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The NIMA-related kinase X-Nek2B is required for efficient assembly of the zygotic centrosome in *Xenopus laevis*

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SUMMARY

Nek2 is a mammalian cell cycle-regulated serine/threonine kinase that belongs to the family of proteins related to NIMA of *Aspergillus nidulans*. Functional studies in diverse species have implicated NIMA-related kinases in G_2/M progression, chromatin condensation and centrosome regulation. To directly address the requirements for vertebrate Nek2 kinases in these cell cycle processes, we have turned to the biochemically-tractable system provided by *Xenopus laevis* egg extracts. Following isolation of a *Xenopus* homologue of Nek2, called X-Nek2B, we found that X-Nek2B abundance and activity remained constant through the first mitotic cycle implying a fundamental difference in Nek2 regulation between embryonic and somatic cell cycles. Removal of X-Nek2B from extracts did not disturb either entry into mitosis or the accompanying condensation of chromosomes providing no support for a requirement for Nek2 in these processes at least in embryonic cells. In contrast, X-Nek2B localized to centrosomes of adult *Xenopus* cells and was rapidly recruited to the basal body of *Xenopus* sperm following incubation in egg extracts. Recruitment led to phosphorylation of the X-Nek2B kinase. Most importantly, depletion of X-Nek2B from extracts significantly delayed both the assembly of microtubule asters and the recruitment of γ-tubulin to the basal body. Hence, these studies demonstrate that X-Nek2B is required for efficient assembly of a functional zygotic centrosome and highlight the possibility of multiple roles for vertebrate Nek2 kinases in the centrosome cycle.

Key words: Nek2, NIMA, Cell cycle, Centrosome, *Xenopus*

INTRODUCTION

Protein phosphorylation plays a major role in eukaryotic cell cycle control. Indeed, a growing number of cell cycle-regulated protein kinases have now been identified. However, in many cases the mechanism by which these kinases regulate cell cycle transitions remains unclear. One such example is the family of kinases that are related to the NIMA serine-threonine kinase of the filamentous fungus *Aspergillus nidulans*. Temperature-sensitive mutants of NIMA become arrested in G_2 of the cell cycle when incubated at the restrictive temperature, while release to the permissive temperature allows a synchronous and rapid entry into mitosis (Morris, 1976; Oakley and Morris, 1983). Dominant-negative mutants of NIMA, consisting of the C-terminal non-catalytic domain alone, also cause a G_2 arrest (Lu and Means, 1994), while high level expression of wild-type NIMA induces premature entry into mitosis from any point in the cell cycle (Osmani et al., 1988). In line with this, NIMA protein abundance and kinase activity are cell cycle-regulated with peak levels in mitosis (Osmani et al., 1991b). Taken together, these results point to an essential role for NIMA in regulating the G_2/M transition in *Aspergillus nidulans*.

However, it is not clear whether all members of the NIMA-related kinase family share this function in mitotic entry. The most closely-related kinase to NIMA is the NIM-1 gene of another filamentous fungus, *Neurospora crassa* (Pu et al., 1995). When expressed in a temperature-sensitive *Aspergillus nim*A strain, NIM-1 can rescue the cell cycle arrest demonstrating that it is a *bona fide* functional homologue of NIMA. Deletion of the fission yeast NIMA-related kinase $p83^{fin1}$ also causes a G_2 arrest, supporting a role for this kinase in the G_2/M transition (Krien et al., 1998). On the other hand, the most closely-related gene to NIMA present in the budding yeast genome, KIN3/NPK1, is non-essential indicating that the product of this gene is clearly not required for mitotic entry (Barton et al., 1992; Jones and Rosamond, 1990; Schweitzer and Philippsen, 1992).

Other functions have been attributed to NIMA-related kinases beyond control of the G_2/M transition. The most broadly studied of these is a potential role in chromosome condensation. Results supporting such a function are: (i) overexpression of NIMA in *Aspergillus* or $p83^{fin1}$ in *Schizosaccharomyces pombe* consistently promotes premature chromatin condensation (O'Connell et al., 1994; Osmani et al., 1988); (ii) ectopic NIMA overexpression in organisms as

diverse as yeast, *Xenopus* and humans also induces premature chromatin condensation (Lu and Hunter, 1995; O'Connell et al., 1994); (iii) *fin1* deletion mutants genetically interact with other *S. pombe* strains defective in chromatin organization (Krien et al., 1998); (iv) murine Nek2 has been reported by one group to associate with meiotic chromosomes (Rhee and Wolgemuth, 1997). However, another group reported no association of murine Nek2 with condensed chromosomes (Tanaka et al., 1997). Moreover, *nim*A5*bim*E7 conditional mutants of *Aspergillus* arrest in mitosis rather than G_2 and exhibit fully condensed chromosomes (Osmani et al., 1991b). Admittedly, this could be due to residual NIMA kinase activity in the double mutant as deletion of NIMA in the presence of the *bimE7* mutation does lead to a G_2 block (Ye et al., 1998). It therefore remains to be proven which, if any, of the NIMA-related kinases are genuinely required for chromosome condensation. Recent studies have also raised the possibility that *Aspergillus* NIMA functions both as a target for S-phase checkpoints and in the correct localization of the $p34^{cdc2}$/cyclin B complex to the nucleus and the spindle pole body in late G_2 (Wu et al., 1998; Ye et al., 1996).

Human Nek2 is the most well characterized of the mammalian NIMA-related kinases. Its substrate specificity and expression pattern is highly reminiscent of NIMA (Fry et al., 1995), while tissue distribution studies show that Nek2 is highly expressed in both meiotic and actively dividing somatic tissues (Arama et al., 1998; Rhee and Wolgemuth, 1997; Tanaka et al., 1997). However, overexpression studies with wild-type and mutant versions of Nek2 have so far not indicated any role in controlling either mitotic entry or chromosome condensation. Rather, they have highlighted a different function for this family of kinases, namely in the organization and regulation of the centrosome. The centrosome is the predominant organizer of the microtubule network throughout the animal cell cycle (Brinkley, 1985; Kellogg et al., 1994) and, at mitosis, separation of the duplicated centrosomes leads to the establishment of the mitotic spindle upon which chromosomes are segregated (Compton, 1998; Merdes and Cleveland, 1997). The basic unit of the centrosome consists of two barrel-shaped centrioles (themselves composed of short, highly stable microtubules) enveloped by a fibrous matrix known as the pericentriolar material or PCM (Zimmerman et al., 1999). It is a highly dynamic piece of cell machinery that undergoes significant changes during the cell cycle in both centriole conformation and PCM organization. Many of these changes are likely to be regulated by reversible protein phosphorylation and a dramatic change in the number of phosphorylated epitopes at the centrosome has been seen upon entry into mitosis (Centonze and Borisy, 1990; Vandré et al., 1984, 1986).

The association of Nek2 with the centrosome is independent of microtubules and occurs throughout the cell cycle (Fry et al., 1998a). Overexpression of Nek2 in cultured cells produces two apparently distinct consequences. Firstly, expression of active Nek2 kinase induces centrosome splitting leading to the hypothesis that Nek2 might be involved in the process of centrosome separation that begins in late G_2 (Fry et al., 1998a). Isolation of a putative centrosomal substrate of the Nek2 kinase, C-Nap1, through a yeast two-hybrid interaction screen has lent support to this hypothesis (Fry et al., 1998b). The second phenotype of Nek2 overexpression is a slow disintegration of the entire centrosome structure which occurs irrespective of whether a wild-type or catalytically-inactive mutant is used (Fry et al., 1998a). The most likely explanation for this phenomenon is that excess Nek2 protein titrates endogenous centrosomal components through either direct or indirect protein-protein interaction. As Nek2 can form homodimers, this titration may be facilitated by dimerization of exogenous protein with endogenous Nek2 (Fry et al., 1999). As well as having a catalytic role, Nek2 may therefore perform a structural role in maintaining the organization of what has been termed the 'centromatrix' (Schnackenberg et al., 1998).

These models of Nek2 function rely heavily on the interpretation of data obtained by overexpressing proteins in cultured cells. We have, therefore, decided to turn to a biochemically-tractable system in order to test the function of Nek2 more directly. Specifically, we have used cell free assays performed in extracts made from the eggs of the African toad *Xenopus laevis* to investigate possible roles for vertebrate Nek2 kinases in mitotic entry, chromosome condensation and/or centrosome regulation. Our results indicate that *Xenopus* Nek2 is not required for mitotic progression or chromosome condensation, at least in embryonic cell cycles, whereas, in contrast, it plays a key role in the assembly of the first zygotic centrosome.

MATERIALS AND METHODS

X-Nek2B cDNA cloning

cDNA clones were obtained from a *Xenopus laevis* oocyte 5′-STRETCH cDNA library (CLONTECH Laboratories Inc., Palo Alto, CA). To generate the initial probe, the full-length human Nek2 cDNA, contained on a *Nae*I-*Xba*I fragment, was excised from pGEM-Nek2 and labeled with [α-^{32}P]dATP using the random-primed DNA labeling kit (Boehringer Mannheim, Mannheim, Germany). For a second round of screening, the full-length X-Nek2B cDNA isolated in the first round was labeled in a similar manner. Positive phages were purified, and confirmed by Southern blotting and sequencing of PCR products amplified with phage-specific primers. Lambda DNA was prepared from agarose plate lysates using the QIAGEN Lambda Kit, and inserts excised and subcloned into the *Eco*RI site of a pBlueScript-SK vector to generate pBS-X-Nek2B. DNA sequencing in both directions was carried out using a 377 DNA Automated Sequencer (Perkin-Elmer Corp., Norwalk, CT).

Antibody production

To generate antibodies, a His_6-tagged fusion protein of the C-terminal 234 amino acids of X-Nek2B was made using the QIAexpress bacterial expression system (QIAGEN). The plasmid pQE11-X-Nek2B was constructed by subcloning an 890 bp *Hin*dIII fragment from the plasmid pBS-X-Nek2B into the *Hin*dIII site of pQE11. Ligation mixes were transformed into competent M15[pREP4] *E. coli* to directly test for His_6X-Nek2B protein expression. Recombinant protein was expressed and purified under denaturing conditions as described by the manufacturer (QIAGEN), before further purification on a preparative 15% SDS-polyacrylamide gel as described by Fry et al. (1998a). For immunizations, 300 μg of the purified His_6X-Nek2B protein was injected subcutaneously into New Zealand white rabbits (Elevage Scientifique des Dombes, Chatillon sur Chalaronne, France) on days 1, 27, 56, 84 and 112. Immune serum was obtained on days 38, 66, 96 and 122 with a final exsanguination on day 130. IgGs were purified on a Protein A-Sepharose column as described by Harlow and Lane (1988).

Cell culture and transfections

Xenopus adult kidney epithelial A6 cells were grown at 23°C in Leibovitz L-15 medium modified as described by Smith and Tata (1991), supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin (100 i.u./ml and 100 μg/ml, respectively). For transient transfection studies, A6 cells were seeded onto HCl-treated glass coverslips at a density of 1×10^5 cells per 35 mm dish. Transfection was carried out by incubating 5 μg of plasmid DNA in 100 μl of 0.25 M $CaCl_2$ for 5 minutes, then mixing with 100 μl of HBS solution pH 7.28 (50 mM Hepes, 1.5 mM Na_2HPO_4, 0.28 M NaCl) and adding dropwise to the cells. After 4 hours the DNA precipitate was removed. Cells were fixed at the times indicated in the text.

Egg extract preparation and immunodepletions

CSF-arrested egg extracts and activated egg extracts (interphase) were prepared as described by Murray (1991), with modifications as described by Descombes and Nigg (1998). For cycling extracts, activated eggs were incubated for 3-4 minutes with calcium ionophore (A23187, Sigma, final concentration 1 μg/ml), followed by a further 15 minute incubation at room temperature in XB buffer before crushing. High speed supernatants were obtained by spinning extracts at 100,000 ***g*** for 30 minutes at 4°C in a benchtop ultracentrifuge (Beckman Instruments, Fullerton, CA). Demembranated sperm nuclei were obtained as described (Murray, 1991). Non-destructible cyclin B (Δ90) was produced as described by Glotzer et al. (1991) and used at 10 μg/ml final concentration. For inspection of chromatin, samples were spotted onto microscope slides and fixed with formaldehyde solution containing Hoechst 33258 (Murray, 1991). For immunodepletion experiments, Protein A beads (Affi-prep, Bio-Rad) were pre-coated with either anti-X-Nek2B R81 antibodies or pre-immune P81 antibodies (equal volume of beads: crude antiserum). After extensive washes in XB buffer, beads were added to extract (20%, v/v) containing 250 μg/ml cycloheximide, and samples incubated for 1 hour at 4°C with rotation. Beads were removed by centrifugation, and the supernatants supplemented with sperm nuclei.

Immunoblotting and protein kinase assays

For immunoblotting of egg extracts, 0.5 μl of extract was resolved by SDS-PAGE and transferred onto nitrocellulose using a Hoefer Semi-Dry blotting apparatus. For immunoblotting of demembranated sperm heads, 5×10^5 sperm were either untreated or incubated in 10 μl CSF extract with 1 μg/ml nocodazole at 22°C for times indicated in the text. Following incubation, samples were mixed with spindle buffer (SB; 10 mM K.Pipes, pH 6.8, 0.3 M sucrose, 0.1 M NaCl, 3 mM $MgCl_2$) to a final volume of 100 μl, layered onto a 1 ml cushion of 25% glycerol in SB and centrifuged at 3300 rpm, 20 minutes, 4°C in a microfuge. After removing 100 μl from the top of the cushion, the interface was washed three times with 100 μl of 1% Triton X-100. The cushion was then removed completely and the pellet washed two times with 1 ml of 1× PBS before resuspending in 15 μl of sample buffer. Samples were resolved by SDS-PAGE and transferred to nitrocellulose as above. Primary antibodies used were R81 anti-X-Nek2B purified IgGs (5.0 μg/ml), anti-γ-tubulin antibodies (1:5000; Sigma) and anti-α-tubulin antibodies (0.3 μg/ml; Amersham). Blots were developed using alkaline phosphatase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:7500; Promega).

X-Nek2B immunoprecipitation and kinase assays were performed as described for human Nek2 (Fry and Nigg, 1997), while histone H1 kinase assays were done as described by Descombes and Nigg (1998).

Sperm aster assays

Fresh CSF-arrested egg extracts, depleted as described above, were used to assemble sperm asters essentially following the procedure described by Sawin and Mitchison (1991). Briefly, CSF extracts were complemented with demembranated sperm (150 sperm/μl) and rhodamine-labelled tubulin (20 μg/ml final concentration) and incubated at 22°C. At the time-points indicated, 1 μl samples were spotted onto a microscope slide and fixed with 2 μl of formaldehyde mix (Murray, 1991) before microscopic inspection.

Immunofluorescence microscopy

For antibody staining of whole cells, A6 cells were grown on HCl-treated coverslips and fixed with methanol at –20°C for 6 minutes. Antibody staining of isolated *Xenopus* sperm was based on previously published methods (Evans et al., 1985; Merdes et al., 1996; Stearns and Kirschner, 1994). In brief, a 15 ml Corex tube (Corning GlassWorks, Corning, NY) was prepared with a coverslip adaptor and round coverslip onto which was pipetted a 5 ml cushion of 25% glycerol in SB. Sperm (3000 sperm in 1 μl) were incubated at 20°C for 10 minutes in 10 μl SB or 10 μl egg extract. Nocodazole was present at 2 μg/ml in both SB and extract. Samples were then diluted with 100 μl SB and gently layered onto the glycerol cushion. Tubes were spun at 3000 rpm, 4°C for 20 minutes in a HB-4 rotor. The top 1 ml of cushion was removed by aspiration and the interface washed with 0.5 ml of 1% Triton X-100. All but the last 1 ml of cushion was then removed by aspiration and the coverslip was lifted out and fixed with methanol at –20°C for 6 minutes.

Immunofluorescence microscopy was performed essentially as described by Fry et al. (1998a). Primary antibodies used in this study were: anti-X-Nek2B R81 purified IgGs (5 μg/ml); anti-myc monoclonal antibody 9E10 (undiluted tissue culture supernatant); anti-γ-tubulin purified IgGs (1.8 μg/ml IgGs; Fry et al., 1998a); anti-α-tubulin monoclonal antibody (3 μg/ml; Amersham). Detection of primary antibodies was carried out using the following secondary reagents: biotinylated anti-rabbit or anti-mouse antibodies (1:50; Amersham) followed by Texas Red-conjugated streptavidin (1:200; Amersham); FITC-conjugated anti-mouse Fab fragment (1:100; Sigma); FITC-conjugated anti-rabbit antibody (1:100; Sigma). None of the secondary antibodies used stained sperm centrosomes in the absence of primary antibody. DNA staining was carried out with Hoechst 33258 (0.2 μg/ml in PBS). Coverslips were mounted in 80% glycerol, 3% *n*-propyl gallate (in PBS).

RESULTS

Isolation of a *Xenopus laevis* Nek2 homologue

To investigate Nek2 function using *Xenopus* cell free assays, we first isolated a *Xenopus* homologue of the mammalian Nek2 kinase by screening a *Xenopus* oocyte cDNA library. Nine independent clones were obtained, all encoding an identical protein sequence, which we termed X-Nek2. Comparison of X-Nek2 protein with human and mouse Nek2 indicated an overall identity of 74% and 73%, respectively. Identity is highest within the N-terminal catalytic domains (86% between *Xenopus* and human), although some sequence conservation is maintained into the C-terminal non-catalytic domain (Fig. 1). Most notably, X-Nek2 possesses the unusual leucine zipper structure present in the C-terminal domain of mammalian Nek2, implying that X-Nek2, like human Nek2, is likely to be regulated by homodimerization (Fry et al., 1999). Homology, however, is lost in the final 30 amino acids of X-Nek2 and, moreover, X-Nek2 is clearly shorter than its mammalian counterpart lacking the second coiled-coil present at the extreme C terminus of human Nek2. The X-Nek2 cDNA thus encodes a protein of 389 amino acids (as compared to 445 amino acids for human Nek2) with a predicted molecular mass of 45.96 kDa and a pI of 8.46.

During the preparation of this manuscript, Sagata and coworkers reported the isolation of a cDNA identical to X-

Nek2 (Uto et al., 1999). Intriguingly, they found that *Xenopus* Nek2 exists as two structural variants, most likely arising from differential splicing (Fig. 1). The longer version, termed Nek2A, comprises 442 amino acids and is thus almost identical in length to human Nek2 (445 amino acids). The shorter version, termed Nek2B, lacks the C-terminal region of Nek2A and is identical to the 389 amino acid version of X-Nek2 described here. By analysing the expression of these two variants, Sagata and colleagues showed that only Nek2B is present during oogenesis and in early embryos, but that a developmental switch to Nek2A expression occurs at the gastrula to neurula transition (Uto et al., 1999). Nek2B therefore represents the appropriate isoform for studies in embryonic systems. To distinguish between human and *Xenopus* Nek2, as well as the two variants, we will refer to the *Xenopus* clone that we have isolated as X-Nek2B.

X-Nek2B abundance is constant in the first embryonic cell cycle

Antibodies were raised in two rabbits (R80 and R81) using a bacterially-expressed fragment of X-Nek2B. Immune serum from R81 reacted with a band of 44 kDa on western blots of extracts prepared from both interphase and CSF-arrested *Xenopus* eggs, while pre-immune serum did not (Fig. 2A, lanes 3-6). This band comigrated with X-Nek2B translated in vitro in the presence of ^{35}S-methionine (Fig. 2A, lane 1). R80 immune serum also recognized the X-Nek2B protein in egg extracts although it showed weak cross-reactivity with a second band at approximately 52 kDa (data not shown) and, hence, for all studies presented here the R81 antibody was used. R81 antibodies were also capable of immunoprecipitating a β-casein kinase activity from wild-type X-Nek2B translated in vitro (Fig. 2B, lane 7). A fraction of the X-Nek2B protein itself became upshifted in this assay indicative of autophosphorylation. A mutant version of X-Nek2B (K37R), did not exhibit either β-casein kinase activity or autophosphorylation (Fig. 2B, lane 8). Furthermore, comparing equivalent amounts of immunoprecipitated protein, X-Nek2B exhibited a very similar level of β-casein kinase activity to human Nek2, confirming that the X-Nek2B cDNA encodes an active protein kinase (Fig. 2B, compare lanes 7 and 9).

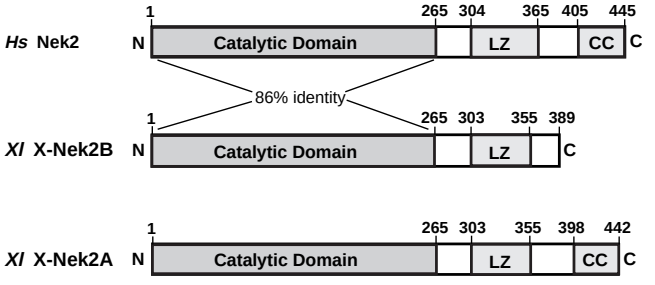


Fig. 1. Structure of X-Nek2B protein. Schematic comparison of human (*Hs*) Nek2 and *Xenopus* (*Xl*) X-Nek2B isolated from a *Xenopus* oocyte cDNA library highlighting the conservation of catalytic domain and leucine zipper (LZ) but not the second coiled-coil (CC) motif present in human Nek2. X-Nek2A, an alternative splice variant isolated by Uto et al. (1999), is identical to human Nek2 in structure. Numbers represent the amino-acid position at which different domains start and end.

During antibody characterization, we noted that there was no change in abundance of X-Nek2B protein between interphase and CSF-arrested egg extracts (Fig. 2A, compare lanes 5 and 6). Equally, we did not observe any obvious difference in electrophoretic mobility of the X-Nek2B protein between these samples. Therefore, we tested whether there was any change in solubility and/or activity in X-Nek2B between interphase and CSF arrest. We found that X-Nek2B was present in the same amount in low and high speed supernatants from both interphase and CSF-arrested extracts (Fig. 2C, top panel). Furthermore, there was no change in the level of β-casein kinase activity (Fig. 2C, bottom panel). These results are surprising when compared with human Nek2 whose abundance and activity are much reduced during mitotic arrest versus interphase in cultured cells (Fry et al., 1995; Schultz et al., 1994). CSF arrest, however, represents a block in metaphase II of meiosis and, as such, is a quite distinct biochemical state from somatic cell mitosis. We therefore assayed X-Nek2B abundance in a cycling extract. However, despite this extract passing through two cell cycles, as indicated by two peaks of H1 kinase activity and nuclear envelope breakdown (Fig. 2D, bottom panel), a change in neither abundance nor migration of the X-Nek2B protein was observed (Fig. 2D, top panel). Thus, X-Nek2B protein remains constant during early embryonic cell cycles.

Comparing the amount of X-Nek2B in egg extracts with known amounts of bacterially-expressed protein, we estimated the concentration of X-Nek2B in *Xenopus* egg extracts to be 0.8 μg/ml (20 nM; data not shown), equivalent to 0.002% of total extract (approx. 40 mg/ml). X-Nek2B is therefore a low abundance protein in egg cytoplasm and is present at significantly lower amounts than many other centrosome or spindle components, e.g. NuMA (8-14 μg/ml; Merdes et al., 1996), Xklp2 (16 μg/ml; Boleti et al., 1996) or Plx1 (10 μg/ml; Descombes and Nigg, 1998). γ-tubulin, the well-characterized centrosomal protein required for microtubule nucleation, is present at 1-10 μg/ml (Stearns et al., 1991; Zheng et al., 1995).

X-Nek2B depletion does not block cell cycle progression or chromatin condensation

Studies on fungal NIMA-related kinases have focused on the role of these proteins in promoting the G_2/M transition and chromatin condensation (Krien et al., 1998; Osmani et al., 1988, 1991a). Indeed, overexpression of *Aspergillus* NIMA can induce aspects of a premature mitosis not only in *Aspergillus* but also in frog, yeast and mammalian cells (Lu and Hunter, 1995; O'Connell et al., 1994). Whether NIMA-related kinases of higher eukaryotes have equivalent roles in mitotic progression and chromatin condensation is therefore an important question. The generation of specific antibodies provided us with the opportunity to test the involvement of X-Nek2B in these processes through an immunodepletion approach.

Incubation of egg extracts with immobilized anti-X-Nek2B antibodies led to complete depletion of the X-Nek2B protein (>95%) as judged by immunoblotting (Fig. 3A). Using these depleted extracts, we tested whether removal of X-Nek2B had an effect on the ability of interphase extracts to enter mitosis either in a cycling extract or following addition of recombinant non-destructible cyclin B (Glotzer et al., 1991). Activation of MPF (Cdk1/cyclin B), assayed by histone H1 kinase activity,

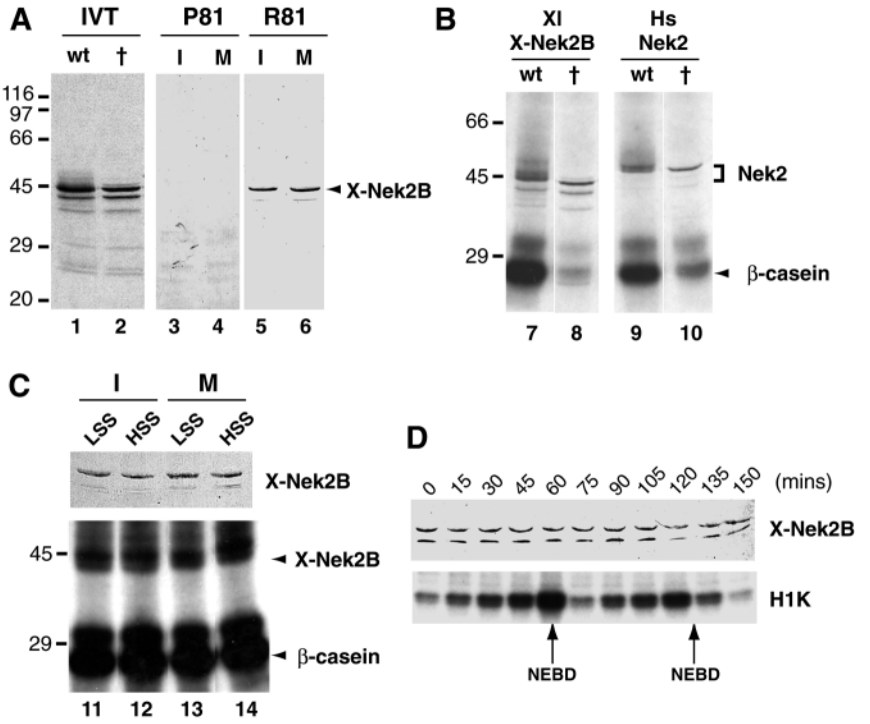


Fig. 2. X-Nek2B abundance and activity are constant during the first embryonic cell cycle. (A) Interphase (I) and CSF-arrested mitotic (M) extracts were immunoblotted with pre-immune (P81) or immune (R81) X-Nek2B antisera (lanes 3-6). Alongside, an autoradiograph of wild-type (wt) and catalytically-inactive (†) X-Nek2B translated in vitro in the presence of [^{35}S]methionine (lanes 1,2). (B) Wild-type or catalytically-inactive versions of *Xenopus* (Xl) X-Nek2B were translated in vitro in the presence of [^{35}S]methionine, immunopreciptated with R81 antibodies and subjected to β-casein kinase assays in the presence of [γ-^{32}P]ATP (lanes 7,8). For comparison, similar reactions were performed with human (Hs) Nek2 immunoprecipitated with R40 anti-Nek2 antibodies (lanes 9,10). Equivalent amounts of X-Nek2B and Nek2 are immunoprecipiated in each lane and a similar level of β-casein phosphorylation is detected. (C) Extracts were prepared from interphase (I) or CSF-arrested (M) eggs either as low speed (LSS) or high speed (HSS) supernatants. Abundance was determined by immunoblotting for X-Nek2B protein (top panel) and activity measured by an IP-kinase assay on β-casein (bottom panel). No change was observed between interphase and CSF extracts. (D) X-Nek2B protein abundance was assayed in cycling egg extracts by immunoblotting with R81 antibodies (top panel). There was no variation in X-Nek2B protein despite extracts passing through two mitoses as indicated by two peaks of H1 kinase activity (bottom panel) and nuclear envelope breakdown (NEBD). X-Nek2B, as also seen for human Nek2, often appears on immunoblots as a doublet that may result from internal initiation or degradation. Molecular mass markers in A-C are indicated on the left (kDa).

was unchanged between mock-depleted and X-Nek2B depleted extracts irrespective of which protocol was used to drive the G_2/M transition (Fig. 3B and C). Likewise, there was no change between the two samples in the time at which nuclear envelope breakdown could be observed. Next we monitored chromatin condensation in extracts driven into M phase by addition of the non-destructible cyclin (Fig. 3D). Microscopic inspection of sperm DNA added to extracts revealed that the condensed chromatin fibers were indistinguishable in control and X-Nek2B-depleted extracts. Taken together, these data provide no support for X-Nek2B as a regulator of MPF activity or chromatin condensation. Furthermore, X-Nek2B depletion did not compromise the ability of MPF to promote entry into M phase during the first embryonic cycle.

Ectopically-expressed X-Nek2B localises to centrosomes in A6 cells

Subcellular localisation studies and biochemical fractionation have previously shown that human Nek2 is a core component of the centrosome. Furthermore, ectopic expression of active Nek2 kinase induced a premature splitting of the two centrosomes in a large proportion of cells (Fry et al., 1998a). Unfortunately, immunoblots of *Xenopus* cultured cells with R81 antibodies gave only weakly reactive bands at various molecular masses implying that endogenous X-Nek2B (and X-Nek2A) is a low abundance protein, as found for mammalian Nek2. Therefore, to determine whether X-Nek2B has a potential role at the centrosome, we overexpressed a myc-epitope tagged version of the wild-type X-Nek2B kinase in *Xenopus laevis* A6 adult kidney cells and observed the cells using anti-myc antibodies. At early times after transfection (8 hours), myc:X-Nek2B protein accumulated at the centrosome as demonstrated by colocalisation with the centrosomal protein γ-tubulin (Fig. 4a,b). At later times, centrosomes in many transfected cells (45% at 40 hours) were separated by more than several microns. Indeed, the fraction of cells with split centrosomes was comparable with the results obtained upon overexpression of human Nek2 (Fig. 4c,d). Microscopic examination of the DNA in these cells confirmed that they were not in the process of undergoing mitosis. We also found that overexpression of human Nek2 in *Xenopus* A6 cells induced centrosome splitting suggesting that the substrates reponsible for this phenotype are sufficiently conserved as to be targeted by both *Xenopus* and human kinases (data not shown).

Recruitment of X-Nek2B to the sperm basal body

In most animals, including *Xenopus*, the centrosome used to generate the first mitotic spindle in the fertilized zygote originates from the basal body of the sperm flagellum (Schatten, 1994). However, this basal body lacks the capacity to nucleate multiple microtubules until it enters the cytoplasm of the egg when, after a short time lag, it becomes competent for nucleation. The conversion of a basal body into a functional centrosome is therefore a useful model for both centrosome assembly and organization and can be studied in vitro by incubating demembranated sperm in soluble egg extracts and observing the formation of microtubule asters (Archer and Solomon, 1994). Based on this assay, centrosome proteins generally fall into two classes: those that are paternally

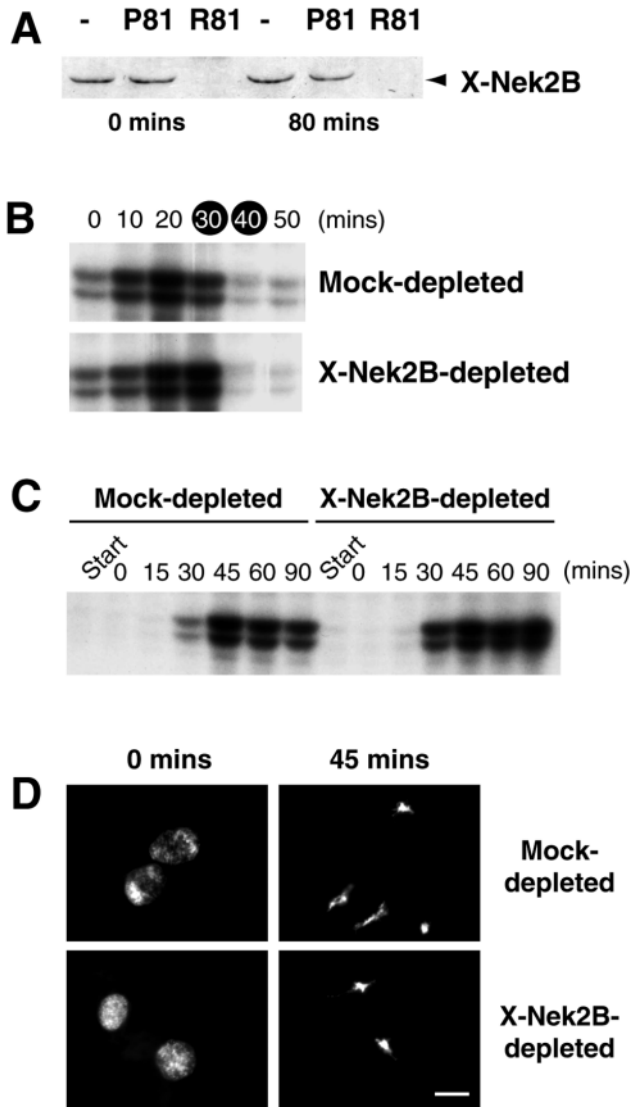


Fig. 3. X-Nek2B depletion does not prevent cell cycle progression or chromosome condensation. (A) Egg extracts were either untreated (-), mock-depleted with P81-Protein A beads (P81) or X-Nek2B-depleted with R81-Protein A beads (R81), then incubated at 22°C with cycloheximide for 0 or 80 minutes and subjected to immunoblot with R81 antibodies. (B) Cycling extracts were immunodepleted, complemented with sperm DNA (100 sperm/μl) and aliquots taken at time points (minutes) indicated. MPF activity was measured by H1 kinase assay and chromatin condensation determined by microscope inspection. Nuclear envelope breakdown was visible in the 30 and 40 minute samples in both control and X-Nek2B-depleted extracts (black circle). Cycloheximide was not added to depleted cycling extracts but immunoblotting confirmed that X-Nek2B did not reappear during the course of the experiment. The doublet indicates the presence of phosphorylated histone H1 protein. (C) The rate at which interphase extracts entered mitosis following addition of non-destructible cyclin B was determined by assaying for H1 kinase activity. Extracts were incubated for 30 minutes (from Start to 0) before cyclin addition. Aliquots were removed at the times indicated (minutes) from either mock- or X-Nek2B-depleted extracts and processed as described. (D) Chromatin condensation was monitored following addition of the non-destructible cyclin B to either mock-depleted extracts (top panels) or X-Nek2B-depleted extracts (bottom panels), before (0) and 45 minutes after (45) cyclin addition. Bar, 5 μm.

contributed through association with the sperm basal body itself, e.g. centrin and pericentrin, and those that are maternally contributed being recruited to the basal body from egg cytoplasm, e.g. γ-tubulin, the γ-TuRC components Xgrip109 and 76p, and MPM-2 epitopes (Fava et al., 1999; Felix et al., 1994; Martin et al., 1998; Stearns and Kirschner, 1994). However, recent results indicate that the situation might not always be so clear-cut as γ-tubulin has been weakly detected on immunoblots of sperm preparations prior to incubation in egg cytoplasm (Merdes and Cleveland, 1998; Simerly et al., 1999; Tassin et al., 1998).

To examine X-Nek2B localisation, demembranated *Xenopus* sperm were incubated in either buffer or egg extract for 30 minutes before costaining with X-Nek2B antibodies and antibodies against α-tubulin to reveal the position of the basal body centrioles. Following incubation in buffer alone, no signal for X-Nek2B protein was detected on sperm (Fig. 5A, c). However, after a 30 minute incubation in CSF-arrested extract, X-Nek2B was strongly and specifically associated with the sperm centrosome (Fig. 5A, f and g). X-Nek2B protein was recruited equally well from interphase and CSF-arrested extract and from both crude low speed supernatants and clarified extracts subjected to 100,000 ***g*** centrifugation (data not shown). Moreover, the recruitment of X-Nek2B to the centrosome was independent of microtubules as it occured despite being performed in the presence of nocodazole.

Recruitment of X-Nek2B was studied in more detail using a biochemical approach whereby spermheads were incubated in nocodazole-containing egg extract, purified through a glycerol cushion and then immunoblotted for X-Nek2 protein (Fig. 5B and C). Equivalent numbers of sperm in all samples was monitored by immunoblotting for α-tubulin. X-Nek2B was not detected in blots of 5×10^5 sperm prior to incubation in extract (Fig. 5B, lane 2). However, a number of faint cross-reacting bands were present at 45-50 kDa when very concentrated sperm samples ($>5\times10^6$) were used which may represent extremely low levels of X-Nek2B (or indeed X-Nek2A) in sperm alone (data not shown). Upon incubation in egg extract at 22°C there was a rapid appearance of X-Nek2B on sperm reaching a maximal level by 10 minutes (Fig. 5B, lanes 3-7). Recruitment was so fast that even the 0 minute sample, when sperm were mixed with extract and then immediately diluted with cold buffer, showed detectable X-Nek2B protein (Fig. 5B, lane 3). Intriguingly, when recruitment was carried out at 4°C, we found that a similar amount of X-Nek2B was recruited as at 22°C (Fig. 5C). However, X-Nek2B that had been recruited at 22°C exhibited a distinct retardation in gel mobility when compared with either X-Nek2B recruited at 4°C or X-Nek2B present in egg extract that had been incubated at 22°C (Fig. 5C). Thus, X-Nek2B recruitment appears to be an energy-independent event, while recruitment itself appears to stimulate post-translational modification of X-Nek2B, possibly through autophosphorylation.

X-Nek2B depletion delays centrosome assembly

The rapid appearance of X-Nek2B on the sperm basal body suggests that it is one of the first proteins recruited during centrosome assembly. We therefore investigated whether X-Nek2B depletion would block the creation of a functional centrosome as measured by microtubule aster formation. Using

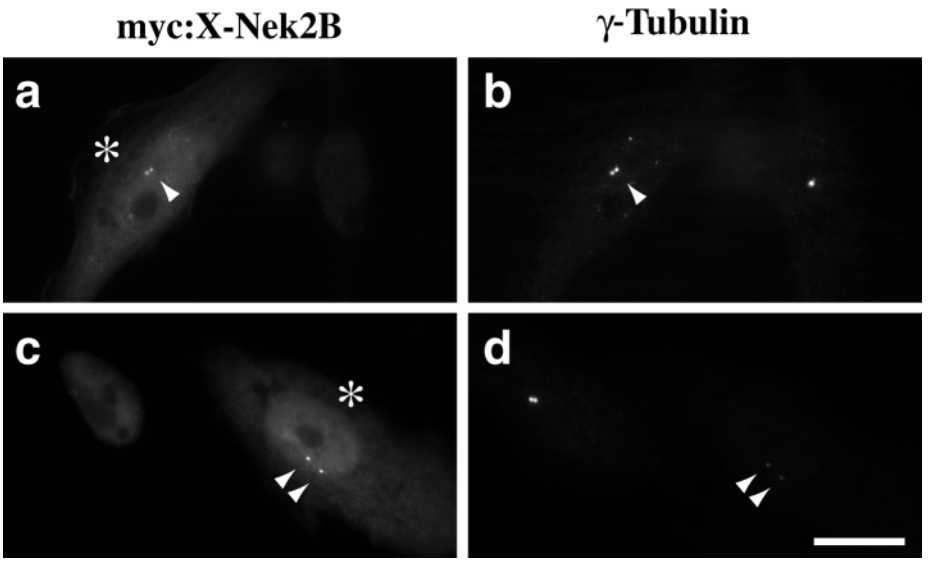


Fig. 4. Localization of ectopically-expressed X-Nek2B to centrosomes of Xenopus A6 cells. Myc-tagged X-Nek2B (myc:X-Nek2B) was transfected into *Xenopus* adult kidney A6 cells. After 6 (a,b) or 16 (c,d) hours, cells were fixed and processed for immunofluorescence microscopy by costaining with anti-myc (a,c) and anti-γ-tubulin (b,d) antibodies. Arrowheads denote the positions of centrosomes in transfected cells as indicated by γ-tubulin stain. Note that in transfected cells (asterisk) myc:X-Nek2B concentrates at centrosomes and, in some cases, induces centrosome splitting (c). Bar, 10 μm.

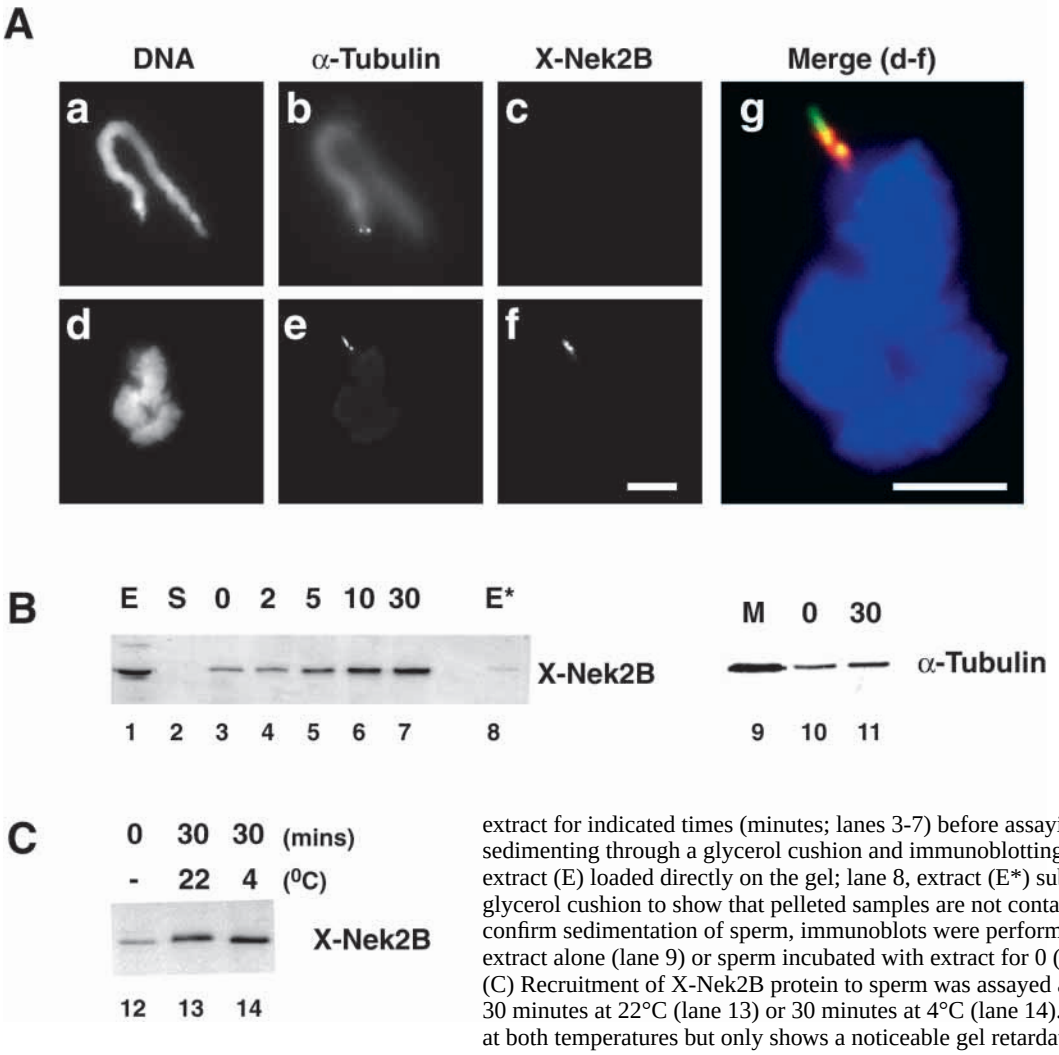
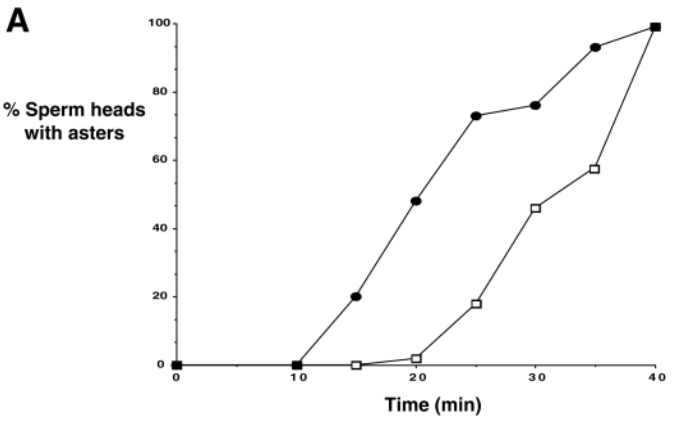


Fig. 5. Recruitment of X-Nek2B to sperm centrosomes. (A) Sperm were incubated for 30 minutes in either buffer alone (a-c) or CSF extract (d-g) before being spun onto coverslips and processed for immunofluorescence microscopy with DNA stain (a,d), α-tubulin antibodies to reveal the centrioles (b,e) and X-Nek2B antibodies (c,f). X-Nek2B is detected on centrosomes only after incubation in extract. While most sperm lose the flagellum during isolation, some retain a remant of the microtubule tightly linked to the proximal centriole; this is revealed by α-tubulin antibodies. By merging the α-tubulin (green), X-Nek2B (red) and DNA (blue) signals from d-f, it is clear that X-Nek2B is present solely around the two centrioles and does not extend along the microtubule (g). Bars, 10 μm. (B) Sperm were either untreated (S, lane 2) or incubated with CSF extract for indicated times (minutes; lanes 3-7) before assaying for X-Nek2B recruitment by sedimenting through a glycerol cushion and immunoblotting with R81 antibodies. Lane 1, extract (E) loaded directly on the gel; lane 8, extract (E*) subjected to sedimentation on the glycerol cushion to show that pelleted samples are not contaminated with egg cytoplasm. To confirm sedimentation of sperm, immunoblots were performed with α-tubulin antibodies of extract alone (lane 9) or sperm incubated with extract for 0 (lane 10) or 30 (lane 11) minutes. (C) Recruitment of X-Nek2B protein to sperm was assayed as in B for 0 minutes (lane 12), 30 minutes at 22°C (lane 13) or 30 minutes at 4°C (lane 14). Note that X-Nek2B is recruited at both temperatures but only shows a noticeable gel retardation when incubated at 22°C.

fresh CSF extracts, we found that sperm incubated in X-Nek2B-depleted extract exhibited a striking delay in aster formation as compared to sperm incubated in a mock-depleted extract (Fig. 6A). For instance, at the 20 minute time-point, asters were associated with the majority of sperm in mock-depleted extract but with very few sperm in X-Nek2B-depleted



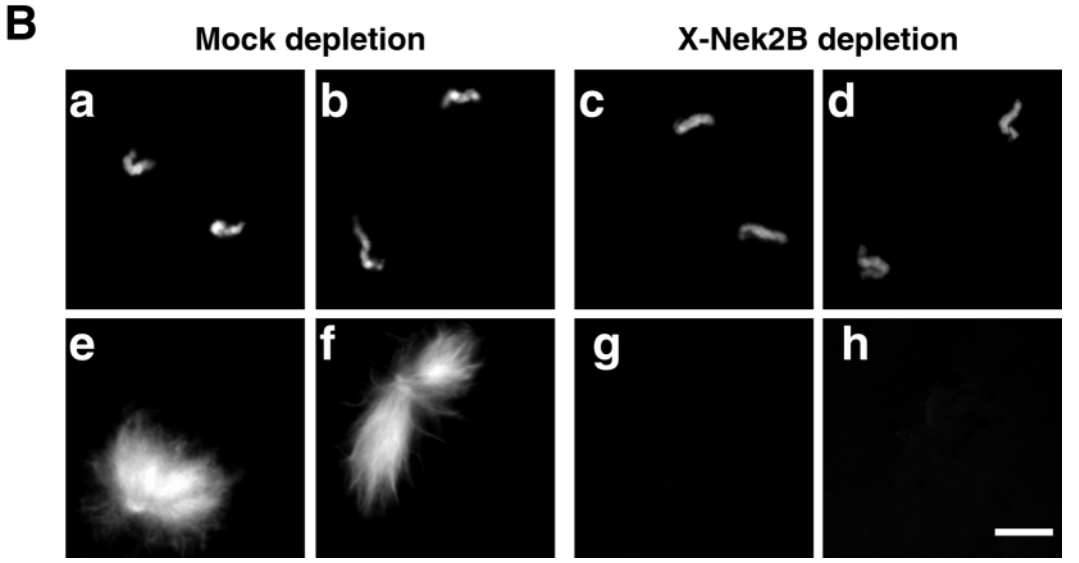


Fig. 6. X-Nek2B depletion inhibits sperm aster formation. (A) The rate of sperm aster formation in either mock-depleted (closed circles) or X-Nek2B-depleted (open squares) CSF extracts as determined by immunofluorescence microscopy. For each time-point, 200-300 sperm heads were counted. (B) Representative microscope images of sperm chromatin (top panels) and corresponding microtubule arrays (bottom panels) following a 20 minute incubation in either mock- or X-Nek2B-depleted extract. Most sperm are associated with microtubule arrays in the mock-depleted sample, whereas the majority of sperm possess few if any microtubules following X-Nek2B depletion. At the time point shown, microtubule arrays are preferentially associated with chromatin and centrosomes are likely to have separated from chromatin consistent with the pathway leading to half-spindle formation (Sawin and Mitchison, 1991). Bar, 5 μm.

extract (Fig. 6B). However, this block was not permanent as eventually all sperm in the X-Nek2B-depleted extracts also formed asters (Fig. 6A).

As the γ-tubulin protein is essential for microtubule nucleation, we tested whether X-Nek2B depletion had altered the ability of the sperm centrosome to recruit γ-tubulin. In agreement with published data (Merdes and Cleveland, 1998; Simerly et al., 1999; Tassin et al., 1998), we were able to weakly detect γ-tubulin in sperm prior to incubation in egg extract by both immunofluorescence microscopy and immunoblotting (Fig. 7A and C). We, therefore, measured the increase in γ-tubulin intensity at the basal body with time following incubation in either mock- or X-Nek2B-depleted extract. A similar delay in recruitment of γ-tubulin was observed following X-Nek2B depletion as was seen for aster formation (Fig. 7A). At early time-points (e.g. 10 minutes), sperm had significantly more intense γ-tubulin staining at the basal body in the mock-depleted extract than in the X-Nek2B-depleted extract (Fig. 7B). However, by 30 minutes sperm in the X-Nek2B-depleted extract also began to show recruitment of γ-tubulin to the centrosome and by 60 minutes there was generally little observable difference between the X-Nek2B- and mock-depleted extracts (Fig. 7A). Immunoblotting for the amount of γ-tubulin recruited to sperm confirmed the slower kinetics of recruitment following X-Nek2B depletion (Fig. 7C, compare upper and lower panels at 10 minutes). As a control, the depleted egg extracts were also immunoblotted for X-Nek2B and γ-tubulin confirming that, while X-Nek2B had been effectively removed, there was no detectable change in the abundance of γ-tubulin in the extract (Fig. 7D). Taken together, these results suggest a model whereby functional centrosome assembly depends upon γ-tubulin recruitment, which in turn is stimulated by the prior recruitment of X-Nek2B (Fig. 8 and see Discussion).

DISCUSSION

This study represents the first investigation into NIMA-related kinase function using cell-free assays in *Xenopus laevis* egg extracts. Other approaches have implicated members of this kinase family in a number of cell cycle processes including mitotic progression, chromatin condensation and centrosome regulation. Here, we have characterised a *Xenopus* NIMA-related kinase, X-Nek2B, which is most closely related to human Nek2. Using an immunodepletion approach, we found no evidence for a requirement for X-Nek2B in progression through the first embryonic cell cycle or chromosome condensation. Rather, X-Nek2B was rapidly recruited to the sperm centrosome upon incubation in cytoplasmic egg extracts and depletion of X-Nek2B from the extracts delayed the functional assembly of the first zygotic centrosome. These

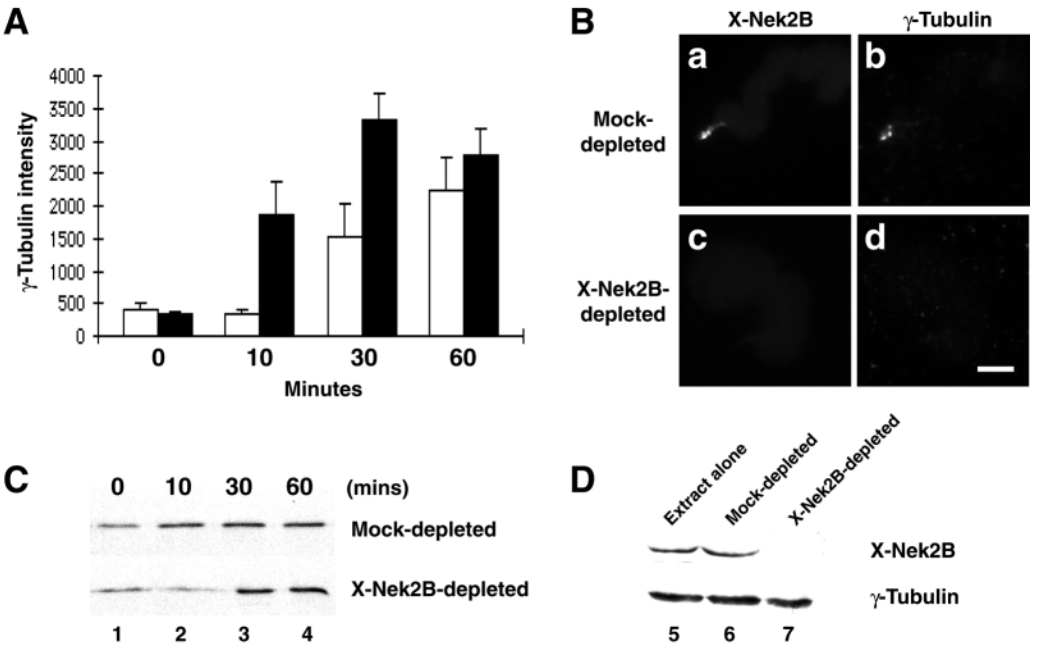


Fig. 7. X-Nek2B depletion inhibits the recruitment of γ-tubulin to the centrosome. (A) Sperm were incubated in either mock- (black bars) or X-Nek2B- (open bars) depleted CSF extracts for times indicated (minutes) before being spun onto coverslips and processed by immunofluorescence microscopy. The amount of γ-tubulin protein present at the sperm basal body was scored by measuring the fluorescence intensity following staining with γ-tubulin antibodies. For this purpose, images were captured using SmartCapture software at fixed exposure times (calculated to avoid pixel saturation). The maximal intensity of centrosomal pixels was then determined in SmartCapture and presented as arbitrary units. Results are the mean of 3 independent experiments; error bars, s.d. (B) *Xenopus* sperm were incubated for 10 minutes in either mock-depleted (a,b) or X-Nek2B-depleted (c,d) CSF extracts. Sperm were then processed for immunofluorescence microscopy with X-Nek2B antibodies (a, c) and γ-tubulin antibodies (b,d). Bar, 10 μm. (C) Immunoblot of *Xenopus* sperm with γ-tubulin antibodies following incubation in either mock (top panel) or X-Nek2B (bottom panel) depleted CSF extracts for times indicated in minutes. (D) Immunoblots of untreated (lane 5), mock- (lane 6) and X-Nek2B- (lane 7) depleted CSF extracts with antibodies against X-Nek2B (top panel) and γ-tubulin (bottom panel). There is no apparent decrease in abundance of γ-tubulin in X-Nek2B-depleted extracts.

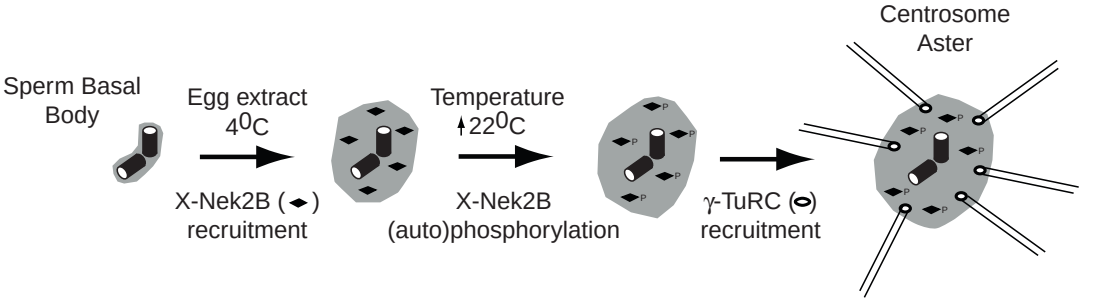


Fig. 8. Model for the role of X-Nek2B in centrosome assembly. This speculative model illustrates how X-Nek2B (diamonds) may play an early role in the assembly of functional centrosomes. Firstly, our results suggest that recruitment to the sperm basal body and phosphorylation of X-Nek2B (P) are independent and separable events. Secondly, they reveal that efficient recruitment of γ-tubulin and formation of a microtubule aster is stimulated by the presence of X-Nek2B kinase. Finally, this model provides one explanation for published data showing that γ-tubulin recruitment is an energy-dependent step and thus might be dependent upon the activity of a protein kinase (Stearns and Kirschner, 1994). It is emphasized that at the present time it is not known whether γ-tubulin is recruited as intact γ-TuRCs, as shown here, or as individual components. Black cylinders represent centrioles surrounded by pericentriolar material or centromatrix in grey.

results complement and extend our earlier studies with human Nek2 and suggest that vertebrate Nek2 kinases may have several distinct roles to play in centrosome organization and regulation.

X-Nek2B represents an embryonic Nek2 variant

X-Nek2B represents a protein that is highly related to human Nek2 across both the N-terminal catalytic domain and the adjacent leucine zipper dimerization motif. However, X-Nek2B is shorter than human Nek2 lacking the C-terminal coiled-coil motif present in the latter. A second protein, X-Nek2A, has been isolated from *Xenopus* that is almost identical in length and primary structure to human Nek2 and analysis of the nucleotide sequence strongly suggests that X-Nek2A and X-Nek2B are splice variants of the same gene (Uto et al., 1999). Expression studies indicate that X-Nek2A, as has been

described for mammalian Nek2, is most highly expressed in testis, whereas X-Nek2B shows highest expression in the ovary (Uto et al., 1999). Indeed, only X-Nek2B is present in maturing oocytes and early embryos explaining why our screen of a *Xenopus* oocyte cDNA library yielded purely clones representing X-Nek2B. At the gastrula to neurula transition, there is a dramatic shift in expression from X-Nek2B to X-Nek2A (Uto et al., 1999). Thus, X-Nek2B is the only isoform present during early embryonic cell cycles and, by extension, must be the relevant kinase for zygotic centrosome assembly.

In our study, we have addressed the abundance of X-Nek2B through an individual cell cycle. Our data reveal that X-Nek2B is not regulated in abundance or activity either upon release from CSF arrest or during the first mitotic cell cycle. The lack of cell cycle variation of X-Nek2B contrasts with results obtained for human Nek2 in somatic cells where Nek2 abundance and activity are high during S and G_2 and low in G_1 and upon mitotic arrest (Fry et al., 1995; Schultz et al., 1994). It is attractive to speculate that this difference in cell cycle regulation between the adult and embryonic forms may relate to the different sequences of X-Nek2A and X-Nek2B. If this were the case, one could expect that *Xenopus* X-Nek2A, like human Nek2 but unlike X-Nek2B, may be cell cycle-regulated. It will be interesting to explore the possibility that a X-Nek2B homologue may be required for early development in mammals.

X-Nek2B is not required for chromosome condensation or mitotic entry

Studies on fungal NIMA-related kinases have indicated essential roles for both mitotic entry and chromosome condensation (Krien et al., 1998; Lu and Hunter, 1995; Morris, 1976; O'Connell et al., 1994). However, experiments in cultured cells have provided no evidence that human Nek2 is required for either process. In this study, depletion of X-Nek2B from egg extracts did not interfere with either condensation or decondensation of chromosomes. One could argue that sperm chromatin already carries some X-Nek2B which fulfills these functions in the first cell cycle. However, this seems unlikely given the lack of signal either by immunofluorescence microscopy or on immunoblots of sperm nuclei prior to incubation in egg extracts. Our results rather argue that Nek2 kinases are not required for chromosome condensation in vertebrates. Equally, we could find no evidence using these cell-free assays that X-Nek2B is required for either MPF activation or mitotic entry. Admittedly, we cannot rule out a role in the adult cell cycle, as it is well known that important differences exist between the control of embryonic and adult cycles. In particular, embryonic cycles are devoid of detectable G_1 and G_2 phases, and the DNA damage and spindle-assembly checkpoints are not effective. However, in line with our results, mutants of human Nek2 have not yet been identified that can act in a dominant-negative fashion with respect to adult cell cycle progression. Furthermore, if a NIMA-related function has been conserved throughout evolution, it remains possible that other NIMA-related kinases have taken over these roles in higher eukaryotes.

X-Nek2B and functional centrosome assembly

The assembly of the zygotic centrosome to produce a functional microtubule nucleating centre represents an excellent model system to investigate centrosome organization (Archer and Solomon, 1994). Sperm basal bodies are only competent for growth of the flagellum from the distal centriole. However, after entering egg cytoplasm, basal bodies are converted into active centrosomes capable of nucleating extensive microtubule asters. The processes involved in this transformation are almost entirely unknown. Importantly, there is a significant time lag of around 7-10 minutes after addition to egg extract before sperm aster formation begins (Felix et al., 1994; Stearns and Kirschner, 1994; also this study). It is assumed that during this time components of the PCM necessary for microtubule nucleation are being assembled around the centrioles. γ-tubulin is present at low levels in sperm alone (Navara et al., 1997; Simerly et al., 1999; Tassin et al., 1998; also this study). However, isolated *Xenopus* and human centrosomes recruit significantly more γ-tubulin after incubation in CSF-arrested extracts and its appearance in significant quantities on the sperm basal body generally coincides with the beginning of aster formation (Felix et al., 1994; Simerly et al., 1999; Stearns and Kirschner, 1994). It is possible that γ-tubulin is recruited to the basal body primarily in the form of complete γ-tubulin ring complexes as other components of this complex which are also recruited are essential for both γ-tubulin recruitment and aster formation (Fava et al., 1999; Martin et al., 1998).

Circumstantial evidence points to protein phosphorylation as an essential regulator of functional centrosome assembly. Sperm centrosome phosphorylation is observed with the MPM-2 phosphoepitope antibody upon incubation in *Xenopus* egg extracts as well as in artificially-inseminated bovine oocytes (Simerly et al., 1999; Stearns and Kirschner, 1994). Moreover, the recruitment of γ-tubulin specifically requires ATP (Stearns and Kirschner, 1994). Here, we found that X-Nek2B is detected at the sperm basal body immediately following incubation in egg cytoplasm suggesting that, if there is a sequential pattern of assembly, X-Nek2B is likely to be one of the earliest proteins recruited. Certainly from a functional perspective, the appearance of X-Nek2B on the centrosome significantly precedes the time at which sperm aster formation is first observed. X-Nek2B recruitment, which occurs in a microtubule-independent manner, is also accompanied by its apparent phosphorylation, yet these are separable events as recruitment still occurs at 4°C. X-Nek2B phosphorylation might result from the action of upstream kinases already present at the basal body or, alternatively, be due to an autophosphorylation reaction that is stimulated by increased local concentration of the active kinase at the centrosome. Clearly, phosphorylation might be expected to change either the activity or substrate targeting of the X-Nek2B kinase and hence lead to its specific regulation within a particular sub-compartment of the cell.

Of most interest was the observation that depletion of X-Nek2B markedly delays the appearance of sperm asters suggesting that X-Nek2B somehow stimulates functional centrosome assembly (Fig. 8). A clue to the possible role of X-Nek2B came from observing that the rate of γ-tubulin recruitment was also delayed to a similar extent. There are several possible interpretations of these results, the most straightforward of which are: (i) X-Nek2B acts in a structural fashion as an essential early building block of the somatic centrosome onto which other proteins dock; (ii) X-Nek2B

kinase activity is required for catalyzing the assembly of the centrosome through phosphorylation of either centrosomal proteins or assembly factors; (iii) X-Nek2B kinase acts as a direct assembly factor for the γ-TuRC itself. A careful series of reconstitution experiments using recombinant wild-type and mutant X-Nek2B proteins will be required to distinguish between these possibilities. It is notable that X-Nek2B depletion only delays and does not prevent the eventual appearance of either γ-tubulin or microtubule asters. Such kinetics are typical of an enzyme-catalysed reaction which proceeds towards equilibrium and, at this stage, one possibility is that the role of Nek2B kinase is to catalyze centrosome assembly through protein phosphorylation. We certainly cannot rule out though that the extracts have not been totally depleted of X-Nek2B protein or that trace amounts of X-Nek2B (and/or X-Nek2A) are present at basal bodies prior to incubation in the extract. These residual populations of kinase might be sufficient to drive centrosome assembly albeit at a slower rate.

In summary, the biochemical approach used here has revealed a novel role for the X-Nek2B kinase in centrosome assembly, a complex process that is critical to the survival of the zygote and development of the early embryo. Our previous expression studies had indicated that human Nek2 might also contribute to the structural integrity of the preformed centrosome in adult cells (Fry et al., 1998a). Hence, vertebrate Nek2 kinases may play a general role in both assembly and maintenance of centrosome architecture. In further support of this hypothesis, inhibition of X-Nek2B function in early *Xenopus* embryos causes dispersal or fragmentation of centrosomes which, in turn, leads to defects in bipolar spindle formation and cleavage (N. Sagata, personal communication). Other data have also implicated Nek2 in regulation of centrosome separation and the induction of centrosome splitting by X-Nek2B expression in adult *Xenopus* cells implies that this putative function is conserved and, moreover, not restricted to the adult isoform of the Nek2 kinase. It seems inevitable then that *Xenopus* cell-free assays will continue to serve as an excellent system in which to further dissect the function of Nek2 kinases in bipolar spindle formation and the centrosome cycle.

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