To monitor the protein amount, the equal amount of cell lysate was subjected to western blot against actin antibody. These results are one of five repeated experiments. The quantification of the pulse-chase experiment by image analysis is shown in (B).

antibody or phospho-threonine-proline mouse monoclonal antibody. Next, because $S^{422}$ of human SGK1 is phosphorylated by Nek6 *in vitro*, we measured the kinase activity with its substrate protein, the inactive SGK1 (Belham et al., 2001; Lizcana et al., 2002). The phospho-(Ser/Thr) PDK1 docking motif (18A2) mouse monoclonal antibody was used to detect the phosphorylation of human SGK1 $S^{422}$ residue. As shown in Fig. 4D, we observed that the kinase activity of the Nek SUMO mutant was reduced to 60% of the Nek6 wt, consistent with its $S^{231}$ and $T^{235}$ phosphorylation results (Fig. 4B and C). Taken together, our data suggest that SUMOylation of Nek6 is required also for its kinase activity, activation, and autophosphorylation. We performed the experiment using NIH-3T3 cells and obtained the same results (data not shown).

### The effect of Nek6 SUMOylation on protein stability

To evaluate the effect of SUMOylation on NEKb protein stability, we performed pulse-chase. Each EGFP-Nek (wt or SUMO mutant) expression vector was transfected into COS-1 cells and immunoprecipitated using EGFP polyclonal antibody following cyclohexamide treatment (Fig. 5A). Nek6 proteins were chased for the indicated time periods (0, 8, 16, and 24 h), and then immunoprecipitated using polyclonal anti-EGFP antibody and subjected to SDS-PAGE followed by western blot with Nek6 antibody. To control for the protein amount, we monitored actin in each sample by western blotting (Fig. 5A). Quantification of the proteins, as determined by image analysis of the dried SDS-PAGE gel, is shown in Fig. 5B. As shown in Fig. 5, the protein stability of the Nek6 wt was twice that of the Nek6 SUMO mutant, suggesting that SUMOylation on $K^{252}$ of Nek6 seems necessary for protein stability.

**Table 1.** Comparison of Nek 6 SUMO mutant cell viability with its wild type

| Nek6 construct | Rate of apoptosis (%) by FACS |
|---|---|
| Nek6 (wt) | 58 +/- 3 |
| Nek6 (K252R) | 13 +/- 4 |
| EGFP (vector only) | 25 +/- 2 |
| | Mean value of 5 repeats |

EGFP-Nek6 wt or its SUMO mutant (K252R) or EGFP vector was transfected, and the rate of apoptosis was measured by FACS. EGFP-Nek6 SUMO mutant was not dominantly localized in the nuclear speckles and was not SUMOylated promoted cell survival significantly compared to Nek6 wt constructs. For details, see the Materials and Methods section.

### The effect of Nek6 SUMOylation on Cell viability

We measured cell viability using FACS analysis to determine whether SUMOylation on $K^{252}$ of Nek6 influenced cell viability. As shown in Table 1, the results indicate that the Nek6 SUMO mutant (K252R) significantly increased the cell survival rate compared to the EGFP-Nek6 wt or EGFP vector alone. Thus, the Nek6 SUMO mutant was less effective on apoptosis than the EGFP-Nek6 wt or EGFP vector alone (Table 1).

In summary, our results indicate that SUMOylation on $K^{252}$ of Nek6 regulates its protein stability, kinase activity, nuclear speckle localization, and cell apoptosis. Therefore, our observations suggest that SUMOylation causes functional modification of Nek6 for its regulation.

## DISCUSSION

The results presented here confirm that Nek6 is modified by SUMO-1 (Fig. 1), based on *in vitro* SUMOyaltion assay (Fig. 1B), co-immunoprecipitation assay (Fig. 1C), and confocal microscopic analysis (Fig. 1D). Although the molecular mechanism underlying Nek6 SUMOylation and its regulation remains unknown, our study is the first to demonstrate that SUMOylation occurs on Nek6 as a novel post-transnational step for regulation of its function. Based on the SUMOylation consensus information (Fig. 1A), we

used site directed mutagenesis to show that the $K^{252}$ residue in $^{251}$F**K**sD$^{254}$ of Nek6 is the SUMOylation site (Fig. 2 and 3). Since Nek6 has a consensus SUMO-1 attachment motif on $K^{252}$, we examined the relationship between $K^{252}$ SUMOylation of Nek6 and its functional regulation. In our effort to understand the functional significance of Nek6 SUMOylation, we tested whether the specific Nek6 SUMOylation affected its roles, such as subcellular localization (Fig. 3), kinase activity (Fig. 4), protein stability (Fig. 5), or cell apoptosis (Table 1). However, further studies are essential to better elucidate the complexity of the molecular mechanism involved in Nek6-mediated cell cycle regulation through SUMOylation. In particular, it will be important to determine exactly when and where Nek6 SUMOylation occurs, how it is regulated and how it contributes to the physiological role of Nek6.

In our experiment, overexpression of the EGFP-Nek6 wt caused cell apoptosis (Table 1). We speculated that overexpression of EGFP-Nek6 induced cell death because it caused disruption of the normal cell cycle. However, we do not currently know how or why the overexpression of the EGFP-Nek6 wt caused cell apoptosis. Interestingly, however, we observed that the EGFP-Nek6 SUMO mutant promoted cell survival but not cell death (Table 1). Thus, our results here suggest that the SUMOyaltion of $K^{252}$ of Nek6 seems to be related with cell survival or apoptosis, and is enough to affect cell survival (Table 1). However, the mechanism by which SUMOylation on $K^{252}$ of Nek6 affects its role for cell survival remains to be elucidated.

Because lysine serves as the attachment site for several modifications, including ubiquitination, acetylation, and methylamine (Melchior, 2002; Gill, 2004; Dohmen, 2004), it seems important that multiple SUMOylation of other lysine residues of Nek6, which is trigged by SUMOylation of the $K^{252}$ residue in $^{251}$F**K**sD$^{254}$ of NEK6, also plays a role by antagonizing other post-translational modifications. However, the relationship between Nek6 SUMOyaltion and its acetylation (or other modification) remains to be characterized.

Until now, four SUMO subtypes (SUMO-1, 2, 3 and 4) have been identified (Melchior and Hengst, 2002; Zhang et al., 2002; Bohren et al., 2004). The SUMOylation consensus sequences for each subtype are known to be the same as SUMO-1 (Φ**K**xE, where Φ represents L, I, V or F and x is any amino acid) (Duprez et al., 1999; Sampson et al., 2001; Yang et al., 2006). Thus, it seems possible that other SUMO subtypes also modify the Nek6 protein. However, Nek6 modification by SUMO subtypes still requires characterization, and each SUMOylation by a different subtype is designated to control the specific Nek6 function.

Nek6, Nek7 and Nek9 are closely related to each other, and Nek9 was identified because of its association with Nek6 and Nek7 (Belham et al., 2003). Because it has been reported that Nek9 binds Ran GTPase and regulates mitotic progression, Nek9, Nek6 and Nek7 might play a role in mitotic regulation (Minoguchi et al., 2003; Yin et al., 2003). Consistent with their similarities, when we evaluated all 11 human Neks, we noticed the presence of SUMOylation motif (F**K**sD) in only Nek6, Nek7 and Nek9. Thus, SUMOylation seems to occur in Nek7 and Nek9, similar to Nek6. Their SUMOylatuion may play a role in mitotic regulation (Roig et al., 2002; Belham et al., 2003; Yin et al., 2003).

It has been reported that SUMO-1 (but not SUMO-2, 3 and 4) monomerically conjugates the e amino group of the K residue in SUMO-1 acceptor consensus sequences (FKxE) (Sampson et al., 2001; Wilson and Rangasamy, 2001; Gill, 2004; Hietakangas et al., 2006). As shown in the right lane of Fig. 3, the SUMOylation of Nek6 wt was detected as several high molecular weight protein bands. The multiple SUMOylation on several other K residues of Nek6 seemed to occur even though $K^{252}$ is one of the consensus sites in the described SUMO-1 acceptor consensus sequences (FKxE). Interestingly, however, SUMOyaltion of the Nek6 SUMO mutant (K252R) was inhibited (Fig. 3). Thus it seems that the $K^{252}$ is the primary site for SUMOylation (Dorval and Fraser, 2006; Yang et al., 2006). Consequently, SUMOyaltion on $K^{252}$ triggers or stimulates different SUMOylation reactions on several other K residues in Nek6 (multiple SUMOylation), including $K^{212}$ in $^{211}$VKLgD$^{215}$. Even though SUMO forms a homo- or hetero- in the cell, SUMOyaltion by other SUMO isotypes (SUMO-2, 3 and 4) is excluded in the experiment because SUMO-1 and its monoclonal antibody were used in this case. We also noticed that Nek6 contains several K residues (total 7 sites) where multiple SUMOylation by SUMO-1 on Nek6 is possible. However, it is currently unclear why SUMOylation of Nek6 wt occurs as several high molecular weight protein bands *in vitro* after SUMOylation assay using SUMO-1 and its specific antibody.

In conclusion, based on point mutagenesis analysis, our result suggests that SUMOylation by SUMO-1 on Nek6 occurs on $K^{252}$ in $^{251}$F**K**sD$^{254}$, which is located at the end of the kinase domain (Fig. 1). By analyzing the functional consequences of impairing Nek6 SUMOylation, this modification of Nek6 was shown to contribute to several functional changes, such as its protein kinase activity, nuclear localization and protein stability, subsequently promoting cell death.

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