Erratum

NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells

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The author Seongjae Kim was mistakenly listed as Sungjae Kim. The correct author list is as given above.

We apologise for this mistake.

NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells

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Summary

The centrosomes in dividing cells follow a series of cyclical events of duplication and separation, which are tightly linked to the cell cycle. Serine/threonine-protein kinase NEK7 (NEK7) is a centrosomal kinase that is required for proper spindle formation during mitosis. In this study, we observed that centriole duplication was inhibited in NEK7-depleted cells. Ectopic expression of centrosome-directed NEK7 led to the formation of extra centrioles in a kinase-activity-dependent manner. We also observed extra centriole formation in centrosome-directed NEK6-expressing cells, suggesting that NEK6 and NEK7 might share biological activities that induce centriole duplication. The centrosomal pericentriolar material (PCM) proteins were significantly reduced in NEK7-depleted cells. The PCM proteins in NEK7-depleted cells did not accumulate at the centrosomes, even if the cells exited mitosis and progressed to the G2 phase. These results revealed that NEK7 is essential for PCM accumulation in a cell cycle stage-specific manner. Furthermore, HeLa cells depleted of NEK7 during S phase retained a higher quantity of PCM proteins and exhibited a less severe mitotic phenotype. On the basis of these results, we propose that NEK7 is involved in the recruitment of PCM proteins, which are necessary for both centriole duplication and spindle pole formation. Our study revealed that NEK7 activity is required for centrosome cycle progression not only at M phase, but also at G1 phase.

Key words: NEK7, Cell cycle, Centriole duplication, Centrosome, Pericentriolar material

Introduction

The centrosome is a major microtubule-organizing center that controls cell morphology, motility and intracellular transport. Each centrosome is composed of a pair of centrioles surrounded by amorphous pericentriolar material (PCM). The centriole pair should be duplicated once per cell cycle for chromosome segregation into two daughter cells to occur during mitosis. Centriole duplication is licensed at the end of mitosis when the two centrioles of a pair are physically freed from each other (Tsou and Stearns, 2006). A procentriole is then generated next to the mother centriole at approximately the time when DNA replication occurs in the nucleus. Two pairs of centrioles are surrounded by PCM, and they eventually separate to form two mitotic spindle poles. An abnormality in centriole duplication can cause defects in bipolar spindle formation, which leads to aneuploidy (Nigg, 2006; Ganem et al., 2009). Therefore, centriole duplication must be precisely controlled.

Genetic and genomic studies conducted in *Caenorhabditis elegans* have identified a group of genes that are involved in centriole assembly. SPD-2 functions at an initial stage in the recruitment of centrosomal components, such as ZYG-1 and SPD-5 (Kemp et al., 2004; Pelletier et al., 2004). ZYG-1 is a kinase that is required for the formation of the central tube with SAS-5 and SAS-6 (Dammermann et al., 2004; Delattre and Gönczy, 2004; Leidel et al., 2005). SAS-4 then plays a role in tethering singlet microtubules to the central tube (Pelletier et al., 2006). SPD-5 is required for the accumulation of PCM during centrosome maturation (Pelletier et al., 2004).

The regulatory mechanisms involved in the centrosome cycle are fundamentally conserved among animal cells (Bettencourt-Dias and Glover, 2007; Nigg and Raff, 2009). For example, CEP192, SAS-6 and CPAP have been identified as the mammalian homologues of SPD-2, SAS-6 and SAS-4, respectively, and are required for human centrosome duplication (Zhu et al., 2008; Strnad et al., 2007; Tang et al., 2009). Additional centriolar and PCM proteins are also required for successful duplication of human centrosomes. Depletion of centriolar components, such as CP110 and centrobin, represses centriole duplication (Chen et al., 2002; Zou et al., 2005). PCM proteins, including NEDD1, γ-tubulin, CG-NAP and pericentrin, are also required for centriole duplication (Haren et al., 2006; Zhu et al., 2008; Keryer et al., 2003; Loncarek et al., 2008). In addition to structural centrosome components, protein kinases are known to be essential for centriole duplication. PLK4, which is known as a functional homologue of ZYG-1, is involved in the initiation of centriole duplication (Habedanck et al., 2005). CDK2 regulates centriole duplication by phosphorylating nucleophosmin, CP110 and MPS1 (Okuda et al., 2000; Chen et al., 2002; Kasbek et al., 2007). PLK2, which is also involved in centriole duplication, phosphorylates CPAP (Chang et al., 2010).

NEK7 is a nimA-related kinase that is crucial for mitotic spindle formation (Yissachar et al., 2006; Kim et al., 2007; O'Regan and Fry, 2009). A recent study using knockout mice

reported the importance of NEK7 during a number of steps of mitosis and cytokinesis (Salem et al., 2010). NEK7 is located at the centrosome, as well as in the cytoplasm (Kim et al., 2007; O'Regan and Fry, 2009). The kinase activity of NEK7, which is controlled by NEK9 phosphorylation, oscillates during the cell cycle, reaching a maximum at M phase (Belham et al., 2003; Richards et al., 2009). To expand our understanding of NEK7 function during the cell cycle, we chose to investigate NEK7 functions in interphase cells. Here, we report that NEK7 is essential for centriole duplication and PCM accumulation during interphase.

Results

Centriole duplication is inhibited in NEK7-depleted cells

It has been reported that NEK7 depletion results in prometaphase arrest with defects in mitotic spindles (Yissachar et al., 2006; Kim et al., 2007; O'Regan and Fry, 2009). In addition to mitotic phenotypes, we have also observed that NEK7 depletion reduces centrosomal γ-tubulin levels in interphase cells (Kim et al., 2007). This led us to investigate the biological functions of NEK7 in interphase centrosomes. We performed immunocytochemistry to confirm the centrosomal localization of NEK7 in U2OS interphase cells (Kim et al., 2007) (supplementary material Fig. S1). The NEK7 signal was also observed in the cytoplasm (supplementary material Fig. S1). The centrosomal and cytoplasmic staining disappeared in NEK7-depleted cells, indicating that NEK7 was located in the cytoplasm, as well as at the centrosome of interphase cells (supplementary material Fig. S1).

We carefully observed the centrosomal phenotypes in NEK7-depleted HeLa cells. NEK7 was depleted by short interfering RNA (siRNA) transfection, and PLK4 was also depleted to allow direct comparison with a kinase involved in centrosome duplication. The cell cycle was arrested at S phase using hydroxyurea to avoid mitotic arrest caused by NEK7 depletion (Fig. 1A). Depletion of NEK7 and PLK4 was confirmed by immunoblot analysis (Fig. 1B). Most of the control cells had four centrioles as a result of centriole duplication during the G1–S transition phase (Fig. 1C,D). By contrast, most of the NEK7- and PLK4-depleted cells had one or two centrioles, which were visualized with the CP110 antibody (Fig. 1C,D). These results indicate that NEK7 is essential for centriole duplication. It is of note that the NEK7-depleted cells had 2 centrosomes, each of which contained a single centriole (Fig. 1C,E). This suggests that NEK7 activity is also required for centriolar linkage, which should be maintained until G2–M phase. In the case of the PLK4-depleted cells, a pair of centrioles was located within a single centrosome in interphase cells, and half of the centrioles were eventually segregated into each spindle pole in mitotic cells (Fig. 1C,E; supplementary material Fig. S2) (Habedanck et al., 2005).

The centrosome is known to be overduplicated in certain cell lines, such as CHO and U2OS cells, when the cell cycle is arrested at S phase using hydroxyurea (Hinchcliffe and Sluder, 2001). However, centrosome overduplication was not induced by hydroxyurea in the NEK7-depleted U2OS cells (Fig. 2B,C). As reported previously, the centrosomes in PLK4-depleted cells were also not overduplicated (Fig. 2B,C) (Habedanck et al., 2005). To rule out a possible off-target effect from the siRNA

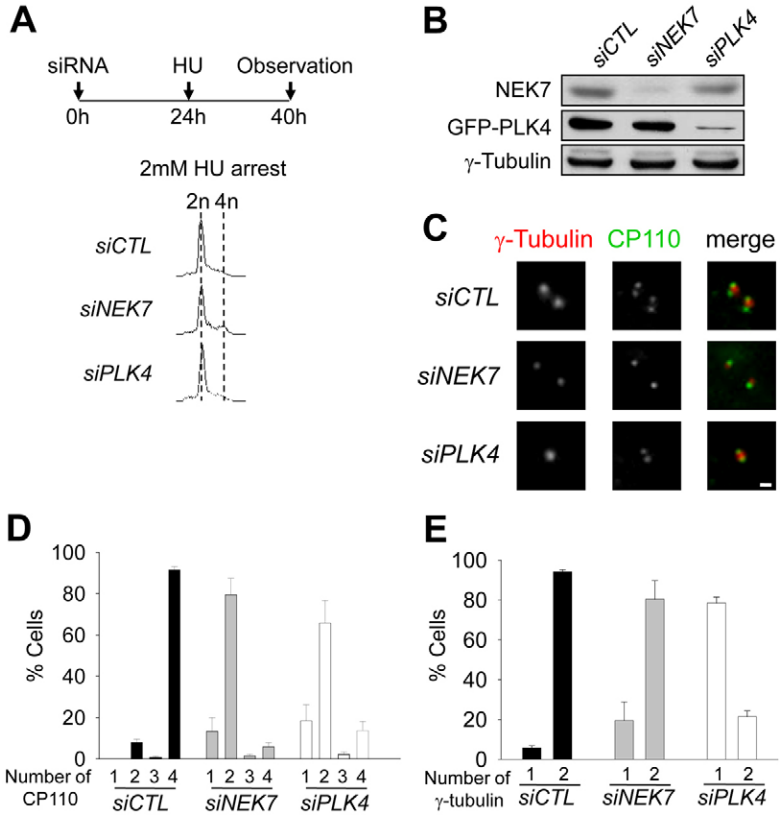

Fig. 1. Centriole duplication was inhibited in NEK7-depleted cells. (**A**) Experimental procedure. HeLa cells were transfected with a non-specific siRNA (*siCTL*) or siRNAs specific to *NEK7* (*siNEK7*) or *PLK4* (*siPLK4*). Twenty-four hours later, the cells were treated with 2 mM hydroxyurea (HU) for 16 hours to arrest the cell cycle at S phase. (**B**) The cell lysates were subjected to immunoblot analysis to confirm depletion of the NEK7 and PLK4 proteins. (**C**) The cells were immunostained with antibodies specific to γ-tubulin (red) and CP110 (green). Insets show enlarged views of the centrosomes. Scale bar: 1 µm. (**D**,**E**) The number of CP110 (D) and γ-tubulin (E) signals was counted in the NEK7- and PLK4-depleted cells and statistically analyzed. A total of 50 cells per experimental group was counted in three independent experiments.

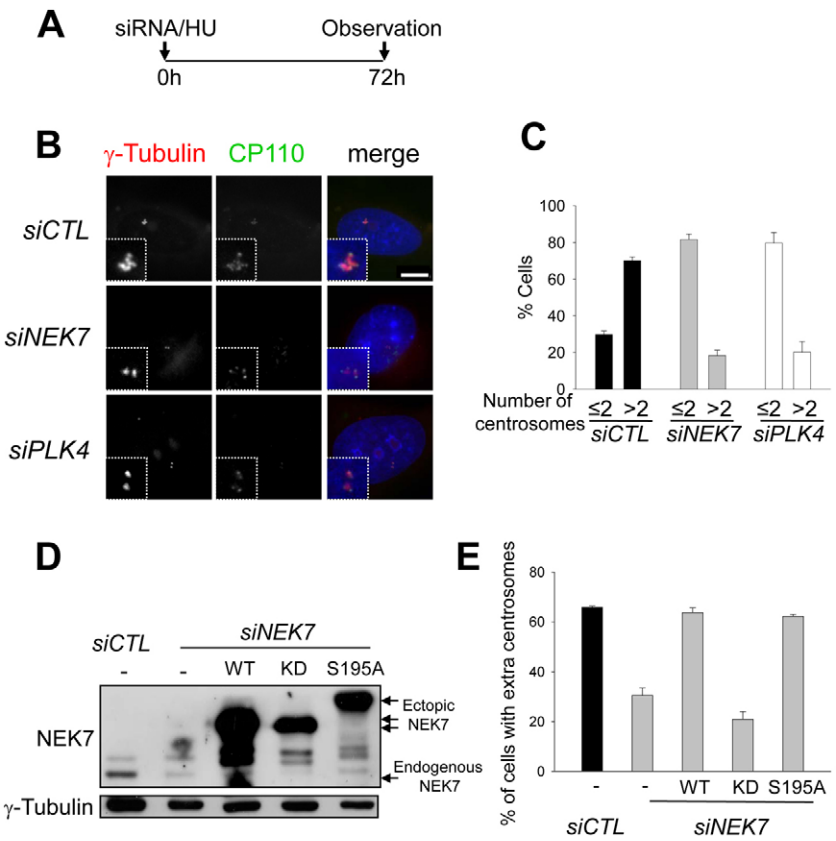

Fig. 2. Centrosome overduplication was inhibited in NEK7-depleted U2OS cells. (**A**) Experimental procedure. U2OS cells were transfected with *siCTL*, *siNEK7* or *siPLK4* and treated with 2 mM hydroxyurea (HU) for 72 hours to induce centrosome overduplication. (**B**) The cells were immunostained with antibodies specific to γ-tubulin (red) and CP110 (green). DNA was stained with DAPI. Scale bar: 10 µm. Insets are magnified views of the centrosomes. (**C**) The number of centrosomes was counted in 50 cells per experimental group, from three independent experiments, and analyzed statistically. (**D**) HeLa cells were transfected with *siNEK7* and subsequently with siRNA-resistant *pFLAG-NEK7*, *pFLAG-NEK7KD* or *pFLAG-NEK7 S195A*. Forty-eight hours later, the cell lysates were subjected to immunoblot analysis with the NEK7 and γ-tubulin antibodies. Ectopic and endogenous NEK7 are indicated with arrows. (**E**) U2OS cells were transfected with *siNEK7* and subsequently with siRNA-resistant *pFLAG-NEK7*, *pFLAG-NEK7KD* or *pFLAG-NEK7 S195A*. The cells were treated with HU for 72 hours, and the number of centrosomes was counted. The number of centrosomes was counted in 150 cells per experimental group, from three independent experiments, and analyzed statistically.

targeted against NEK7, we performed a knockdown-rescue experiment with RNA interference (RNAi)-resistant forms of wild-type and kinase-dead NEK7 constructs (Fig. 2D) (O'Regan and Fry, 2009). As expected, wild-type NEK7 successfully rescued the knockdown phenotypes, whereas kinase-dead NEK7 (NEK7KD) did not (Fig. 2E). These results indicate that NEK7 activity is required for centriole overduplication in S-phase-arrested U2OS cells. The kinase activity of NEK7 has been reported to be controlled by NEK9 phosphorylation at serine 195 of NEK7 (Belham et al., 2003). Therefore, we tested whether the knockdown phenotype could be rescued by NEK7 S195A, which is resistant to phosphorylation by NEK9. The results showed that NEK7 S195A successfully rescued the centriole overduplication phenotype in the NEK7-depleted U2OS cells, suggesting that NEK9 activation might not be essential for the function of NEK7 in centriole duplication (Fig. 2E).

Formation of extra centrosomes due to ectopic expression of centrosome-directed NEK7 proteins

To confirm the involvement of NEK7 in centriole duplication, we ectopically expressed a NEK7 protein coupled with the PACT (for pericentrin–AKAP–450 centrosomal-targeting) domain, which is the centrosome localization signal of pericentrin B (Gillingham and Munro, 2000). After transfection, the HeLa cells were synchronized at G2 phase with a thymidine block and release, then immunostained with antibodies specific to FLAG and γ-tubulin (Fig. 3A). FLAG–NEK7–PACT was concentrated at the centrosome and concurrently induced extra centrosome formation (Fig. 3B). By contrast, FLAG–NEK7KD–PACT was properly located at the centrosome but did not generate extra centrosomes (Fig. 3B).

We immunostained the FLAG–NEK7–PACT-expressing cells with the centrin-2 antibody, a centriole marker. The results showed two centrin-2 dots at the pre-existing mother and daughter centrioles of the parent centrosome (Fig. 3C, white square). Additionally, overduplicated centrosomes with a single centrin-2 dot were detected in over 30% of the cells (Fig. 3C, green square). We also immunostained the FLAG–NEK7–PACT-expressing cells with an antibody to centrobin, which is a daughter centriole marker. A single centrobin dot was detected in the overduplicated centrosomes, as well as in parent centrosomes (Fig. 3C). These results indicate that all of the extra centrosomes in the FLAG–NEK7–PACT-expressing cells are composed of single daughter centrioles.

To determine whether the centriole overduplication phenotype is specific to NEK7, we generated a number of constructs with NEK kinases and introduced them into HeLa cells. After transfection, the cells were synchronized at S phase with hydroxyurea and immunostained with antibodies specific to FLAG and CP110 (Fig. 3D). We observed overduplicated centrioles not only in cells with FLAG–NEK7–PACT but also in cells with FLAG–NEK6–PACT (Fig. 3D). By contrast, FLAG–NEK2–PACT did not induce any centriole overduplication (Fig. 3D). This suggests that NEK6 and NEK7 might share biological activities that induce centriole duplication. We also observed that centriole overduplication occurred only when the PACT domain was linked to the NEK fusion proteins, indicating the importance of centrosomal localization of NEK6 and NEK7 for centriole duplication (Fig. 3D). The involvement of NEK6–PACT and NEK7–PACT in centriole overduplication was confirmed by immunostaining using CPAP and centrin-2 antibodies (Fig. 3E).

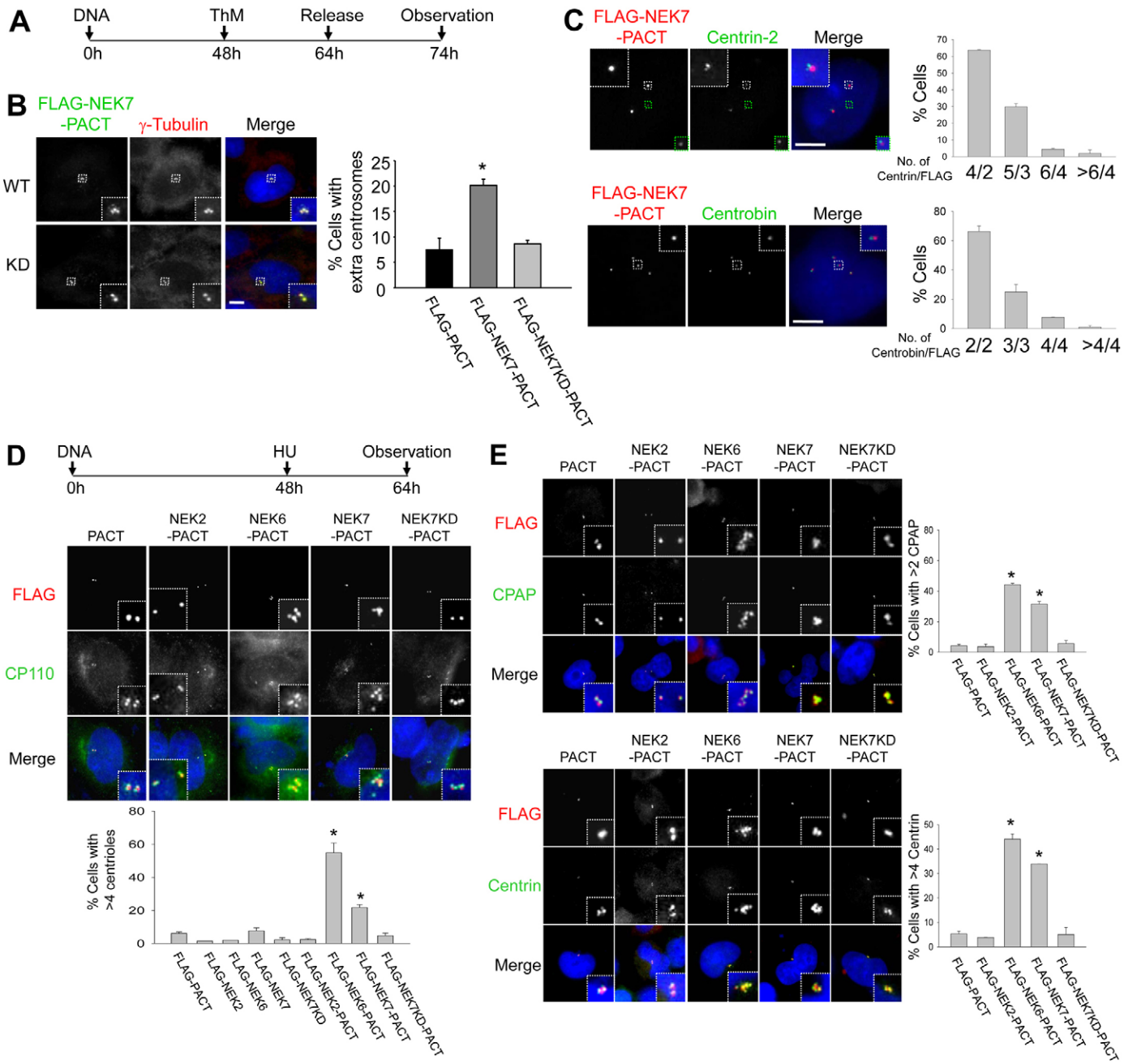

Fig. 3. Centrosome-directed NEK7 induced extra centrosomes. (**A**) Experimental procedure. HeLa cells were transfected with *pFLAG-NEK7-PACT* or *pFLAG-NEK7KD-PACT* and synchronized at G2 phase with a single thymidine block and release. (**B**) FLAG–NEK7–PACT- and FLAG–NEK7KD–PACT-expressing cells were immunostained with FLAG (green) and γ-tubulin (red) antibodies. DNA was stained with DAPI. The boxed regions (the centrosomes) are magnified in the insets. The number of cells with extra centrosomes was counted. The number of centrosomes was counted in 40 cells per experimental group, from three independent experiments, and analyzed statistically. *$P<0.01$ in comparison with the control group. (**C**) FLAG–NEK7–PACT-expressing cells were immunostained with antibodies specific to centrin-2 and centrobin (green), along with FLAG antibody (red). The boxed regions (the centrosomes) are magnified in the insets: the regions within the white dotted lines have two centrin-2 signals per FLAG signal, whereas those within the green dotted line have a single centrin-2 signal per FLAG signal. Scale bars: 10 µm. The number of dots per a cell for the indicated antibodies was counted and is shown in the graphs. At least 50 cells per experimental group, from two independent experiments, were analyzed statistically. (**D**) HeLa cells were transfected with the indicated *NEK* constructs. Forty-eight hours later, the cells were treated with hydroxyurea (HU) to arrest the cell cycle at S phase. The cells were co-immunostained with antibodies specific to the FLAG tag (red) and CP110 (green). DNA was stained with DAPI. The insets are magnified views of the centrosomes. The number of cells with extra centrioles was counted. At least 50 cells per experimental group, from three independent experiments, were analyzed statistically. *$P<0.01$ in comparison with the control group. (**E**) HeLa cells were transfected with the indicated *NEK-PACT* constructs, cultured as in D, and co-immunostained with antibodies specific to CPAP and centrin-2 (red), along with FLAG antibody (green). The number of cells with extra centrioles was counted. At least 50 cells per experimental group, from two independent experiments, were analyzed statistically. *$P<0.01$ in comparison with the control group.

Reduction of PCM in NEK7-depleted centrosomes

We previously reported that centrosomal γ-tubulin levels are reduced in both interphase and mitotic phase cells following NEK7 depletion (Kim et al., 2007). Suppression of γ-tubulin is known to cause a defect in centriole duplication (Haren et al., 2006). Therefore, we hypothesized that NEK7 might be involved in centriole duplication through regulation of PCM accumulation in interphase centrosomes. To test this hypothesis, we examined the constituents of centrosomes in the NEK7-depleted cells. To avoid cell cycle stage-dependent fluctuations of centrosomal proteins, we arrested the cells at S phase with hydroxyurea and carried out immunocytochemistry experiments with selected antibodies specific to centrosomal proteins (Fig. 4A). Centriolar proteins, including CEP135, centrobin and CP110, were present as distinct dots at the centrosomes (Fig. 4B). As shown previously, the number of CP110 and centrobin dots was reduced in the NEK7- and PLK4-depleted cells, but the fluorescence intensity of the individual dots was not reduced to any extent (Fig. 4B). The immunostaining patterns of PCM proteins, such as γ-tubulin, pericentrin, CG-NAP, CEP215 and NEDD1, appeared as larger dots (Fig. 4C). The fluorescence intensities of the centrosomal PCM proteins were reduced significantly in the NEK7-depleted cells, whereas no significant reduction was observed in the PLK4-depleted cells (Fig. 4C). These results revealed that, unlike PLK4 depletion, NEK7 depletion resulted in a reduction in PCM protein levels.

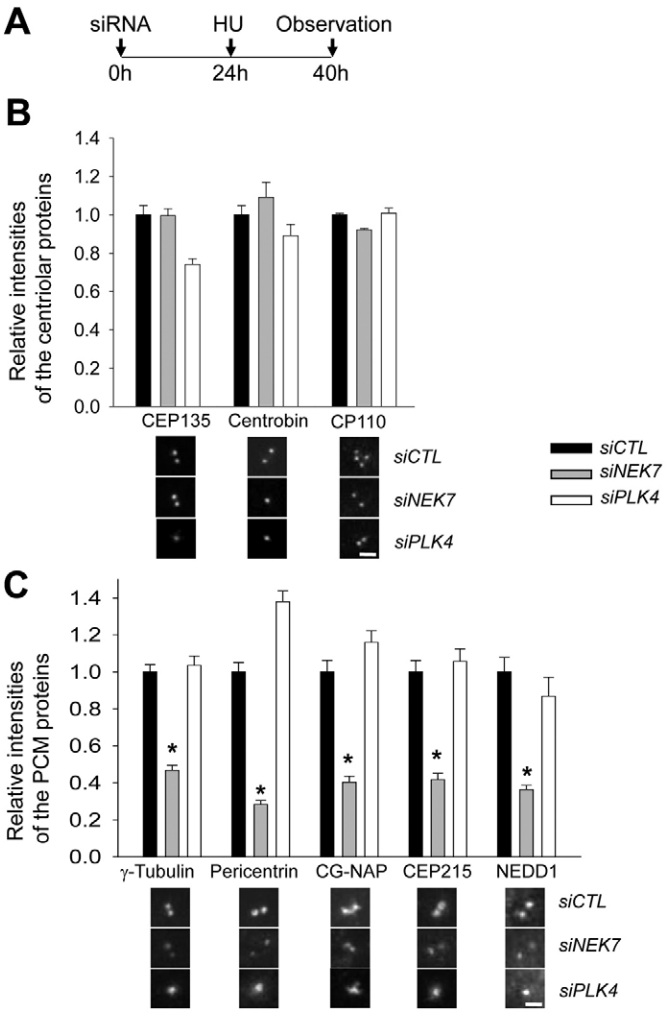

Fig. 4. Accumulation of centrosomal proteins in NEK7-depleted cells. (**A**) Experimental procedure. HeLa cells were transfected with *siCTL*, *siNEK7* or *siPLK4*. Twenty-four hours later, the cells were treated with 2 mM hydroxyurea (HU) for an extra 16 hours to arrest the cell cycle at S phase. (**B**,**C**) The cells were immunostained with antibodies specific to centrioles (CEP135, centrobin and CP110; B) and PCM (γ-tubulin, pericentrin, CG-NAP, CEP215 and NEDD1; C) proteins. Representative images of the centrosomes are shown. Scale bars: 1 μm. The relative fluorescence intensities of the centrosomal proteins were determined densitometrically from 30 cells per experimental group and analyzed statistically. $*P<0.01$ in comparison with controls.

To confirm that NEK7 is required for PCM accumulation, we carried out knockdown-rescue experiments. Immunoblot analysis revealed that siRNA-resistant FLAG–NEK7 and FLAG–NEK7KD were effectively expressed in the NEK7-depleted cells (Fig. 5A). Furthermore, FLAG–NEK7 rescued the level of centrosomal γ-tubulin significantly, whereas FLAG–NEK7KD did not (Fig. 5B,C). These results indicate that the kinase activity of NEK7 is required for PCM accumulation at the centrosome.

To confirm the requirement of NEK7 for PCM accumulation at interphase centrosomes, we determined the centrosomal γ-tubulin levels in NEK7-overexpressing cells. Ectopic expression of FLAG–NEK7 increased centrosomal γ-tubulin levels (Fig. 5D). In FLAG–NEK7–PACT-expressing cells, an increase in centrosomal γ-tubulin levels was detected only when extra centrosomes were formed (Fig. 5D). Furthermore, the centrosomal γ-tubulin levels were significantly reduced in the FLAG–NEK7KD–PACT-expressing cells (Fig. 5D). These results are consistent with the hypothesis that centrosomal NEK7 is essential for PCM accumulation. We also examined NEK6, which induces extra centriole formation when overexpressed. The centrosomal γ-tubulin levels in the FLAG–NEK6–PACT-expressing cells were lower than those in the control cells, suggesting that NEK6 might induce centriole duplication in a different way to NEK7 (Fig. 5D).

The importance of centrosomal γ-tubulin levels in centriole duplication was examined by counting the number of centrioles in the FLAG–NEK7KD–PACT-expressing cells. The number of cells with two centrioles increased in the cell population expressing FLAG–NEK7KD–PACT (Fig. 5E). This suggests that kinase-dead NEK7 might function in a dominant-negative manner in centriole duplication, as well as in bipolar spindle formation (O'Regan and Fry, 2009).

NEK7 is essential for G1-phase-specific accumulation of PCM proteins

The centrosomes in dividing cells follow a series of cyclic events of duplication and maturation that are tightly linked to the cell cycle. Interphase cells initiate PCM accumulation to provide a proper environment for centriole duplication and, eventually, for the robust microtubule-organizing activity of the spindle pole. Therefore, we decided to observe the effect of NEK7 depletion on PCM accumulation during cell cycle progression. First, we had to define the experimental conditions under which NEK7-depleted cells were able to proceed to M phase and then advance to G1 phase. To overcome M phase arrest, we controlled the siRNA transfection period so that cellular NEK7 levels were sufficiently maintained to allow progression through M phase and were then fully depleted at G1 phase. Most of the *siNEK7*-transfected cells were arrested at M phase if the transfection

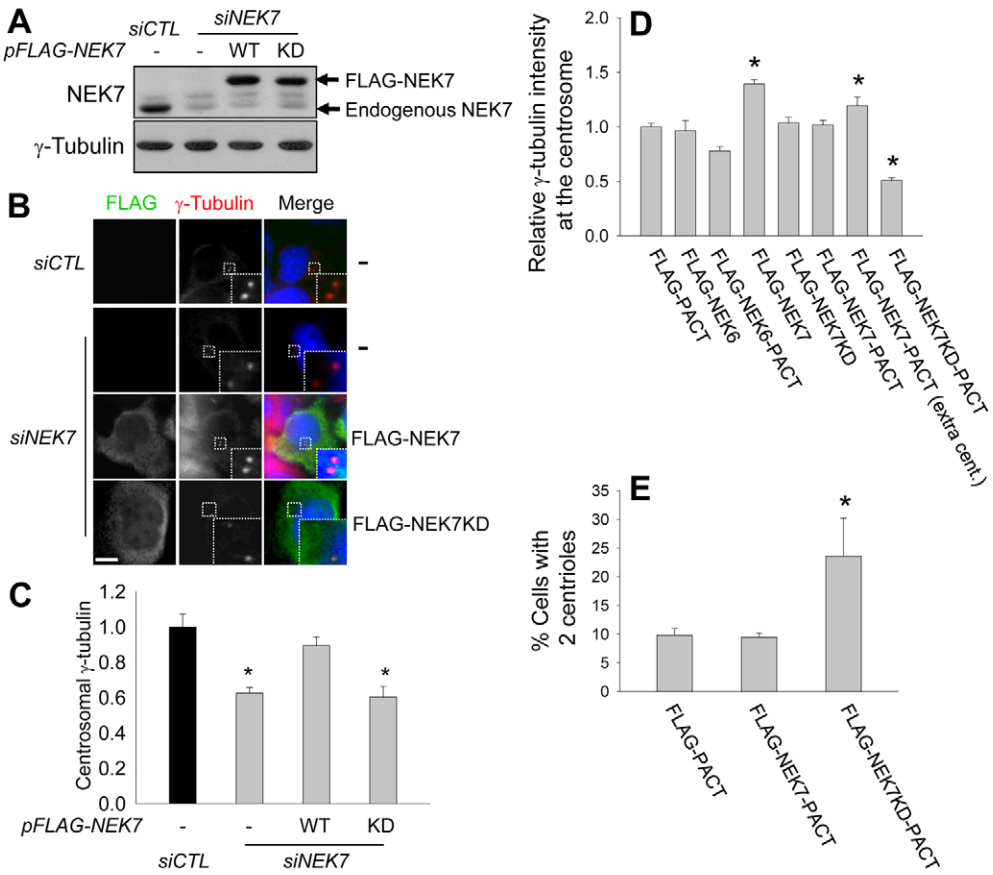

Fig. 5. The kinase activity of NEK7 was required for centrosomal accumulation of γ-tubulin. (**A**) HeLa cells were transfected with *siNEK7* and subsequently with siRNA-resistant *pFLAG-NEK7* or *pFLAG-NEK7KD*. Forty-eight hours after transfection, the cell lysates were subjected to immunoblot analysis with NEK7 antibody. γ-Tubulin was detected as a loading control. (**B**) The cells were fixed and co-immunostained with FLAG (green) and γ-tubulin (red) antibodies. DNA was stained with DAPI. Scale bar: 10 µm. The boxed regions (the centrosomes) are magnified in the insets. (**C**) The staining intensities of centrosomal γ-tubulin were measured densitometrically and analyzed statistically using 20 cells for each experimental group. $*P<0.01$ in comparison with the control group. (**D**) The indicated NEK6 and NEK7 constructs were transfected into HeLa cells. Forty-eight hours after transfection, the cells were treated with hydroxyurea to arrest the cells at S phase, and they were then cultured for 16 hours. The cells were co-immunostained with FLAG and γ-tubulin antibodies. The intensity of centrosomal γ-tubulin staining was measured in approximately 40 cells for each experimental group. In the case of the FLAG–NEK7–PACT group, the centrosomal γ-tubulin intensities were differentially determined in cells with and without extra centrosomes. $*P<0.05$ in comparison with the control group. (**E**) HeLa cells were transfected with *pFLAG-PACT*, *pFLAG-NEK7-PACT* or *pFLAG-NEK7KD-PACT*. Forty-eight hours later, the cells were treated with HU for 16 hours. The cells were co-immunostained with FLAG and CP110 antibodies. The centriole numbers were counted and analyzed statistically. The experiments were repeated twice. Approximately 50 cells were counted in each experimental group. $*P<0.01$ in comparison with the control group.

period was longer than 37 hours (supplementary material Fig. S3A). However, the *siNEK7*-transfected cells were able to progress to M phase if the transfection period was less than 32 hours (supplementary material Fig. S3A). It is probable that cellular NEK7 levels have to be above a crucial level to progress to M phase within the 32-hour culture period. The results of our FACS and immunoblot analyses support this interpretation (supplementary material Fig. S3B,C). Therefore, we adapted the M phase progression scheme to determine G1-phase-specific accumulation of γ-tubulin and pericentrin in NEK7-depleted cells. Immediately after HeLa cells were transfected with *siNEK7*, the cells were synchronized using a thymidine–nocodazole block (Fig. 6A). As a result, the NEK7-depleted cells were able to synchronously progress to G1–S phase, as determined by FACS analysis (Fig. 6B). Cyclin A expression started 11 hours after release from nocodazole and reached a maximum 17 hours later (Fig. 6C). Furthermore, the rate of BrdU incorporation reached a maximum at 11 hours after release in both control and NEK7-depleted cells (Fig. 6D). These results indicate that the NEK7-depleted cells progress to G1–S phase without a significant delay, as occurs in control cells. However, we cannot rule out the possibility that NEK7-depleted cells might progress to G1–S phase with a slight delay compared with control cells, because the rate of BrdU incorporation of the NEK7-depleted group was 15% lower than that of the control group.

When the cells exited mitosis and entered G1 phase, γ-tubulin and pericentrin began to accumulate at the centrosomes (Fig. 6E). The centrosomal levels of the PCM proteins in the NEK7-depleted cells were almost identical to those of the control cells at the beginning of G1 phase (Fig. 6E). However, centrosomal accumulation of the PCM proteins was hampered in the NEK7-depleted cells when the cell cycle approached S phase (Fig. 6E). We also determined the centrosomal γ-tubulin and pericentrin levels after S phase and observed a significant reduction of both

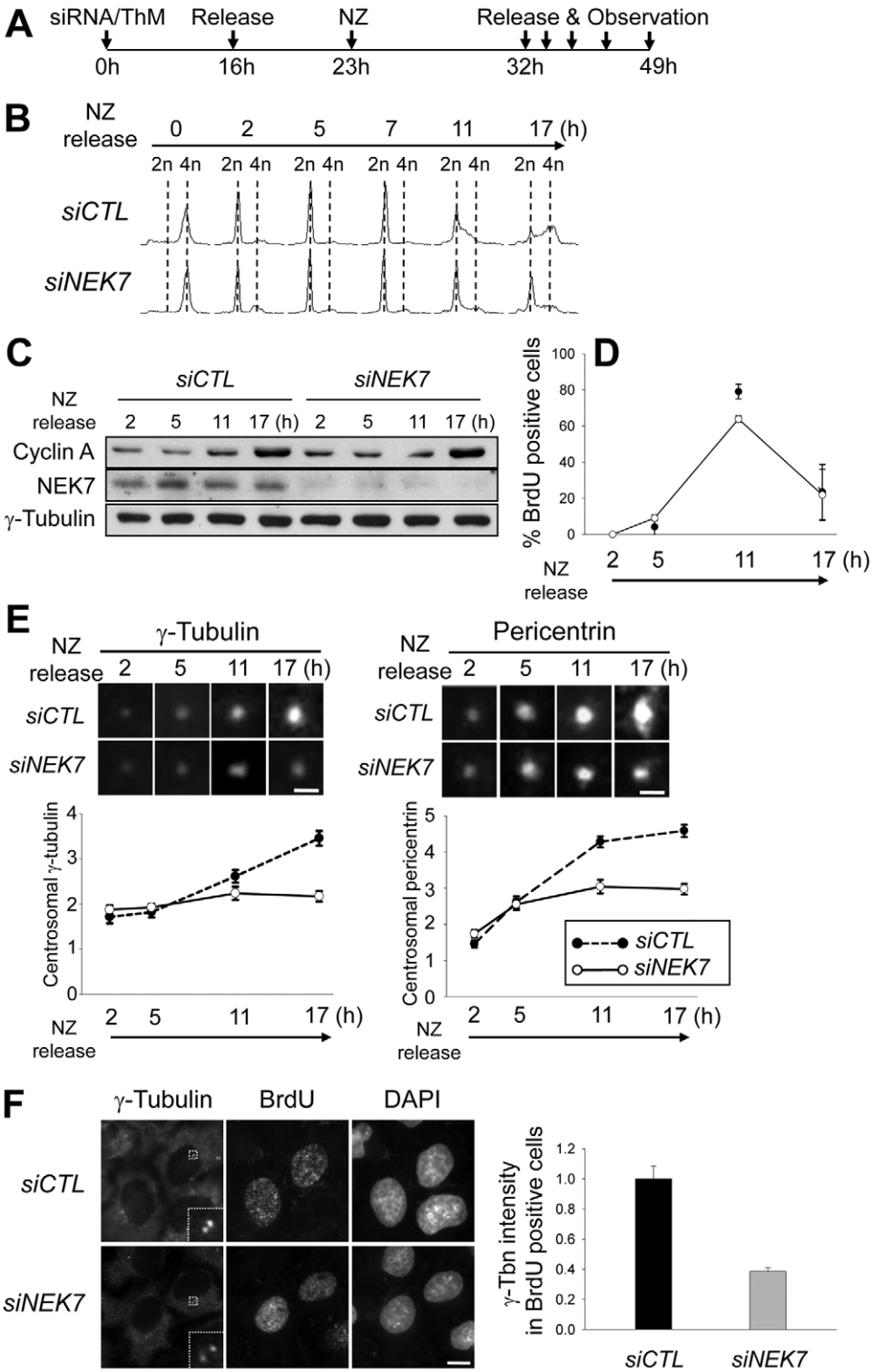

Fig. 6. G1–S-specific accumulation of PCM proteins was hampered in NEK7-depleted cells. (**A**) Experimental procedure. HeLa cells were transfected with *siCTL* or *siNEK7* and immediately treated with thymidine (ThM) and subsequently with nocodazole (NZ) to synchronize the cell cycle at M phase. The cell cycle was then synchronously released into G1 phase from the thymidine–nocodazole block. At the indicated time points, the cells were fixed and analyzed. (**B**) Cell cycle progression was confirmed by FACS analysis. (**C**) At the indicated time points, the levels of NEK7 and cyclin A were determined by immunoblot analysis. γ-Tubulin was used as a loading control. (**D**) At the indicated time points, BrdU was added to the medium, and the cells were cultured for 15 minutes. The cells were then fixed and immunostained with γ-tubulin and BrdU antibodies. The BrdU-positive cells were counted and analyzed statistically. At least 50 cells per experimental group were analyzed in three independent experiments. (**E**) Images of γ-tubulin and pericentrin at the centrosome. Scale bars: 1 µm. The centrosomal levels of γ-tubulin and pericentrin were determined by immunocytochemistry. The intensities of the immunostaining signals were measured densitometrically in 20 cells for each experimental group. Values are arbitrary units. (**F**) The intensity of the centrosomal γ-tubulin staining was determined in the BrdU-positive cells 11 hours after release from nocodazole arrest. Insets are magnified views of the centrosomes. Scale bar: 10 µm. Approximately 30 centrosomes were analyzed for each experimental group. The graph gives the relative γ-tubulin intensity in BrdU-positive cells.

proteins in NEK7-depleted cells (supplementary material Fig. S4). These results suggest that NEK7 is required for centrosomal accumulation of the PCM proteins, beginning from late G1 phase. To confirm that such differences did not originate from a delay in cell cycle progression, we determined the centrosomal γ-tubulin levels in BrdU-positive cells. As shown in Fig. 6D, the rates of BrdU incorporation in both the control and NEK7-depleted cells reached maximal levels at 11 hours after release from nocodazole. At this time point, the centrosomal γ-tubulin levels in the NEK7-depleted cells were significantly lower than those in the control cells, even if both control and NEK7-depleted cells entered S phase (Fig. 6F). This confirms that the reduction in PCM protein levels was due to NEK7 depletion, rather than to a delay in cell cycle progression.

The importance of NEK7-dependent PCM accumulation for proper bipolar spindle formation

Having determined that NEK7-dependent accumulation of PCM proteins occurred during late G1 phase, we subsequently examined the importance of NEK7 in later stages of interphase. Immediately after *siNEK7* transfection, HeLa cells were treated with hydroxyurea for 48 hours to arrest the cell cycle at S phase (Fig. 7A). When NEK7 was depleted while cells were arrested at S phase, the centrosomal levels of γ-tubulin and pericentrin were not reduced to any extent (Fig. 7B,C). These results suggest that NEK7 is important for PCM protein accumulation at G1 phase, but not in later phases of the cell cycle.

Additionally, we examined the mitotic spindle formation of cells in which NEK7 was depleted at S phase (Fig. 8A). As expected, the centrosomal γ-tubulin levels were not reduced in these cells (supplementary material Fig. S5). Nevertheless, defects in mitotic spindle formation were still observed (Fig. 8B,C). The severity of the mitotic phenotypes was reduced, so that fewer monopolar spindles and more abnormal bipolar spindles were observed in the cells depleted of NEK7 during S phase (Fig. 8B,C). These results suggest that the centrosome maturation defects in the NEK7-depleted cells affected proper bipolar spindle formation to some degree.

Discussion

No interphase-specific defects have been reported for either NEK7-depleted cells or *Nek7*-deleted mouse embryonic fibroblasts (Yissachar et al., 2006; Kim et al., 2007; O'Regan and Fry, 2009; Salem et al., 2010). In fact, NEK7-depleted cells are arrested at M phase, exhibiting defects in spindle formation and cytokinesis. Therefore, it is unlikely that interphase defects of NEK7-depleted cells will be prominently revealed unless the cell cycle is carefully manipulated. The current study showed that NEK7 activity is required for centrosome cycle progression during interphase. The centrioles were not duplicated in the NEK7-depleted cells, and PCM proteins did not accumulate concomitantly. Overexpression of centrosome-directed NEK7 induced the formation of extra centrioles. On the basis of these results, we propose that NEK7 is involved in the recruitment of PCM proteins, which are necessary for centriole duplication (supplementary material Fig. S6).

The importance of the PCM proteins in centriole duplication has been confirmed in *C. elegans* and higher organisms. The best example of their importance is probably the requirement of SPD-2 and its mammalian homologue, CEP192, for centriole duplication (Dammerman et al., 2004; Zhu et al., 2008). NEDD1 is another example of a PCM protein that is important for the recruitment of γ-tubulin and, eventually, for centriole duplication in mammalian cells (Haren et al., 2006). Recently, SADB kinase was shown to phosphorylate γ-tubulin and to enhance tubulin polymerization within the centriole (Alvarado-Kristensson et al.,

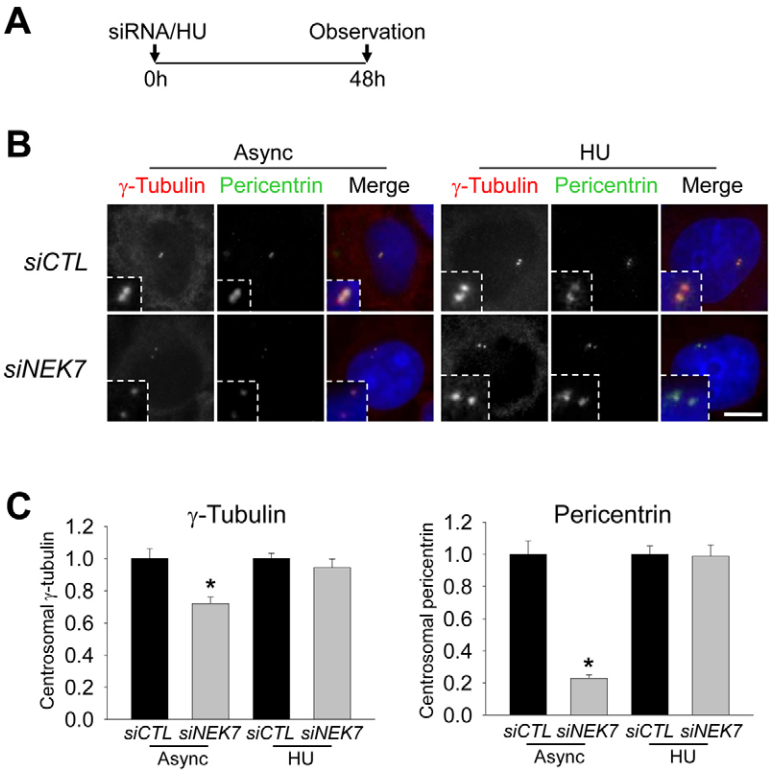

Fig. 7. The centrosomal levels of γ-tubulin and pericentrin were not reduced when NEK7 was depleted at S phase.
(**A**) Experimental procedure. HeLa cells were transfected with *siCTL* or *siNEK7* and simultaneously treated with hydroxyurea (HU) to arrest the cell cycle at S phase. As a control, the transfected cells were cultured without HU. (**B**) Forty-eight hours later, the cells were fixed and co-immunostained with antibodies specific to γ-tubulin (red) and pericentrin (green). DNA was stained with DAPI. Scale bar: 10 μm. Insets are magnified views of the centrosomes. (**C**) The intensities of centrosomal γ-tubulin and pericentrin stainings were measured densitometrically (the left graph shows relative γ-tubulin intensity at the centrosome, and the right graph shows relative pericentrin intensity at the centrosome) and analyzed statistically in 30 cells per experimental group. $*P<0.01$ in comparison with the control group.

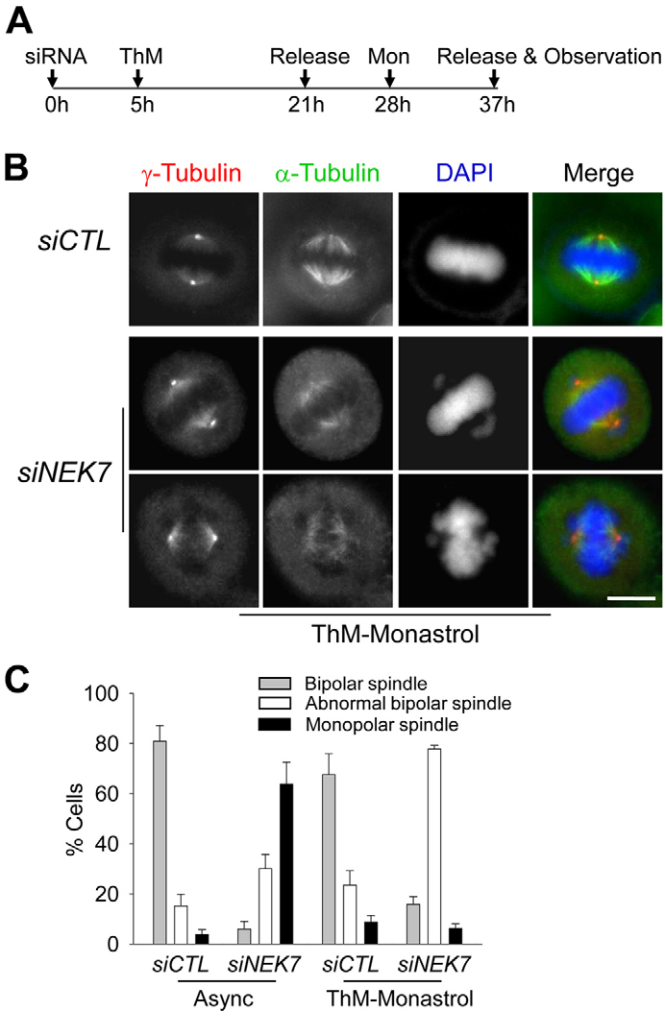

Fig. 8. Effect of NEK7 depletion at S phase on mitotic spindle phenotypes. (**A**) Experimental procedure. HeLa cells were transfected with *siCTL* or *siNEK7*. The cell cycle was synchronized with a thymidine (ThM)–monastrol (Mon) block and synchronously released to metaphase for 1 hour in the presence of MG132. (**B**) The cells were co-immunostained with antibodies specific to γ-tubulin (red) and α-tubulin (green). DNA was stained with DAPI. Scale bar: 10 μm. (**C**) The mitotic spindle pattern was categorized as bipolar, abnormal bipolar or monopolar, and the averages of three independent experiments were analyzed statistically.

2009). It was proposed that these proteins provide an ideal environment for centriole assembly by recruiting essential factors for procentriole formation (Strnad and Gönczy, 2008). Our results showed that several PCM proteins were not sufficiently recruited to the centrosome at G1–S phase in the NEK7-depleted cells. This suggests that the kinase activity of NEK7 is required for the accumulation of PCM at the centrosome prior to centriole duplication. The accumulated PCM would provide an environment in which PLK4 can initiate centriole duplication (supplementary material Fig. S6). In support of this hypothesis, depletion of PLK4 blocked centriole duplication but did not affect PCM accumulation at G1 phase (Fig. 4).

We previously reported that NEK7 depletion results in monopolar spindles with reduced spindle pole activities (Kim et al., 2007). Therefore, it is likely that the reduction of PCM accumulation at G1 phase might be attributed to the mitotic phenotype of the NEK7-depleted cells. Consistent with this interpretation, the centrosomal pericentrin and γ-tubulin levels were not reduced at the mitotic spindle poles of cells in which NEK7 was depleted at S phase. At the same time, the severity of the mitotic defects was significantly alleviated, suggesting that PCM accumulation and the interphase functioning of NEK7 are essential for spindle assembly at M phase. The remaining mitotic defects in the NEK7-depleted cells might result from a lack of essential PCM proteins with relatively short half-lives. We do not rule out the possibility that there is a NEK7-specific substrate for mitotic spindle formation at M phase.

NEK6 exhibits the closest structural homology to NEK7 among the NEK kinases (Quarmby and Mahjoub, 2005). The kinase activities of both NEK6 and NEK7 are induced by NEK9 phosphorylation (Belham et al., 2003). Therefore, it is to be expected that NEK6 and NEK7 might share common biological functions. In fact, depletion of either NEK6 or NEK7 causes defects in mitotic progression (O'Regan and Fry, 2009). In this study, we observed extra centriole formation in both centrosome-directed NEK6- and NEK7-expressing cells, suggesting that both NEK6 and NEK7 are required for centriole duplication. However, their mechanisms of action might be slightly different because NEK6 overexpression did not induce PCM accumulation. Further experiments are necessary to clarify the biological functions of NEK6 and NEK7 in centriole duplication.

We observed a slight delay in G1 phase progression in the NEK7-depleted cells, in addition to the centrosomal phenotypes. In fact, several NEK kinases are known to be essential for cell cycle progression during interphase. For example, NEK11 is involved in DNA damage checkpoints and NEK9 is important for interphase progression in association with FACT, a chromosome structure modulator (Noguchi et al., 2002; Melixetian et al., 2009; Tan and Lee, 2004). NEK7 might be involved in interphase cell cycle progression. Alternatively, the defects in PCM accumulation observed in NEK7-depleted cells might be linked to the delay in G1 phase progression. It is known that centrosomal localization of CDK2–cyclin complexes is an initial step for entry into S phase (Matsumoto et al., 2004; Pascreau et al., 2010). NEK7 depletion might delay centrosomal localization of the CDK2–cyclin complex and, as a result, delay S phase entry.

We propose a novel function of NEK7 in the centrosomal recruitment of PCM proteins that affect centriole duplication in interphase and subsequent spindle assembly in M phase. The next step is to identify the substrates of NEK7 that are responsible for PCM accumulation and centriole duplication.

Materials and Methods

Cell culture, treatment and transfection

HeLa and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and McCoy's 5A medium with 10% fetal bovine serum, respectively. To induce S-phase arrest, the cells were treated with 2 mM hydroxyurea for 16 hours. Centrosome overduplication was induced by treating U2OS cells with 2 mM hydroxyurea for 72 hours. For M phase arrest, the cells were treated with 100 ng/ml nocodazole or 0.1 mM monastrol for 7 hours after a single thymidine block (16 hours) and release (7 hours). Transient transfection into HeLa cells was carried out using LipofectAMINE Plus reagent (Invitrogen). For RNAi suppression, siRNAs specific to *NEK7* (5′-CTC CGA CAG TTA GTT AAT ATT-3′) and *PLK4* (5′-CTA TCT TGG AGC TTT ATA ATT-3′) were transfected into HeLa and U2OS cells with LipofectAMINE RNAi max (Invitrogen) according to the manufacturer's instructions. A non-specific control siRNA (5′-AAG TAG CCG AGC TTC GAT TGC-3′) was also used.

Flow cytometry

The cells were trypsinized, suspended in 300 μl phosphate-buffered saline (PBS) and fixed by adding 700 μl cold ethanol, dropwise. The fixed cells were washed

with 0.1% PBS containing 0.5% Triton X-100 (PBST) and incubated with 100 μg/ml RNase A for 30 minutes. The cells were then incubated with 10 μg/ml propidium iodide and analyzed using FACScalibur (BD Biosciences) and CellQuest Pro software.

Antibodies

NEK7 antiserum was raised against a bacterially expressed His–NEK7 fusion protein. The antibodies were affinity purified using strips of nitrocellulose membranes blotted with the NEK7 antigens and eluted with 100 mM glycine, pH 2.5. Polyclonal antibodies against pericentrin, NEDD1 and centrin-2 were raised against GST–pericentrin (amino acids 1–582), GST–NEDD1 (279–660) and GST–centrin-2 (1–172), respectively. Antibodies against CP110 (Kim et al., 2008), centrobin (Jeong et al., 2007), CEP135 (Kim et al., 2008), CEP215 (Lee and Rhee, 2010) and CPAP (Chang et al., 2010) were used for immunoblot and immunocytochemical analyses. Commercially available antibodies against γ-tubulin (Santa Cruz) and FLAG (Sigma) were also used. CG-NAP antibody was a gift from Y. Ono (Takahashi et al., 1999).

Immunoblot analysis

Immunoblot analysis was carried out as described previously (Yoo et al., 2004). Briefly, cells were lysed with SDS sample buffer [50 mM Tris pH 6.8, 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 0.1% Bromophenol Blue, and 10% glycerol], subjected to electrophoresis on an SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk for 0.5–1 hour and then incubated with a primary antibody. Next, the membrane was washed with 0.3% TBST and incubated with a horseradish peroxidase-conjugated secondary antibody for 30 minutes. After incubation, the blot was washed with 0.3% TBST, incubated with enhanced chemiluminescence (ECL) solution, and exposed to X-ray film.

Immunocytochemistry

Immunocytochemistry experiments were carried out as described previously (Jeong et al., 2007). Briefly, 3.0×10^4 cells were seeded into four-well dishes and transfected the following day. The cells were fixed 48 hours after transfection with cold methanol for 10 minutes, permeabilized, and blocked with 3% bovine serum albumin (BSA) in 0.5% PBST for 20 minutes. The cells were then incubated with a primary antibody for 1 hour, washed with 0.3% PBST three times, and incubated with either fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies for 30 minutes. After the cells were washed with 0.3% PBST, they were incubated with 4′-6-diamidine-2-phenyl indole (DAPI) solution for 5 minutes, washed with 0.3% PBST, and observed using an Olympus IX51 fluorescence microscope equipped with a CCD (Qicam fast 1394, Qimaging) camera. Images were obtained and quantified with ImagePro software (Media Cybernetics, Inc.) and statistically analyzed using SigmaPlot. For co-staining with two polyclonal antibodies, Zenon Alexa Fluor 555 (Z25305, Invitrogen) dye was conjugated to one of the primary antibodies according to the manufacturer's instructions.

Determination of the fluorescence intensities of centrosomal proteins

Determination of the fluorescence intensity of centrosomal proteins was carried out as described previously (Kim et al., 2008). Briefly, the fluorescence intensity was measured and analyzed using ImagePro software (Media Cybernetics, Inc.). The fluorescence intensity of each protein at the centrosome was determined based on the sum of the fluorescence intensity minus the background intensity. For comparison with the control group, the intensity in each cell was normalized by dividing the mean value for the cell with the mean value of the control group.

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