Nek9 Phosphorylation of NEDD1/GCP-WD Contributes to Plk1 Control of γ-Tubulin Recruitment to the Mitotic Centrosome

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Summary

The accumulation of γ-tubulin at the centrosomes during maturation is a key mechanism that ensures the formation of two dense microtubule (MT) asters in cells entering mitosis, defining spindle pole positioning and ensuring the faithful outcome of cell division ([1] and references herein; [2]). Centrosomal γ-tubulin recruitment depends on the adaptor protein NEDD1/GCP-WD [3, 4] and is controlled by the kinase Plk1 [5–8]. Surprisingly, and although Plk1 binds and phosphorylates NEDD1 at multiple sites [9, 10], the mechanism by which this kinase promotes the centrosomal recruitment of γ-tubulin has remained elusive. Using Xenopus egg extracts and mammalian cells, we now show that it involves Nek9, a NIMA-family kinase required for normal mitotic progression and spindle formation [11, 12]. Nek9 phosphorylates NEDD1 on Ser377 driving its recruitment to the centrosome in mitotic cells. This role of Nek9 requires its activation by Plk1-dependent phosphorylation [13] but is independent from the downstream related kinases Nek6 and Nek7 [14]. Our data contribute to understand the mechanism by which Plk1 promotes the recruitment of γ-tubulin to the centrosome in dividing cells and position Nek9 as a key regulator of centrosome maturation.

Results

Nek9 Is Required for Microtubule Nucleation at the Centrosome in Xenopus Mitotic Egg Extract

To understand the role of Nek9 during M-phase, we re-examined the consequences of depleting Nek9 on spindle assembly in Xenopus egg extracts. Confirming previous results [12], xNek9 depletion resulted in a lower percentage of bipolar spindles and an increase of monopolar spindles (see Figure S1A available online). Because Nek9 is activated at centrosomes in G2/M [12], we then examined microtubule (MT) aster formation by sperm nuclei (associated to an immature centrosome) upon short incubation in xNek9 depleted extract (to avoid the influence of the RanGTP gradient generated by the chromatin). xNek9 depletion resulted in a reduced ability of the sperm nuclei to form MT asters, with almost 20% of them totally unable to nucleate MTs (Figure 1A), a phenotype rescued by addition of FLAG-Nek9 to the depleted extract (Figure 1A). We then looked at MT formation by purified centrosomes in mitotic egg extracts. In the absence of xNek9, centrosomes nucleated significantly less and shorter MTs than in control extract (Figure 1B, top two graphics). These effects were specific since they were rescued by addition of FLAG-Nek9 to the depleted extract.

Nek9 interacts with γ-tubulin and components of the γ-TuRC [12]. We therefore examined whether the reduced capacity of centrosomes to nucleate MTs in the absence of Nek9 could be due to defects in γ-tubulin recruitment. Indeed, we observed a significant reduction in the amount of centrosomal γ-tubulin in xNek9-depleted extracts that was in large part rescued upon addition of FLAG-Nek9 to the depleted extract. Interestingly, similar results were obtained for the γ-TuRC targeting factor NEDD1 (Figure 1B, bottom two graphics; note that neither γ-tubulin nor xNEDD1 levels change appreciably in xNek9-depleted extracts, Figure S1B). In contrast to a previous report [15], we found that centrosomal γ-tubulin levels strongly depended on NEDD1 in Xenopus egg extracts (Figure S1C), suggesting that the defects in γ-tubulin recruitment observed in xNek9-depleted extracts could be attributed to defects in NEDD1 recruitment.

Because Nek9 has been shown to act in a signaling cascade activating Nek6/7 that in turn phosphorylate effector proteins [13, 14], we decided to examine whether the phenotypes observed so far could involve Nek6/7. Surprisingly, using antibodies generated against the respective recombinant proteins, we could not detect Nek6 and Nek7 (xNek6 and xNek7) in Xenopus egg extracts or xNek9 immunoprecipitates (Figure S1D), although the corresponding mRNAs were present in the egg extract (data not shown). Moreover, anti-xNek6 antibodies recognized a band at the expected size in XL177 cell lysates, confirming their specificity and suggesting that Nek6 expression is developmentally regulated. Similar results were found using another anti-xNek6 antibody raised against a peptide (data not shown). We conclude that Nek9 has a role in promoting MT nucleation at the centrosome in mitotic egg extract and that it most likely exerts this function directly and not through the activation of Nek6/7.

Nek9 Plays a Direct Role in NEDD1 and γ-Tubulin Recruitment to the Centrosome in Human Cells

To further explore a direct role of Nek9 in the recruitment of NEDD1 and γ-TuRC to the centrosome, we independently downregulated Nek9, Nek6, and Nek7 levels in HeLa cells using small interfering RNAs (siRNAs). As expected, in control cells, γ-tubulin and NEDD1 centrosomal levels increased 2– to 4-fold in prometaphase compared to interphase (Figures 2A and 2B and [1, 8]). Nek6 or Nek7 silencing had no consequence on the recruitment of γ-tubulin and NEDD1 to prometaphase centrosomes (Figures 2A and 2B). By contrast, Nek9 silencing by two independent siRNAs interfered with γ-tubulin and

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Figure 1. xNek9 Depletion Impairs Normal Aster Formation in Xenopus Mitotic Egg Extracts by Reducing MT Density as Well as Centrosomal γ-Tubulin and xNEDD1

(A) Aster formation induced by addition of sperm nuclei to control, xNek9-depleted and xNek9-depleted extract containing 10 nM purified FLAG-Nek9. Scale bar represents 10 μm. Graph shows mean ± SEM (n = 3 experiments, 30 structures counted for each experimental condition; statistical
NEDD1 accumulation at prometaphase centrosomes (Figures 2A and 2B; Figure S2B) without altering the content of the core centriolar protein centrin, or the pericentriolar material (PCM) protein pericentrin indicating that general centrosome structure was intact (Figure S2C). Moreover, Nek9 silencing had no observable effect on the amount of γ-tubulin and NEDD1 at interphase centrosomes (Figures 2A and 2B; Figure S2B). The specificity of Nek9 requirement was demonstrated by the full rescue of γ-tubulin recruitment to prometaphase centrosomes in Nek9-silenced cells expressing recombinant wild-type Nek9 (Figure S2B).

We then examined whether the capacity of prometaphase centrosomes to generate MT asters was impaired by quantifying MT regrowth after cold-induced MT depolymerization (Figure 2C; NEDD1 was used as positive control). β-tubulin fluorescence intensity around prometaphase centrosomes was clearly diminished in Nek9-silenced cells but not in Nek6- or Nek7-silenced cells. Altogether these data suggest that Nek9 is directly required for NEDD1 and γ-tubulin recruitment to the mitotic centrosomes and as a consequence for efficient centrosomal MT assembly.

Nek9 Acts Downstream of Plk1 and Upstream of NEDD1 for the Recruitment of γ-Tubulin to the Centrosome

We have previously shown that Nek9[869A] does not bind to the Nek9 activator Plk1 [13]. To test whether this interaction is required for Nek9 function in centrosomal γ-tubulin recruitment, we examined whether Nek9[869A] could rescue the defects observed upon Nek9 silencing. In contrast to the wild-type form, Nek9[869A] was unable to promote the rescue (Figure S2B). We then examined the ability of a constitutively active form of Nek9 (Nek9[346–732]) [11], to compensate for the downregulation of Plk1 during this process. Figure 3A shows that expression of FLAG-Nek9[346–732] had no effect on the amount of centrosomal γ-tubulin in interphase but slightly increased the pool of centrosomal γ-tubulin in mitotic cells, an effect abrogated by Nek9 silencing. Remarkably, it was Plk1-independent, as FLAG-Nek9[346–732] was capable of rescuing the centrosomal levels of γ-tubulin in Plk1-depleted mitotic cells (in contrast, recombinant wild-type Nek9 slightly increased γ-tubulin centrosomal levels in Plk1-depleted cells but not to control levels; data not shown).

Altogether, our results indicate that downstream of Plk1, Nek9 contributes to control the recruitment of γ-tubulin to mitotic centrosomes and thus their MT nucleating capacity, by regulating NEDD1 localization.

NEDD1 interacts with and Is a Substrate of Nek9

We next tested whether Nek9 and NEDD1 interact. Figure S3A shows that FLAG–NEDD1 coimmunoprecipitated Nek9. Moreover, yeast-two hybrid assays showed that the N-terminal domain of NEDD1 (NEDD1[1–371]) directly interacts with the RCC1 (Nek9[347–726]) and C-terminal (Nek9[721–979]) domains of Nek9 (Figure S3B). Endogenous NEDD1 and Nek9 did not coimmunoprecipitate in mammalian cells, but they coimmunoprecipitated efficiently in Xenopus egg extracts (Figure S3C).

NEDD1 is phosphorylated at multiple sites during mitosis, and both its interaction with γ-tubulin and centrosomal recruitment are regulated by phosphorylation [8–10]. To determine whether NEDD1 is a substrate of Nek9, we performed in vitro phosphorylation assays on xNEDD1 fragments expressed as GST-fusions in bacteria (GST-xNEDD1[1–370] and GST-xNEDD1[371–655]). As shown in Figure S3D, xNek9 exclusively phosphorylated the C-terminal domain of xNEDD1. LC/MS/MS tryptic peptides analysis identified two phosphorylation sites, one in a peptide containing Ser376, Thr377, and Ser378 and one corresponding to Ser444. Both phosphopeptides were also identified in endogenous xNEDD1 immunoprecipitated from mitotic Xenopus egg extracts. Whereas Ser444, Thr377, and Ser378 are not conserved in other organisms, Ser376 is conserved in different vertebrates, including humans where it corresponds to Ser377, a residue that we found by mass spectrometry analysis to be phosphorylated in mitotic HeLa cells (Figure S3E). Moreover, Ser376 is part of a motif ([LF]xx[ST]) that has been shown to be preferred by members of the NIMA kinase family [16, 17] (Figure S3F).

Nek9 regulates Centrosomal γ-TuRC Recruitment by Phosphorylating NEDD1

We then examined the functional consequences of NEDD1 phosphorylation by Nek9. As we could not express a functional recombinant full-length xNEDD1 for rescue experiments in Xenopus egg extracts, we used HeLa cells. We first generated a form of NEDD1 in which Ser377 was replaced with a phosphomimetic residue. FLAG-NEDD1[377D] or NEDD1[377E] (but not wild-type FLAG-NEDD1 expressed at similar levels) rescued the mitotic recruitment of γ-tubulin in Nek9-silenced cells (Figure 3B; however, NEDD1[377D] was not able to rescue Plk1 downregulation, data not shown). These results strongly suggested that Nek9 controls the centrosomal recruitment of γ-tubulin mainly through the phosphorylation of NEDD1 at Ser377.

We next generated a form of NEDD1 that could not be phosphorylated, NEDD1[377A] and tested its ability to support γ-tubulin recruitment to the mitotic centrosome in NEDD1-silenced cells. Expression of NEDD1 wild-type in NEDD1-silenced cells restored γ-tubulin recruitment to the prometaphase centrosomes to levels similar to control cells. By contrast, expression of NEDD1[377A] under similar conditions did not (Figure 4A). Furthermore, it did not restore normal progression through mitosis (Figure 4B): although cells entered into mitosis and progressed into prometaphase with a timing similar to that of control or FLAG–NEDD1-expressing cells, they failed to progress further and accumulated in prometaphase with diminished MT asters. Substitution of endogenous NEDD1 by the NEDD1[377A] in nonsynchronized cells similarly increased the mitotic index (Figure S4D).

Because NEDD1[377A] interacted with γ-tubulin in a similar manner as wild-type NEDD1 (Figure S4E), we examined whether Ser377 phosphorylation could regulate the mitotic recruitment of NEDD1 to centrosomes. In contrast to wild-type NEDD1, NEDD1[377A] failed to accumulate at centrosomes in prometaphase cells (Figure 4C). We conclude that the phosphorylation of NEDD1 on Ser377 by Nek9 controls NEDD1 phosphorylation, t test). Levels of endogenous and recombinant xNek9 in the extracts are shown in Figure S1B. In this and subsequent figures *p < 0.05, **p < 0.01, ***p < 0.001. (B) Aster formation induced by addition of purified centrosomes. Scale bar represents 10 μm. The distribution of MT length and MT fluorescence, γ-tubulin and NEDD1 fluorescence intensities are shown as box plots (50 structures counted for each experimental condition; statistical analysis, t test). AU, arbitrary units.
Figure 2. Nek9 Is Necessary for Normal Centrosomal MT Nucleation as Well as for \(\gamma\)-Tubulin and NEDD1 Recruitment in Mitosis Independently of Nek6/7

(A and B) Effect of different siRNA transfections on \(\gamma\)-tubulin (A) and NEDD1 (B) contents in interphase and prometaphase HeLa cells. Representative examples of the observed phenotypes in prometaphase are shown (scale bar represents 10 \(\mu\)m). The distribution of intensities is shown in each case as a box plot (three independent experiments, 20 cells counted for each experimental condition of each experiment). Asterisks indicate a statistically significant difference with the corresponding controls in prometaphase. For each condition, a representative prometaphase centrosome was LUT coded to represent staining intensity (scale bar represents 1 \(\mu\)m).
recruitment and thereby γ-tubulin recruitment to the centrosome in the early phases of mitosis.

Discussion

Centrosome maturation in G2/M involves the recruitment of different proteins to the PCM resulting in a notable increase in centrosome size and MT-nucleating activity. Key among the recruited proteins is γ-tubulin as part of the γ-TuRC, the major MT nucleation-promoting complex in the cell [18]. The centrosomal recruitment of γ-TuRC is tightly regulated by the protein kinase Plk1 [5] and depends in vertebrates on the adaptor protein NEDD1 [3, 4]. However, somehow surprisingly, the link that connects Plk1 and the centrosomal

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Figure 3. Nek9 Is Downstream of Plk1 and Upstream of NEDD1 in the Control of γ-Tubulin Recruitment to the Mitotic Centrosomes

(A and B) NEDD1[S377D] is able to rescue Nek9 downregulation during γ-tubulin recruitment to the centrosome. Effect of different siRNA and plasmid transfections on γ-tubulin contents in interphase and prometaphase HeLa cells. Representative examples of the observed phenotypes in prometaphase are shown (scale bar represents 10 μm). FLAG-positive cells were scored and the distribution of γ-tubulin intensities is shown as a box plot (three independent experiments, 20 cells counted for each experimental condition of each experiment). Endogenous and recombinant protein levels are shown in Figures S4A and S4B.

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(C) Effect of different siRNA transfections on centrosomal MT nucleating activity. Twenty-four (NEDD1) or 48 hr after transfection HeLa cells were cold-treated to depolymerize MTs and following incubation for 20 s in warm medium were fixed and stained. Representative examples of the observed phenotypes in prometaphase are shown (scale bar represents 10 μm). The distribution of intensities is shown as a box plot (three independent experiments, 50 cells counted for each experimental condition of each experiment). Statistical analysis was performed with the Mann-Whitney U-test. Protein levels after the different siRNA transfections are shown in Figure S2B.
Figure 4. NEDD1[S377] Is Necessary for γ-Tubulin Recruitment to the Centrosome and Normal Mitotic Progression and Controls NEDD1 Recruitment to the Centrosome during Mitosis

(A) As in Figures 3A and 3B. FLAG epitope (cyan) labeled using Zenon labeling technology. Total and FLAG-NEDD1 levels in exponentially growing and mitotic cells are shown in Figure S4A.

(B) Effect of different siRNA and plasmid transfections on cell-cycle progression of HeLa cells. Twenty-eight posttransfection cells were treated with 9 μm RO-3306 for 20 hr. Synchronization in G2 was confirmed by FACS (not shown). Cells were released, and at the indicated times fixed and stained. Prometaphase nuclei were counted and represented as a line-histogram (100 cells counted for each condition; n = 2, one significant experiment is shown). Representative examples of the observed phenotypes at 60 min after release are shown (scale bar represents 10 μm).

(C) Importance of NEDD1 Ser377 for NEDD1 centrosomal localization as assessed by transfection in HeLa cells. Representative examples of the observed phenotypes are shown (scale bar represents 10 μm). The distribution of centrosomal FLAG intensities in FLAG-positive cells is shown as a box plot (three independent experiments, 20 cells counted for each experimental condition of each experiment).
accumulation of NEDD1 and γ-tubulin during mitosis has not yet been completely elucidated [8, 19]. We now show that Nek9, a Plk1-activated kinase [13], controls the mitotic levels of NEDD1 and γ-tubulin at the centrosome. We also show that Nek9 phosphorylates NEDD1 at a single residue and thereby controls its recruitment to the centrosome downstream of Plk1.

In somatic mammalian cells, Nek9 has been shown to act through the activation of Ne6 and Ne7 [11, 13, 14]. Nek6/7 then regulates mitotic players like Eg5, controlling centrosome separation [13, 20]. The apparent absence of these two kinases in Xenopus eggs suggests a novel direct mechanism of action of Nek9 that we have confirmed using HeLa cells. This suggests that the spindle phenotypes reported for Nek6/7-silenced cells [21] may be related to yet to be described Nek6/7 functions (we found no evidence for a role of Nek7 in centriole duplication [22], data not shown).

Nek9 was previously shown to coimmunoprecipitate with γ-tubulin and other γ-tuRC components [12]. Here, we found that Nek9 interacts directly with NEDD1, suggesting that this could mediate the reported interaction of Nek9 with the γ-tuRC. The finding that the WD40 N-terminal domain of NEDD1 and the RCC1 domains of Nek9 are involved in the interaction raises the possibility of an intermolecular binding between the two γ-propeller domains.

We show that in vitro Nek9 phosphorylates NEDD1 at a conserved site: Ser376 in xNEDD1, Ser377 in hNEDD1. Interestingly, Ser377 phosphorylation was reported to occur in mitotic human cells [23], and we could confirm these data. Although this phosphorylation is Plk1-dependent, it is achieved by a different kinase [23]. Our data and the fact that Ser377 falls in a NIMA kinase consensus motif strongly suggest that Nek9 (downstream of Plk1) is the kinase responsible for the phosphorylation of Ser377 in mitotic cells.

NEDD1 is highly phosphorylated during mitosis and has been shown to be a substrate for CDK1 and Plk1 [8–10]. Plk1 controls the centrosomal localization of several PCM proteins including NEDD1 and others more proximal to the centrosome thereby initiating maturation [8, 24] but the precise mechanism controlling NEDD1 recruitment to the centrosome in G2/M was not understood. We now show that the single phosphorylation of NEDD1 on Ser377 by Nek9 is essential for this process. NEDD1[Ser377A] is not recruited to the centrosome in mitosis and it does not support γ-tubulin recruitment to the mitotic centrosome thereby interfering with spindle formation and mitotic progression. Overall, this single phosphorylation event on NEDD1 Ser377 appears to explain fully the role of Nek9 in centrosome maturation (Figure 3B). However, some of our results (Figure 3A and the inability of NEDD1[S377D] to fully rescue Plk1 downregulation) suggest the existence of additional Nek9-dependent and -independent roles of Plk1 during this multistep process, possibly involving the recruitment of additional PCM components to the centrosome [8].

The inability of NEDD1[Ser377A] to accumulate at centrosomes not only highlights the importance of Ser377 for NEDD1 localization and physiological function but also suggests that centrosomes having a low γ-tubulin content and MT-nucleating activity impair mitotic spindle assembly and normal mitotic progression in mammalian cells. This highlights the importance of centrosome physiology and regulation for spindle assembly and cell division.

In summary, we describe a novel role for the NIMA-family kinase Nek9 in the control of γ-tubulin recruitment to the centrosome in M-phase through the phosphorylation of NEDD1. Our data positions Nek9 as a major Plk1 effector in the control of the centrosome cycle contributing both to the regulation of centrosome MT nucleation activity and to centrosome separation [13] during the entry into mitosis.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.06.027.

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