**Xenopus NEDD1 is required for microtubule organization in Xenopus egg extracts**

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Summary

The centrosome serves as the major microtubule-nucleating and -organizing center in animal cells. It is composed of hundreds of proteins. The molecular details of how centrosomal proteins contribute to centrosome function are only beginning to emerge. Members of the neuron-precursor-cell-expressed developmentally downregulated protein 1 (NEDD1) family of conserved proteins have recently been implicated in recruiting γ-tubulin and its associated proteins, which together make up the γ-tubulin ring complex (γTuRC), to the centrosome. Human NEDD1 and its Drosophila ortholog Dgp71WD are WD-repeat proteins that interact with the γTuRC. Experimental knockdown of human NEDD1 was recently shown to result in loss of γ-tubulin from the centrosome. By contrast, however, Dgp71WD knockdown has no effect on targeting the γTuRC to the centrosome in flies. Using Xenopus egg extracts, we show that Xenopus NEDD1 is mostly dispensable for targeting γ-tubulin to centrosomes, but that microtubule organization is disrupted in NEDD1-depleted extracts. We show that NEDD1 exists in a complex that is distinct from the γTuRC, suggesting that NEDD1 may not be a bona fide subunit of the Xenopus γTuRC. We propose that the main function of NEDD1 in Xenopus is in microtubule organization.

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Key words: Xenopus NEDD1, γ-tubulin, γTuRC, Microtubule organization, Centrosome

Introduction

Microtubules serve important roles in cellular organization both during interphase – when they help to position cellular organelles and facilitate directional transport of proteins and vesicles – and during cell division – when they provide the framework for the segregation of the chromosomes. The overall arrangement of microtubules in a cell – and their dynamic behavior – is regulated by microtubule-stabilizing and -destabilizing activities as well as microtubule-crosslinking and -motor proteins. In most cell types microtubules are nucleated and organized by microtubule-organizing centers (MTOCs). The major MTOC in animal cells is the centrosome. Although proteomic approaches have contributed valuable information to our knowledge of the components of the centrosome, a detailed understanding of how these components interact to allow the centrosome to function is still missing.

Centrosome function requires a ring-shaped protein complex that contains γ-tubulin, a specialized member of the tubulin superfamily (Zheng et al., 1995; Moritz et al., 1995). In metazoa, γ-tubulin associates with at least six additional proteins that are highly conserved across phylogenetically distant species (Wiese and Zheng, 2006). One of these, Dgp71WD (also known as Grip71), is a tryptophan-aspartate (WD)-repeat-containing protein of ~71 kDa first identified as part of the Drosophila γ-tubulin ring complex γ(TuRC) (Gunawardane et al., 2003). Dgp71WD was later shown to probably be a peripheral subunit of the γTuRC because its depletion does not disrupt the complex (Vérollet et al., 2006). Subsequent work identified a human ortholog of Dgp71WD as a member of the neuron-precursor-cell-expressed developmentally downregulated protein 1 (NEDD1) family of conserved proteins. Like Dgp71WD, human NEDD1 is a centrosomal protein that interacts with the γTuRC but is not required for the integrity of the complex, suggesting that the peripheral association of the WD-containing protein with the γTuRC is evolutionarily conserved (Lüders et al., 2006; Haren et al., 2006).

Despite these similarities, important functional differences between Dgp71WD and human NEDD1 were revealed by experiments performed in tissue culture cells. For example, knockdown of human NEDD1 by RNA interference (RNAi) resulted in the reduction of the amount of γ-tubulin associated with the centrosome (Lüders et al., 2006; Haren et al., 2006), which suggests that NEDD1 is required for γTuRC recruitment to the centrosome in human cells. Surprisingly, however, knockdown of Dgp71WD had no effect on the amount of γ-tubulin associated with the centrosome in Drosophila tissue culture cells, suggesting that Dgp71WD is dispensable for γTuRC recruitment (Vérollet et al., 2006).

To gain a better understanding of the molecular details of the function of the WD-containing γTuRC subunit we turned to the Xenopus member of this family. Xenopus egg extracts, pioneered by Lohka and Masui (Lohka and Masui, 1983), have been used extensively to study spindle assembly and γTuRC recruitment. These extracts have the advantage that they can be experimentally manipulated such that centrosome-dependent and centrosome-independent processes can be studied independently. Moreover, the extracts can be made to support interphase or mitotic processes. Thus, the contributions of a given protein of interest to interphase or mitotic processes (most notably, centrosome- and microtubule-assembly) can be examined in the absence of other cell cycle stages. In a process that mimics fertilization, addition of
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Demembranated sperm heads to the egg extracts initiates centrosome assembly from sperm-derived centrioles and egg-derived pericentriolar material (including γTuRC) (Felix et al., 1994). The centrosomes, once competent to nucleate microtubules, initially assemble microtubule asters; subsequent structural rearrangements of the asters result in ‘half-spindles’, which then fuse to form bipolar spindles (Lohka and Masui, 1983; Murray, 1989; Sawin and Mitchison, 1991). These processes can be used to functionally characterize proteins for their contribution to γTuRC recruitment, centrosome assembly, microtubule stability or spindle assembly. More recently, *Xenopus* egg extracts have also been used to assemble asters and spindle-like structures in the absence of centrosomes by the addition of RanGTP or RanGTP mimicking mutant proteins (reviewed in Kahana and Cleveland, 1999; Dasso, 2002). Moreover, protein requirements for microtubule organization in the absence of centrosomes can also be studied in microtubule asters induced by the addition of the microtubule stabilizing reagent, taxol (e.g. Verde et al., 1991).

In this study, we used *Xenopus* egg extracts to investigate the functions of *Xenopus* NEDD1 in γTuRC recruitment to centrosomes and in centrosome-independent processes. Our results suggest that NEDD1 plays a minor (and possibly indirect) role in recruiting γTuRC during sperm-centriole-templated centrosome assembly. However, our data also strongly suggest that, in *Xenopus*, NEDD1 has functions in microtubule organization that are independent of its role in recruiting the γTuRC to the centrosome.

**Results**

An alignment of the NEDD1 primary sequence with its human and *Drosophila* (Dgp71WD) orthologs (supplementary material Fig. S1) shows that *Xenopus* NEDD1 shares sequence similarity with human NEDD1 and Dgp71WD over large portions of the N-terminal WD-40 domain. Except for a ~100 amino acid stretch of unknown function near the C-terminus that is shared by *Xenopus* NEDD1 and human NEDD1 there is only limited similarity among human NEDD1, Xenopus NEDD1 and Dgp71WD outside of the WD domain.

To learn more about the function of *Xenopus* NEDD1, we began our analysis by raising polyclonal antibodies in rabbits against the C-terminal half of NEDD1 (amino acids 301–655) and using these antibodies to characterize NEDD1 by western blotting and immunofluorescence microscopy (Fig. 1). The affinity-purified antibodies recognized a band of ~75 kDa in interphase *Xenopus* egg extracts and a tight doublet migrating at ~85 kDa in mitotic extracts (Fig. 1A). The slower migration of NEDD1 from mitotic extracts is, at least in part, due to phosphorylation, because treatment with phosphatase resulted in faster migration of NEDD1 (Fig. 1Ab). This is consistent with a previous report that human NEDD1 is phosphorylated during mitosis (Lüders et al., 2006). We speculate that the doublet in mitotic extracts probably represents two NEDD1 gene products that are less well resolved in interphase extracts, because *Xenopus laevis* is pseudo-tetraploid. Consistent with a role for NEDD1 in centrosome function, our antibodies stained centrosomes in *Xenopus* egg extracts (Fig. 1B,C) and in *Xenopus* tissue culture cells during all stages of the cell cycle (Fig. 1D,E). The NEDD1 antibodies also stained spindle microtubules in mitotic extracts (Fig. 1B) and cells (Fig. 1D,E). Similar centrosome and microtubule staining was seen in tissue culture cells transiently expressing GFP-tagged NEDD1 (supplementary material Fig. S2).

Human NEDD1 has been proposed to function in centriole assembly, because disruption of NEDD1 in human cells in culture leads to centriole defects (Haren et al., 2006). Consistent with the idea that NEDD1 family members might function at the centriole, antibodies against *Xenopus* NEDD1 stained two distinct spots on sperm heads that presumably correspond to sperm-associated centrioles (supplementary material Fig. S3).

NEDD1 disruption causes defects of aster- and spindle-assembly in *Xenopus* egg extracts

To test whether NEDD1 is required for the assembly of microtubule structures, we immunodepleted NEDD1 from mitotic *Xenopus* egg extracts and examined the depleted extracts (supplemented with a small amount of rhodamine-labeled tubulin to allow visualization of microtubules formed in the extract; see Materials and Methods) for their ability to assemble microtubule asters around exogenously added sperm heads within 15 minutes of incubation. Two rounds of immunodepletion reduced the levels of NEDD1 to ~10–20% (Fig. 2A). This reduction in NEDD1 levels had profound effects on aster assembly: the number of sperm heads able to assemble asters (‘firing’ sperm heads) was reduced to 34% of control levels. In addition, the microtubule density per aster (aster size; measured by the amount of rhodamine fluorescence associated with each aster) was reduced by ~80% compared with controls (Fig. 2C,D). Both types of defect (number of sperm heads that fired and aster size) could be rescued by the addition of bacterially expressed recombinant *Xenopus* NEDD1 (Fig. 2C–E), suggesting that the defects were caused by the absence of NEDD1 rather than by the absence of co-depleted proteins. Indeed, the defects could be rescued by XNEDD1-C, a truncation mutant that lack the N-terminal WD-40 repeats (lacking amino acid residues 1–300), but not by XNEDD1-N, which comprises the WD-40 domain (lacking amino acid residues 301–655) (Fig. 2B–E; supplementary material Fig. S4), suggesting that the WD domains are dispensable for the ability of XNEDD1 to function in microtubule-aster assembly.

To determine whether NEDD1 was also required for spindle assembly, we examined the microtubule structures formed in egg extracts at later time points. Spindles assembled within 90 minutes in NEDD1-depleted extracts showed one of two phenotypes: they either had seemingly normal numbers of disorganized microtubules and misaligned chromosomes, or they had substantially fewer microtubules that were organized into bipolar spindles but whose chromosomes were misaligned (Fig. 2F,G). This suggests that NEDD1 function is required for spindle assembly.

Addition of 300 nM full-length NEDD1 to egg extracts – equivalent to a sixfold excess of NEDD1 compared with ~50 nM of endogenous NEDD1 levels – resulted in spindle disruption but had little effect on asters. Consistent with the inability of XNEDD1-N to rescue the NEDD1 depletion, addition of NEDD1-N to egg extracts had no detectable effect on microtubules. By contrast, overexpression of NEDD1-C showed a dominant-negative phenotype: both microtubule asters (Fig. 3A,C) and spindles (Fig. 3B) were severely disrupted. Interestingly, the microtubules appeared highly bundled, suggesting a role for NEDD1 in microtubule crosslinking. This is further discussed below. Overexpression of XNEDD1-C also partially disrupted the centrosomes assembled around sperm centrioles, because the amount of γ-tubulin recruited to sperm heads was reduced by 50% (Fig. 3D,E). Together, these observations suggest that NEDD1 is required for the correct organization of microtubules assembled in egg extracts.
NEDD1 is not strictly required for γTuRC recruitment to centrosomes

Smaller and fewer sperm aster in NEDD1-depleted extracts could result from defects in either microtubule nucleation or stability, or both. A decrease in the amount of γ-tubulin at the centrosome in the presence of exogenous XNEDD1-C suggested that NEDD1 might be involved in recruiting γ-tubulin to the centrosome. To test this more directly, we first examined whether NEDD1 depletion affected γ-tubulin recruitment to sperm centrosomes. Reactions were supplemented with the microtubule-destabilizing drug nocodazole to eliminate γ-tubulin staining along the microtubules to facilitate visualization of centrosome-associated γ-tubulin. Sperm heads were incubated with mock-depleted or NEDD1-depleted extracts, spun onto coverslips and then processed for γ-tubulin immunofluorescence to measure the amount of γ-tubulin present at the reconstituted sperm centrosome. We found that the...
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The total amount of γ-tubulin at sperm centromeres in NEDD1-depleted extracts varied significantly between experiments and ranged between 54% in one experiment and 96% in another, with an average of 78±6.5% (±s.e.m.; n=6 independent experiments, with 50 sperm tips measured per sample per experiment) of the amount of γ-tubulin at centromeres assembled in mock-depleted extracts (Fig. 4).

Because the presence of γ-tubulin on sperm centrioles prior to the experiment (supplementary material Fig. S3) potentially introduces ‘background noise’ to the experiment, we sought an

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**Fig. 2.** *Xenopus* NEDD1 (XNEDD1) depletion from *Xenopus* egg extract leads to disruption of microtubule structures. (A) Western blot analysis to determine the extent of XNEDD1 depletion. Relative amounts of mock-depleted and XNEDD1-depleted extract loaded are indicated above the lanes. (B) Schematic diagram of the constructs (XNEDD1-FL, XNEDD1-N and XNEDD1-C) used in this study. (C) Examples of sperm-induced asters assembled in mock-depleted (top panels) or XNEDD1-depleted extracts (all other panels). XNEDD1-depleted extracts were supplemented with buffer or full-length XNEDD1, XNEDD1-N or XNEDD1-C, as indicated. All extracts were supplemented with small amounts of rhodamine-tubulin to visualize microtubules (red in overlays). Signals in the rhodamine channel (left panels) or UV to visualize DNA (middle panels) were acquired separately and digitally overlaid. Scale bar, 10 μm. (D,E) Quantification of the (D) fluorescence intensity and (E) asters assembled around sperm centrioles for the experiments shown in C, expressed as percent of mock-depleted control. Error bars give the standard error (± s.e.). (F) Examples of spindles assembled in mock-depleted (top two rows of panels) or XNEDD1-depleted extracts (all other panels). Scale bar, 10 μm. (G) Quantification of the relative abundance of bipolar spindles, spindles with at least one disorganized pole and spindles with greatly reduced microtubule numbers in mock-depleted (light bars) and XNEDD1-depleted (dark bars) egg extracts. Error bars give the standard error (± s.e.).
independent way to measure the amount of γ-tubulin recruited to centrosomes in NEDD1-depleted extracts. We therefore tested NEDD1-depleted extracts for their ability to complement salt-extracted centrosomes. In this centrosome-complementation assay, centrosomes are rendered unable to nucleate microtubules in vitro by exposing them to a chaotropic agent such as 1 M potassium iodide (KI) (Moritz et al., 1998). The microtubule-nucleating capacity of the centrosome can then be restored by incubating the salt-extracted centrosomes in *Xenopus* egg extract (Popov et al., 2002). We found that NEDD1-depleted extracts were able to restore full activity to salt-stripped centrosomes (Fig. 4C). Consistent with this observation, the amount of γ-tubulin recruited to centrosomes complemented with control mock-depleted extracts was indistinguishable from the amount of γ-tubulin recruited to centrosomes complemented with control mock-depleted extracts (Fig. 4D). Interestingly, the most noticeable effect of NEDD1 depletion was on the amount of γ-tubulin associated with microtubules, which was reduced to ~30% in NEDD1-depleted extracts (Fig. 4F,G).

Although we could not rule out that the small amount of NEDD1 that remains in the extract after immunodepletion is sufficient to recruit γ-tubulin to centrosomes, or that NEDD1/Dgp71WD was not efficiently removed from the centrosomes by salt extraction, our results suggest that γ-tubulin can be recruited to the centrosome in the absence of most of the endogenous NEDD1. Consistent with this idea, depletion of NEDD1 from the egg extracts had no effect on the levels of γ-tubulin that remained in the extracts (Fig. 4E).

Recombinant NEDD1 can recruit the γTuRC from extracts

Next, we wanted to further investigate a potential interaction between NEDD1 and the γTuRC. We began by asking whether NEDD1 can recruit the γTuRC. For this, we reasoned that recombinant NEDD1 that had been immobilized on beads should gain the ability to nucleate microtubules when incubated in untreated egg extracts, but should be unable to do so when incubated in γTuRC-depleted extracts. Furthermore, γTuRC subunits should be able to associate with immobilized NEDD1 incubated in egg extract. Using the NEDD1-coated beads as bait, we found that beads coated with full-length NEDD1 or XNEDD1-C gained the ability to nucleate microtubules in vitro from purified bovine brain tubulin when incubated in egg extract and isolated again (Fig. 5A), but not if the extracts had been depleted of γTuRC. Western blot analysis showed that full-length NEDD1 and XNEDD1-C were able to recruit the γTuRC subunits γ-tubulin, Xgrip210 and Xgrip109 from

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**Fig. 3.** Addition of recombinant *Xenopus* NEDD1 (XNEDD1) to egg extracts disrupts microtubule structures. (A) Micrographs of microtubule asters formed in egg extracts in the presence of buffer, 300 nM full-length XNEDD1, XNEDD1-N or XNEDD1-C (all expressed and purified as GST fusion proteins; the GST portion was proteolytically removed prior to the experiment), as indicated. Microtubules (red in the overlays) and DNA (blue) are visualized as described for Fig. 2. Scale bar, 10 μm. (B) Micrographs of spindles formed in the egg extracts in the presence of buffer, 300 nM full-length XNEDD1 or XNEDD1-C, as indicated on the left. Microtubules (red in the overlays) and DNA (blue) are visualized as described for Fig. 2. Scale bar, 10 μm. (C) Quantification of aster intensity for the experiment shown in A, expressed as percent of mock-depleted control. Error bars give the standard error (± s.e.). (D) Micrographs of sperm heads incubated in egg extracts with buffer (top panels) or 300 nM XNEDD1-C (bottom panels) and labeled with antibodies against γ-tubulin (green in the overlays) or acetylated tubulin (to mark the position of the sperm centrioles; red in the overlays), and stained to visualize the DNA (blue in the overlays). Scale bar, 1 μm. (E) Quantification of the amount of γ-tubulin immunofluorescence associated with sperm heads for the experiment shown in D, expressed as percent of mock-depleted control. Error bars give the standard error (± s.e.).
the extracts (Fig. 5B). The interaction between NEDD1 and the γTuRC was further confirmed by reciprocal immunoprecipitation experiments, which showed that γ-tubulin immunoprecipitates contained NEDD1, and NEDD1 immunoprecipitates contained γ-tubulin and Xgrip109 (Fig. 5C). These observations suggested that NEDD1 recruited the γTuRC to nucleate microtubules in vitro.

Fig. 4. Xenopus NEDD1 (XNEDD1) depletion reduces the amount of γ-tubulin that associates with the centrosome but does not abolish γ-tubulin recruitment. (A,B) Micrographs of γ-tubulin staining at sperm heads incubated in buffer (top panels), mock-depleted extract (middle panels), or XNEDD1-depleted extract (bottom panels). Signals from the individual fluorescein (γ-tubulin, left column; green in overlay), rhodamine (α-tubulin, center column; red in overlay), or UV (DNA stain; blue in overlay) are shown as overlays in the right column. Scale bar, 1 μm. (B) Quantification of the amount of γ-tubulin associated with sperm centrioles for the experiment shown in A, normalized against the γ-tubulin immunofluorescence for sperm tips incubated in mock-depleted extract. Error bars, SE. (C) Drosophila centrosomes were rendered inactive by treatment with KI, and were then incubated with mock-depleted (top row) or XNEDD1-depleted (bottom row) extract (see text for detail). The extract was then washed off and reconstituted centrosomes were incubated with a solution of pure tubulin (containing a small amount of rhodamine-tubulin) to assay their ability to nucleate microtubules. Microtubules were fixed, and visualized under the microscope. Centrosomes reconstituted with mitotic (left panels) or interphase (right panels) extract are shown. Scale bar, 5 μm. (D) Quantification of γ-tubulin recruited to reconstituted centrosomes treated as described in C, except that they were fixed and stained for γ-tubulin immunofluorescence instead of being tested in the microtubule nucleation assay. γ-tubulin immunofluorescence intensity is reported as percent of mock-depleted control. Error bars give the standard error (± s.e.). (E) Western blot showing the extent of XNEDD1 depletion (top panel) and lack of co-depletion of γ-tubulin (bottom panel). (F) Microtubule asters assembled in mock-depleted (top panel) or XNEDD1-depleted (bottom panel) extracts are stained for γ-tubulin (left row; green in overlay), α-tubulin (second row; red in overlay) or DNA (third row; blue in overlay). (G) Quantification of γ-tubulin immunofluorescence relative to the amount of α-tubulin fluorescence for the experiment shown in F. Error bars give the standard error (± s.e.).
NEDD1 exists in a complex that is distinct from the γTuRC

Our results thus far suggested that NEDD1 can indeed interact with the γTuRC, but our experiments could not determine whether this interaction was direct. Our finding that γ-tubulin recruitment to sperm centrosomes was only partially impaired in NEDD1-depleted extracts suggested that the interaction between NEDD1 and γ-tubulin might not be important. Furthermore, we noticed that, although NEDD1 and γ-tubulin colocalized to centrosomes they generally did not colocalize very well within the centrosome (Fig. 1C; for more examples, see supplementary material Fig. S3). Taken together with the observation that depletion of NEDD1 had little effect on the levels of γ-tubulin remaining in the egg extract (Fig. 4E), we wondered whether NEDD1 is truly a subunit of the γTuRC, as has been suggested for its homologs in flies and humans (Gunawardane et al., 2003; Vérollet et al., 2006; Lüders et al., 2006; Haren et al., 2006). To examine this question further, we fractionated untreated or XNEDD-depleted Xenopus egg extracts on 5%–40%-sucrose gradients and probed the fractions for NEDD1 and γ-tubulin (Fig. 6B,D). This analysis revealed that depletion of NEDD1 had no effect on the migration of γ-tubulin on the gradient, confirming earlier reports that the NEDD1 subunit is not an integral part of the γTuRC (Vérollet et al., 2006; Lüders et al., 2006; Haren et al., 2006). We were surprised to find that the distributions of NEDD1 and γ-tubulin were largely non-overlapping; NEDD1 peaked in fractions 11-13, whereas γ-tubulin peaked in fractions 14-16 (Fig. 6C). This suggested that NEDD1 exist in a complex that is distinct from the γTuRC. A comparison of NEDD1 and γ-tubulin immunoprecipitates by Coomassie staining supported this idea (Fig. 6A). Although NEDD1 antibodies co-immunoprecipitated faint bands that presumably corresponded to γTuRC subunits, these antibodies also co-precipitated proteins that are not part of the γTuRC (marked by asterisks in Fig. 6A). The molecular identity of these NEDD1-interacting proteins is not yet known.

NEDD1 is required for centrosome-independent microtubule organization

Our finding that the majority of NEDD1 exists outside of the γTuRC led us to examine whether NEDD1 was required for microtubule-dependent processes that do not involve recruiting the γTuRC to the centrosome. For this, we tested the effects of NEDD1 depletion or overexpression on microtubule assembly induced by the addition of the RanGTP mimetic RanL43E, which does not require centrosomes, to Xenopus egg extracts. Microtubule aster formation without centrosomes can also be induced by addition of the microtubule stabilizing drug taxol to egg extracts. Surprisingly, we found that NEDD1 depletion had no effect on microtubule formation in the egg extract, but completely abolished the ability of the microtubules to organize into focused arrays (Fig. 7B; see also supplementary material Fig. S5B). Addition of recombinant XNEDD1-C (but not full-length NEDD1 or XNEDD1-N) to RanGTP-treated extracts similarly disrupted microtubule organization and led to formation of highly bundled microtubule arrays (Fig. 7C). This indicates that NEDD1 is dispensable for microtubule nucleation, but is required for microtubule organization in the egg extracts. Consistent with a role in focusing microtubule arrays, NEDD1 was concentrated at the centers of RanGTP or taxol-induced asters, as well as along astral microtubules (Fig. 7A, supplementary material Fig. S5A). Based on these observations, we conclude that NEDD1 is required for microtubule organization independent of its activity in recruiting γ-tubulin to the centrosome.

XNEDD1-C bundles microtubules

To test whether NEDD1 interacted directly with microtubules, we examined the effects of adding bacterially expressed NEDD1 to in vitro microtubule-assembly reactions. Microtubules assembled in the presence of 300 nM XNEDD1-C but not full-length NEDD1 or XNEDD1-N (both at 3 μM), and appeared highly bundled when examined under the light microscope (Fig. 8A). XNEDD1-C, 

![Image](https://example.com/image.png)

**Fig. 5.** Xenopus NEDD1 (XNEDD1) interacts with the γTuRC in egg extracts. (A) Beads coated with recombinant GST, full-length GST-XNEDD1, GST-XNEDD1-N, GST-XNEDD1-C, or γ-tubulin antibodies were incubated with pure tubulin to assay their ability to nucleate microtubules. Beads were either used directly (left panels; not exposed to extract) or were first incubated in extract (middle panels, exposed to extract) or in γ-tubulin-depleted extract (right panels, exposed to extract but γTuRC−Δ). Representative pictures are shown for each condition. Beads were detected by auto-fluorescence; microtubules appear as spiky halos surrounding the beads. Scale bar, 10 μm. (B) Western blot of proteins that associate with the beads coated as in A and incubated in extract. The blot was probed with antibodies directed against the γTuRC subunits Xgrip210, Xgrip109, or γ-tubulin, as indicated on the right. Protein ‘baits’ are indicated above the lanes. Extr, extract control; GST, GST-coated beads; FL, full-length GST-XNEDD1; N, GST-XNEDD1-N; C, GST-XNEDD1-C; IP, beads coated with antibodies against γ-tubulin. (C) Western blot of immunoprecipitations from egg extracts using antibodies directed against γ-tubulin or XNEDD1, as indicated above the blot. Input represents 1.5 μl of extract; immunoprecipitations were from 100 μl of extract.
similarly bundled preformed taxol-stabilized microtubules (Fig. 8C), and we determined that it bound to microtubules with an apparent $K_d$ of $\sim 1.43$. Consistent with a role in crosslinking microtubules, XNEDD1-C localized along the length of bundled microtubules and pelleted with tubulin in pelleting assays (Fig. 8B). These results suggest that XNEDD1-C can bundle both polymerizing and already polymerized microtubules. We propose that NEDD1 affects microtubule organization through its bundling activity.

**NEDD1 localizes to kinetochore fibers**

In addition to localizing to the centrosome, NEDD1 also localized to spindle microtubules during mitosis (Fig. 1). The microtubule-bundling activity of NEDD1 prompted us to test whether NEDD1 is involved in organizing kinetochore fibers, a highly bundled subset of spindle microtubules that are resistant to cold treatment (reviewed by Compton, 2000). To visualize kinetochore fibers, we placed *Xenopus* tissue culture cells on ice to depolymerize non-kinetochore microtubules. The cells were then fixed and processed for immunofluorescence with NEDD1-specific antibodies. This showed that NEDD1 localized to fibers that are stable in the cold (‘cold-stable fibers’; Fig. 8D,E). To confirm that these cold-stable fibers are indeed kinetochore fibers, we double-stained cold-treated cells with NEDD1 antibodies and antibodies against the kinetochore protein Bub1 (Boyarchuk et al., 2007). We found that many NEDD1-positive fibers ended at the kinetochores (Fig. 8E). Based on these observations, it is very intriguing to hypothesize that NEDD1 functions as a microtubule bundling protein at the kinetochore fibers.

**Discussion**

Centrosomal targeting of the $\gamma$TuRC presumably requires at least two components: a $\gamma$TuRC ‘receptor’ on the centrosome, and a $\gamma$TuRC subunit (or set of subunits) that act as ligand(s) for this receptor. To identify the molecular mechanisms that bring the...
γTuRC to the centrosome, we thought it reasonable to first identify the centrosome-targeting subunit of the γTuRC and to then use this subunit as bait to identify potential γTuRC receptor(s). Five of the six known γTuRC subunits are members of a conserved protein family that share one or two regions of sequence similarity known as ‘grip motifs’ (reviewed in Wiese and Zheng, 2006). The sixth γTuRC subunit, NEDD1 (initially identified in flies as Dgp71WD), lacks both grip motifs but instead possesses a WD domain. Being unrelated to the other γTuRC subunits, it was tempting to speculate that the WD-domain protein may be the centrosome-targeting subunit of the γTuRC. The work presented here suggests that the Xenopus ortholog of Dgp71WD, NEDD1, is a centrosomal protein that contributes to microtubule organization. We were surprised to find, however, that the WD-domain protein may not be a bona fide subunit of the γTuRC in frogs. This hypothesis is supported by several observations: for one, depletion of NEDD1 from egg extracts results in co-depletion of only small amounts of γ-tubulin, and vice versa. Second, NEDD1 exists in a complex that does not contain γ-tubulin. And, third, NEDD1 is not required for the assembly or the maintenance of the γTuRC.

The molecular mechanisms that regulate how γ-tubulin is targeted to various sites within the vertebrate cell remain poorly understood. The γTuRC resides mainly at the centrosome during both interphase and mitosis, but centrosomal γTuRC dynamically exchanges with a much larger cytoplasmic pool (Khodjakov and Rieder, 1999; Wiese and Zheng, 2006). γ-Tubulin has been reported to associate with both the centrioles and the pericentriolar material (Fuller et al., 1995; Moritz et al., 1995), which together make up the centrosome. During mitosis, γ-tubulin also localizes within the spindle (Lajoie-Mazenc et al., 1994; Stearns and Kirschner, 1994), presumably either by lateral interactions with centrosomal microtubules or by minus-end capping of non-centrosomal microtubules, or both. Moreover, γ-tubulin is found at non-centrosomal microtubule anchoring sites in polarized cells (Buendia et al., 1990; Rizzolo and Joshi, 1993; Meads and Schroer, 1995; Salas, 1999; Oriolo et al., 2007). How recruitment and anchoring of γ-tubulin complexes differs under these different conditions remains a mystery.

Depletion of 80-90% of the endogenous NEDD1 from egg extracts resulted at most in a 50% reduction of centrosomal γ-
tubulin – often substantially less. One explanation for this reduction is the possibility that γ-tubulin is depleted together with NEDD1. However, NEDD1 depletion had little effect on the overall levels of γ-tubulin that remained in the extracts, making co-depletion a very unlikely possibility. It is possible that the small amount (10-20%) of endogenous NEDD1 that remains in the depleted extract can efficiently recruit γ-tubulin to the centrosome. However, it is worth considering that only a small fraction of the total NEDD1 appears to be associated with γ-tubulin in the egg extract, and the amount of NEDD1 available for γ-tubulin recruitment is likely to be very small. Thus, we interpret our results to suggest that NEDD1 depletion results in only minor defects in γTuRC recruitment. Consistent with this, RNAi of Dgp71WD in flies does not inhibit γ-tubulin recruitment to the centrosome (Vérollet et al., 2006).

Why does NEDD1 depletion affect sperm-centrosome assembly more strongly than centrosome reconstitution after salt extraction in our in vitro assay? Salt extraction of centrosomes leaves an insoluble ‘centromatrix’ onto which centrosomal proteins can assemble to reform a functional centrosome. Sperm centrioles, however, recruit virtually all of the pericentriolar-material proteins from the egg extract and thus, presumably, start from an earlier step in the assembly process. These observations raise the interesting possibility that NEDD1 may be involved in the early stages of centrosome assembly. Once past this point in the assembly process, NEDD1 is no longer required to recruit the γTuRC. Its depletion, however, fails to provide the scaffold onto which other centrosomal components can be attached. Two observations support this idea: (1) human NEDD1 is required for centriole assembly in human cells (Haren et al., 2006) and, (2) NEDD1 is associated with the sperm centrioles (this study). However, a potential role for NEDD1 in centriole assembly needs yet to be explored.

γ-Tubulin localizes to spindle microtubules in animal cells (Lajoie-Mazenc et al., 1994) and along microtubules in Xenopus egg extracts (Stearns and Kirschner, 1994). Although NEDD1 depletion had only minor effects on γ-tubulin recruitment to centrosomes, it strongly affected overall microtubule organization. The most noticeable effects of NEDD1 disruption were a 70% reduction in the amount of γ-tubulin associated with astral microtubules and, in the case of NEDD1 overexpression, highly bundled microtubules. Interestingly, knockdown of Dgp71WD or human NEDD1 in RNAi experiments was also reported to abolish the association of γ-tubulin with spindle microtubules (Haren et al., 2006; Lüders et al., 2006), suggesting that the NEDD1 family of proteins might mediate an interaction between the γTuRC and microtubules. A role for NEDD1 in microtubule organization is further supported by our observations that microtubules assembled by addition of RanGTP or taxol to egg extracts are unable to organize into asters but instead form disorganized heaps of microtubules. Finally, our observation that NEDD1 binds to kinetochore fibers in mitotic cells suggests a mechanism by which the microtubule-bundling activity of NEDD1 is involved in organizing kinetochore fibers. Alternatively, NEDD1 might recruit a subset of γ-tubulin to kinetochore fibers to nucleate and organize microtubule microtubules. In support of a role for NEDD1 in kinetochore-microtubule organization, chromosomes were often misaligned on spindles assembled in NEDD1-depleted extracts.

Consistent with a direct interaction between NEDD1 and microtubules, we found that XNEDD1-C binds to taxol-stabilized microtubules with an apparent $K_d$ of 1.43 μM. Association of γ-tubulin with astral and/or spindle microtubules as a mechanism to nucleate new microtubules has been described in fission yeast and plants. For example, the S. pombe protein mto2p recruits γ-TuCs to the side of existing microtubules to nucleate new microtubules (Janson et al., 2005), and γ-tubulin has been proposed to localize to existing microtubules to nucleate new ones in plant cells (Murata et al., 2005). However, it is also possible that γ-tubulin localization to astral microtubules is an indirect effect of the microtubule bundling activity of NEDD1. In this scenario, γ-tubulin localizes to the side of one microtubule because it is associated with the minus end of a second microtubule that is held close to the first one by the bundling activity of NEDD1. Although γTuRC-independent roles for Dgp71WD or human NEDD1 have not been described, our findings are consistent with the findings that Dgp71WD is required for γTuRC recruitment to spindle microtubules but not to centrosomes. This hypothesis is further supported by the observations by Lüders et al. that phosphorylation of human NEDD1 is required for γTuRC recruitment to spindle microtubules but not for centrosome recruitment (Lüders et al., 2006).

In summary, we draw four main conclusions from this work in Xenopus: (1) NEDD1 exists in a complex that is distinct from the γTuRC and includes only a small, if any, amount of γ-tubulin. (2) NEDD1 depletion has only minor effects on recruitment of γ-tubulin to centrosomes. (3) NEDD1 binds directly to microtubules. (4) NEDD1 is required for the organization of microtubules. However, several major questions regarding NEDD1 remain. For example, it is not yet clear whether the centrosome-independent functions of NEDD1 involve γ-tubulin, or who the binding partners of NEDD1 are. The interaction between NEDD1 and the γTuRC is entirely unexplored and it might be indirect. It will be interesting to see whether members of the NEDD1 family of proteins in different species serve conserved or distinct functions, and what role the WD repeats play in NEDD1 function.

Materials and Methods

Recombinant proteins

Xenopus NEDD1 was identified in database searches as the ortholog of Dgp71WD (Gunawardane et al., 2003) and human NEDD1 (Lüders et al., 2006; Haren et al., 2006). Full-length Xenopus NEDD1 cDNA was generated by PCR amplification of an expressed sequence tag (EST; IMAGE clone 6633797; purchased from Open Biosystems, Huntsville, AL). To generate a fusion protein with glutathione-S-transferase (GST), full-length Xenopus NEDD1 was subcloned directionally into pGEX-6P2 (Amersham Biosciences, Piscataway, NJ) using BamHI and XhoI as cloning sites. The following primers (restriction sites are underlined) were used: 5’-ATGTGATCCAGGATACAGATATGACC-3’, 5’-ATGGATCCATGATATGACC-3’. Association of xenopus NEDD1 (amino acid residues 301-655) was generated using primers 5’-ATGGATCCACAGCAGAATTTAGGGCGG-3’, 5’-ATGGATCCCAAGGAATACATCAG-3’, 5’-ATGGATCCAAATAATGCCCCG-3’, and 5’-ATGGATCCAAATAATGCCCCG-3’.

To generate a fusion protein with green-fluorescent-protein (GFP), full-length Xenopus NEDD1 was subcloned into the XhoI-BamHI site of the pEGFP-C3 vector (Clontech). A fusion protein in which GFP is fused to the C terminus of Xenopus NEDD1 (amino acid residues 301-655) was generated using primers 5’-ATGGATCCCAAGGAATACATCAG-3’, 5’-ATGGATCCAAATAATGCCCCG-3’, and 5’-ATGGATCCAAATAATGCCCCG-3’. Association of xenopus NEDD1 (amino acid residues 301-655) was generated using primers 5’-ATGGATCCCAAGGAATACATCAG-3’, 5’-ATGGATCCAAATAATGCCCCG-3’, and 5’-ATGGATCCAAATAATGCCCCG-3’.

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The following fusion proteins were expressed in E. coli strain C41(DE3) (Miroux and Walker, 1996) and purified by affinity chromatography using standard protocols for purification of GST-fusion proteins: GST-RanL43E (see Wilde and Zheng, 1999), GST-XNEDD1-FL, GST-XNEDD1-N and GST-XNEDD1-C (diagrammed in Fig. 587).
2B). All purified recombinant proteins were concentrated to >10 mg/ml, dialyzed against XB (10 mM K-HEPES, 100 mM NaCl, 1 mM MgCl₂, 0.1 mM EGTA, pH 7.6). Washed nuclei, pH 7.6), flash-frozen in liquid nitrogen as small aliquots, and stored at −80°C for later use. Full-length XNEDD (aa 1-655), XNEDD-N (aa 1-300) or XNEDD-C (aa 301-655) were expressed as glutathione S-transferase (GST) fusion proteins and purified using GST agarose. For the experiments shown in Figs 2, 3, 7, 8A and 8C, the GST portion of the fusion protein was proteolytically removed prior to the addition of the purified protein to the reaction. The uncleaved protein was used for the experiments shown in Figs 5 and 8B.

**Antibodies**

Polyclonal antibodies against Xenopus NEDD1 were generated in rabbits using bacterially expressed, purified XNEDD1-C as antigen. Polyclonal rabbit antibody against γ-tubulin (Xenc) was generated as described by Zheng et al. (Zheng et al., 1995). Antibodies against Bub1 were a kind gift from Mary Dasso (NIH, MD). Monoclonal antibodies against γ-tubulin (GTU88), acetylated-tubulin (6-11B-1) and alkaline-phosphatase-linked anti-mouse or anti-rabbit secondary antibodies (for western blots) were obtained from Sigma (St Louis, MO). Alexa-Fluor-488 and Alexa-Fluor-594 anti-mouse or anti-rabbit secondary antibodies (for immunofluorescence) were purchased from Molecular Probes (Eugene, OR).

**Xenopus egg extract preparation**

CSF-arrested Xenopus egg extracts were prepared as described (Murray, 1991) and were supplemented with Δ90-cyclinB to arrest them in mitosis (Murray et al., 1989).

**Cell culture**

XLKWG cells (Martin et al., 1998) were cultured in R1640 medium (GIBCO) supplemented with 10% FBS and 2% penicillin and streptomycin at 32°C in a 5% CO₂ atmosphere. For immunofluorescence, cells were fixed in 4% formaldehyde (in PBS) for 20 minutes at room temperature and permeabilized in TBS with 0.1% Triton X-100 for 5 minutes.

**Microtubule polymerization assays**

Microtubule polymerization assays were performed as previously described (Oegema et al., 1999) with the following modifications: microtubule-assembly reactions (5 μl) containing various concentrations of purified XNEDD1-C (0 mg/ml, 0.1 mg/ml, 0.2 mg/ml and 0.8 mg/ml) and 32.5 μM bovine brain tubulin (supplemented with a small amount of rhodamine-labeled tubulin) were incubated at 30°C for 10 minutes, fixed with ten volumes of 1% glutaraldehyde in BRB80 at 23°C for 3 minutes, and diluted with 250 μl of 70% glycerol in BRB80. A 3-μl aliquot of the reaction was then mounted on a microscope slide and viewed in the microscope.

**Covalent attachment of GST fusion proteins to activated Dynabeads**

GST-fusion proteins or GST alone were covalently linked to Dynabeads (Dynal Co). GST-fusion proteins or GST alone were covalently linked to Dynabeads (Dynal Co) via a short linker and the GST fusion proteins were resolved by SDS-PAGE. The GST fraction were loaded on a 10% SDS-PAGE gel. Proteins were visualized by western blotting with antibodies to GST. For covalent attachment of GST fusion proteins to activated Dynabeads, GST-fusion proteins were mixed with activate Dynabeads in a molar ratio of 2:1 (Beads:GST) and the mixture was incubated at room temperature for 1 h. The GST-fusion proteins were covalently linked to the activated Dynabeads.

**Aster- and spindle-assembly assays**

Xenopus egg extracts were supplemented with energy mix (7.5 mM phosphocreatine, 1 mM ATP, 100 μM EGTA, 1 mM MgCl₂) and Δ90-cyclinB, and incubated at room temperature for 30 minutes. To induce aster assembly, either RanL3E (1 mg/ml) or sperm chromatin (150 sperm per μl) were added to extracts and incubated at room temperature for 15 minutes. To induce taxol asters, taxol was added to the extract to a final concentration of 0.1 μM and the taxol-treated extract was incubated at room temperature for 30 minutes. To induce cyclin B asters, spindle, fresh mitotic extract was driven into interphase by addition of sperm chromatin and 0.3 mM Ca²⁺, and were then cycled back into mitosis by addition of 100 mM NaCl, 20 mM Tris-HCl, pH 7.4) for 20 minutes at room temperature. Samples were fractionated on parallel 2-ml-sucrose gradients in a 4 hour spin at 55,000 rpm in a Beckman TLS 55 rotor. Sixteen 125-μl fractions were collected and 25 μl of each fraction were loaded on a 10% SDS-PAGE gel. Proteins were visualized by western blotting.

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