CHARACTERIZATION OF NLP, A NOVEL CENTROSONAL SUBSTRATE OF THE NEK2 KINASE

Thesis submitted for the degree of
Doctor of Philosophy
at the
University of Leicester

by

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February 2004
DECLARATION

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled Characterization of Nlp, a novel centrosomal substrate of the Nek2 kinase, is based on work conducted by the author in the Department of Biochemistry at the University of Leicester mainly during the period between October 2000 and February 2004. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

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CHARACTERIZATION OF NLP, A NOVEL CENTROSOMAL SUBSTRATE OF THE NEK2 KINASE

JOSEPH RAPLEY

SUMMARY

The centrosome is a non-membraneous organelle composed of two centrioles surrounded by pericentrolar material. The primary function of the centrosome is to act as the dominant microtubule organizing centre in animal cells. It therefore contributes to formation of both the interphase cytoskeleton and bipolar mitotic spindle. Both centrosome structure and microtubule organization are controlled in a cell cycle dependant manner by protein phosphorylation. The centrosomal kinase Nek2 regulates centrosome organization, mitotic progression and bipolar spindle assembly. However, the only core centrosomal substrate of this kinase so far identified is C-Napl, a structural protein required for centriolar cohesion. The aims of this project were therefore to isolate a *Xenopus laevis* homologue of C-Napl in order to study its function using *Xenopus* based cell free assays and identify and characterize novel centrosomal substrates of Nek2. By database screening, we identified a *Xenopus* protein of high similarity to C-Napl called rootletin, while using the yeast two hybrid system we identified the *Xenopus* Nip protein as a novel substrate of Nek2. Human Nlp is a recently characterized centrosomal protein involved in microtubule organization and which is regulated by another centrosomal kinase, Plk1. Antibodies were raised to *Xenopus* Nlp and used to confirm subcellular localization to the centrosome in *Xenopus* cells. Further localization and expression studies revealed that Nlp is a mother centriole specific protein that is displaced from the centrosome, but not degraded, during mitosis. These data suggest that Nlp is involved in interphasic microtubule anchorage. By transfection into *Xenopus* and human cells, we found that although Nek2 and Plk1 phosphorylate Nlp at distinct sites, they can both trigger Nlp displacement from the centrosome at the onset of mitosis. Finally, data were obtained raising the possibility that Nek2 may act as a novel priming kinase for recruitment of Plk1 to its substrate Nlp.
ACKNOWLEDGEMENTS

For the duration of my PhD I have received much support from many friends and family. I would like to say a big thank you to my supervisor Dr Andrew Fry for all his help and guidance throughout the duration of this work, as well as to all members of lab 201 for their help and suggestions. Thank you to my mum who has always been supportive wherever I am, and late father for his enthusiasm during this period.
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ABBREVIATIONS

A<sub>260</sub> Absorbance at 260 nanometers
AD Activation domain
APC/C Anaphase promoting complex/cyclosome
ATP Adenosine triphosphate
BCA Bicinchoninic acid
BCIP 5-bromo-4-chloro-3-indolyl phosphate
bp base pairs
BSA Bovine serum albumin
C- Carboxy-
CDK Cyclin dependant kinase
cDNA Complementary deoxyribonucleic acid
CEP250 Centrosomal protein 250
CG-NAP Centrosome and Golgi localized PKN-associated protein
Ci Curie
cm Centimeter
CMFM Calcium magnesium free media
C-Nap1 Centrosomal Nek2 associated protein 1
CSF Cytostatic factor
dATP Deoxyadenosine triphosphate
DBD DNA binding domain
dCTP Deoxycytidine triphosphate
D-box Destruction box
dGTP Deoxyguanosine triphosphate
DMEM Dulbeco’s modified eagle’s medium
DMSO Dimethylsulfoxide
DNA Deoxyribonucleic acid
dNTP Deoxynucleotide triphosphates
DTT Dithiothreitol
dTTP Deoxythymidine triphosphate
EDTA Ethylene diamine tetra acetic acid
EGTA Ethylene glycol-bis (β-aminoethylether) N, N, N’ N’-tetraacetic acid
EST Expressed sequence tag
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<td>FACS</td>
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<td>FACT</td>
<td>Facilitates chromatin transcription</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>g</td>
<td>Gravity</td>
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<td>g/l</td>
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<td>IMS</td>
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<td>mm</td>
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<td>Mitotic phosphorylation motif (epitope)</td>
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<td>mRNA</td>
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<td>Msps</td>
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<td>MTOC</td>
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<tr>
<td>ng</td>
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<td>PBS</td>
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<td>rpm</td>
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<td>SPB</td>
<td>Spindle pole body</td>
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<td>TACC</td>
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XIV
CHAPTER 1

INTRODUCTION
1.1 The eukaryotic cell cycle

For the duplication of a cell, the genetic content must first be replicated then co-ordinately segregated to the two daughter cells. Failure to perform these procedures correctly will lead to a daughter cell containing a genetic complement with an additional or reduced number of chromosomes, ultimately providing an excess or absence of specific gene products. The consequences resulting from an abnormal DNA complement can cause apoptosis or contribute to the progression and development of tumorigenesis in adult cells. During development, embryonic abnormalities would occur resulting in either an aborted foetus or offspring with birth defects. Therefore, to ensure an organism’s survival and propagation DNA must be replicated and segregated in a timely controlled manner.

Cell division is executed through a highly regulated process known as the “cell cycle” (Alberts et al., 2002). The cell cycle can be split into four distinct sections referred to as G1, S, G2 and M, all of which have discrete events and regulation. Cells that are non-proliferative enter a quiescence stage referred to as G0 (Figure 1.1). Cells entering the proliferative cycle must first pass through G1, when extracellular signals trigger growth and preparation for DNA replication. Following on from G1 is S phase when the important event of DNA replication takes place, giving a diploid cell a 4n DNA content. G2 then proceeds when an increase in cell mass takes place to prepare the cell for its final step M phase, when the duplicated DNA is separated evenly into two daughter cells (2n again). This may seem a straightforward system, but it is subject to many levels of regulation, with protein phosphorylation and degradation being major factors.

During interphase, chromosomes are decondensed and individually undetectable using a light microscope. In contrast, the dynamic events of mitosis are clearly visible by light microscopy and allow mitosis to be divided into discrete stages (Pines and Rieder, 2001). Mitosis begins with chromosomes condensing into discrete compact structures while the nuclear envelope remains intact, an event called prophase (Figure 1.2). Prometaphase begins with the fragmentation of the nuclear envelope allowing chromosomes to become attached to the emerging mitotic spindle. The captured chromosomes become positioned upon the cell’s equator, a state called metaphase. Once all chromosomes are positioned upon the metaphase plate and attached to the mitotic spindle, anaphase begins with sister chromatids pulled apart by the action of microtubules. The progression of anaphase causes
Figure 1.1 Stages of the cell cycle

The cell cycle can be divided into four stages of which three make up interphase (G1, S and G2) and one mitosis (M). Cells that receive no signal to grow become quiescent (G0), whereas upon growth stimulation quiescent cells re-enter G1.
chromosomal movement towards each cell pole creating two discrete chromosome populations. The process of telophase sees the chromosomes decondense while a nuclear envelope reassembles around the DNA. Telophase completes the mitotic segregation of chromosomes. However, to complete the separation of cytoplasm and physical division of the cell another process called cytokinesis is required. This involves the formation and constriction of an actin-based contractile ring causing an invagination of the plasma membrane that leads to the formation of a cleavage furrow. Cell migration and further constriction causes the cleavage furrow to thin and form a microtubule dense structure called the midbody. Abscission occurs within the midbody resulting in two daughter cells marking the end of cell division.

1.2 Microtubule organizing centers (MTOCs)

MTOCs are found in all eukaryotic systems but are not present in prokaryotic cells. The MTOC is a cellular organelle that lies at the heart of the microtubule network during interphase, while during mitosis it is involved in formation of the bipolar spindle. MTOCs have been studied in various organisms with the mammalian centrosome having the most extensive dissection. The yeast MTOC (spindle pole body, SPB) has also undergone extensive studies and although the yeast SPB shows similarities in function, it contains many different structures when compared to the mammalian centrosome. One may presume that during evolution the MTOC has adapted to suit its host species’ requirements.

1.2.1 The mammalian centrosome: structure and function

The mammalian MTOC is called the centrosome and consists of distinct organized structures associated with a vast interchangeable protein pool totaling a combined volume of 1 μm³. The composition of the centrosome is not static and alters throughout the cell cycle to integrate with its environment. The molecular composition of the centrosome is still incompletely defined but the bank of identified protein candidates with known functions is growing rapidly (Andersen et al., 2003; Andersen, 1999; Mack et al., 2000). The centrosome consists of two core centrioles which lie perpendicular to each other and are surrounded by an electron dense protein meshwork called the pericentrolar material (PCM) (Bornens, 2002; Doxsey, 2001; Marshall, 2001) (Figure 1.3). The two centrioles are called the mother and daughter and are distinguishable by appendage structures.
Figure 1.2 The stages of mitosis

Interphase chromosomes are decondensed and remain within the nuclear envelope. Prophase sees chromosome condensation occur. Prometaphase sees the breakdown of the nuclear envelope and chromosome attachment to the mitotic spindle. Metaphase chromosomes line up on the cell equator. Anaphase begins with chromosome splitting and poleward movement. Telophase cells begin chromosome decondensation, nuclear envelope reformation and plasma membrane invagination. Cytokinesis results in the formation of the cleavage furrow, followed by abscission resulting in two separate daughter cells. Images were taken from the University of Arizona website “The cell cycle and mitosis tutorial” @ http://www.biology.arizona.edu/cell_bio/tutorials/cell_cycle/cells3.html
associated exclusively with the mother. Both centrioles are composed of nine triplets of microtubules that form the central microtubule scaffold to which other components adhere.

The nine triplet microtubules within the core centriole structure are composed primarily of $\alpha/\beta$-tubulin dimers. Astral microtubules have a similar composition but are less stable than those within the centriole. The $\alpha/\beta$-tubulin dimers of the centrioles become heavily modified through post-translational mechanisms, including polyglutamylation, which increases their stability (Bobinnec et al., 1998). The stability of the centrioles is critical for providing a stable structure that will resist depolymerization under conditions where microtubules are undergoing dynamic instability. Both centrioles display polarity with a proximal (minus end of centrioles) and distal (plus end of centrioles) end that are defined by the growth of the primary cilium from the distal end of the mother centriole. The appendages of the mother centriole are located towards the distal end while a cartwheel structure is detectable at the proximal lumen. The composition of the cartwheel structure located within the nine triplet microtubules is unknown, but a protein called centrin is located at the distal lumen (Paoletti et al., 1996). The appendages upon the mother centrosome fall into two types, distal appendages and subdistal appendages, and are thought to have distinct functions (Bornens, 2002). The distal appendages are required for the anchoring of the basal body to the membrane, this allows the distal lumen to point outwards from the cell in turn allowing the polymerization of a cilium/flagellum into the external environment. Two proteins forming the distal appendage have been identified, cenexin (Lange and Gull, 1995) and p210 (Lechtreck et al., 1999). The subdistal appendages are involved in microtubule anchorage and are absent during mitosis, ninein is a component of these structures (Mogensen et al., 2000). Other less well characterized proteins intimately associated with the centrioles include Sp97, Sp83 (Hinchcliffe and Linck, 1998) and tektins (Stephens and Lemieux, 1998).

The two centrioles are maintained in close proximity during interphase by a poorly defined structure called the intercentriolar linkage. Interconnecting fibers extend between the proximal end of each centriole (Bornens et al., 1987) and may be attached to the centrioles via a protein called C-Nap1 which has been shown to be involved in centriolar cohesion (Fry et al., 1998a). The precise composition of the linkage is unknown but the proteins $\delta$-tubulin (Chang and Stearns, 2000), p160ROCK (Chevrier et al., 2002) and $\theta$-tubulin (Galvani et al., 2002) localize in this vicinity.
Figure 1.3 The centrosome structure
Simplified diagram highlighting the main structural features of the centrosome. The two centrioles are connected by an intercentriolar linkage and surrounded by the PCM. The mother centriole contains the characteristic additional appendages. Image was adapted from Urbani and Stearns (1999).
Additional proteins associate directly with the centriole but are not core components: many show a "loose" association with the centrosome and can be located within the PCM (Bornens, 2002; Doxsey, 2001). Centriolar satellites also exist within and beyond the PCM (Vorobjev and Chentsov, 1982) and recently have been implicated in protein recruitment to the centrosome via microtubules (Dammermann and Merdes, 2002). The only known component of the satellites is PCM-1 (Balczon et al., 1994) which may bind to other centrosomal proteins and facilitate their recruitment. The PCM harbours the site of microtubule nucleation which is catalyzed by the γ-TuRC (Moritz et al., 1995; Zheng et al., 1995) and associated proteins (see section 1.3.2). The centrosome structure is therefore well adapted to perform the complex task of microtubule organization. Having a stable scaffold allows for the recruitment and concentration of additional proteins that permit the centrosome to nucleate and anchor microtubules making it the dominant MTOC within the cell.

The primary function of the centrosome within the cell is the polymerization of microtubules from α- and β-tubulin heterodimers. Polymerization occurs from centrosome associated complexes called γ-tubulin ring complexes (γ-TuRCs) (Moritz et al., 1995; Zheng et al., 1995; Zimmerman et al., 1999). Microtubules have an array of functions within the cell, including the formation of the bipolar spindle during mitosis (see section 1.3.4). However, experiments in which the centrosome was removed from cells have recently shown that the centrosome has multiple functions apart from microtubule nucleation. Cells lacking centrosomes show a failure in the rate of cytokinesis, that may result from an inadequate repositioning of the spindle (Khodjakov and Rieder, 2001; Piel et al., 2001). Acentrosomal cells that do complete mitosis arrest in G1 and never enter S phase (Hinchcliffe and Miller, 2001; Khodjakov and Rieder, 2001), suggesting a critical role for the centrosome in cell cycle progression. How centrosomes promote this signal is currently unknown. A speculative model is that during the cell cycle the centrosome acts as a scaffold or platform bringing enzymes and substrates into close proximity (Fry and Hames, 2004). Alternatively, a monitoring system or checkpoint may exist to detect the presence of the centrosome and allow progression. In support of this model, centrosomes lacking a recently identified mother centriole protein called centriolin have cytokinetic defects and arrest in G1; the presence of centriolin at the centrosome may thus be monitored for cell cycle progression (Gromley et al., 2003). The centrosome can also take on specialized functions in differentiated cells and become a basal body. Here, centrioles
localize to the cell membrane and promote the polymerization of a cilium or flagellum in different cell types.

1.2.2 The centrosome duplication cycle
Centrosomes must duplicate in a cell cycle specific manner in order to form a bipolar spindle and ensure inheritance of a single centrosome by each daughter cell (Figure 1.4). As the cell enters G1, the centrioles lose their perpendicular orientation and move independently despite remaining connected by the intercentriolar linker, an event known as centriole disorientation. Centrioles begin duplication at the onset of S phase coincident with initiation of DNA replication. A pro-centriole emerges perpendicular to the proximal lumen of the older centrioles and elongates throughout S phase and G2. During G2 centrosome maturation occurs with the recruitment of additional proteins in preparation for mitosis. At the end of G2, centriole cohesion is lost between the mother and daughter centriole by the removal of the intercentriolar linker allowing duplicated centriole pairs to move towards the cell poles and establish the bipolar spindle.

During the cell cycle, specific behavioral patterns are observable for both the mother and daughter centriole. Disorientation has been reported to occur before mitotic exit, during or before telophase (Piel et al., 2000). Piel et al. also showed that the mother centriole remains near the cell centre during G1, while the daughter can move extensively throughout the cytoplasm. During the G1/S transition, the movement becomes attenuated and by mitosis the behavior of both is identical. It has also been reported that the mother centriole moves into the midbody between cells during cytokinesis, while abscission causes its relocation to the cell centre (Piel et al., 2001). This would coincide with the centrosome having a role in cytokinesis as previously mentioned.

1.2.3 Yeast MTOCs
The yeast MTOC is called the spindle pole body (SPB) and shares some similarities but also has noticeable differences with the centrosome. Many components of the SPB from the budding yeast _Saccharomyces cerevisiae_ have been identified and characterized by mass spectrometry, biochemical and genetic techniques (Francis and Davis, 2000; Helfant, 2002; Wigge et al., 1998). The SPB structure and composition of the fission yeast _Schizosaccharomyces pombe_ is less well defined (Hagan and Petersen, 2000). The structure of the budding yeast SPB reveals it is a cylindrical organelle consisting of distinct
Figure 1.4 The centrosome duplication cycle

The centrosome duplicates in a semi-conservative manner co-coordinated with the initiation of DNA synthesis. During G1, the mother (with appendages) and daughter centrioles (blue) lose their perpendicular arrangement but remain connected by the intercentriolar linker. During S phase, the procentrioles (yellow) emerge from the proximal end of both preceding centrioles. Progression through G2 allows the procentrioles to elongate, then the centrosomes begin to mature (with the recruitment of additional PCM (purple) and the linkage is removed. The centrosomes migrate towards the spindle poles and continue maturing into M phase. The bipolar spindle can then be established.
layers called plaques (Figure 1.5A); no centrioles are present within the SPB which is embedded in the nuclear envelope throughout the cell cycle (O'Toole et al., 1999). Of the SPB substructure, the central plaque is the only area directly embedded within the nuclear envelope and comprises two core structural proteins called Spc29p and Spc42p. The remaining four core structural proteins called Nudlp, Cnm67p, Cmdlp and Spc110p are located in either the cytoplasm or nucleoplasm (Figure 1.5A) (Adams and Kilmartin, 1999). As the SPB is embedded within the nuclear envelope and the nuclear envelope never breaks down during yeast mitosis, two discrete populations of microtubules exist which never come into contact with each other. The yeast homologue of γ-tubulin called Tub4p is responsible for microtubule nucleation on either side of the nuclear envelope (Sobel and Snyder, 1995) and exists in a complex with Spc97p and Spc98p (Geissler et al., 1996; Knop et al., 1997).

The SPB duplicates in a cell cycle controlled manner that is distinct compared to the centrosome, but the mother SPB is also required for the synthesis of the daughter SPB. Duplication initiation occurs during G1 with a half bridge structure that emanates from the SPB on both sides of the nuclear membrane. Two priming proteins called Kar1p and Cdc31p then appear upon the distal end of the half bridge in the cytoplasmic domain only. This allows a satellite to form which contains the core proteins Cnm67p, Nudlp, Spc42p and Spc29p which elongate to form a duplication plaque. Finally, the outer and inner plaque form while the daughter SPB fuses with the nuclear envelope, this process is completed during S phase. Once assembled, the half bridge is removed and both SPBs separate for migration towards the cell poles (Figure 1.5B)(Adams and Kilmartin, 1999; Francis and Davis, 2000). During mitosis, the mother SPB moves into the bud neck and relocation into the daughter cell allows cytokinesis to occur producing two daughter cells, the interaction between the mother SPB and cortex microtubules with the bud neck aids this selection (Pereira et al., 2001). There is growing evidence for a SPB role in cytokinesis regulation. As the SPB moves into the bud neck, mitotic exit network factors associate with it permitting abscission to occur once the DNA is segregated correctly. The mitotic exit network ultimately ends mitosis by the release of the phosphatase Cdc14p from the nucleolus which causes dephosphorylation of CDK substrates (Pereira and Schiebel, 2001; Simanis, 2003).
Figure 1.5 The yeast spindle pole body (SPB)

(A) The main components of the SPB, adapted from Helfant (2002). (B) SPB duplication cycle. (1) The satellite forms at the distal end of the half-bridge. (2) Assembly of Spc42p and probably Spc29p enlarges the satellite to form the duplication plaque, the half bridge elongates. (3) Insertion into the nuclear envelope, nucleoplasmic SPB components assemble. (4) Side-by-side SPBs connected by the bridge. The SPBs then move through the nuclear envelope to form the spindle poles. Images and text taken from Adams and Kilmartin (1999).
Another useful model organism with growing information on the centrosome is the slime mould *Dictyostelium discoideum*, which can be used to examine multicellular development, as well as the somatic cell cycle. The *D. discoideum* centrosome structure is evolutionary somewhere between the yeast SPB and the centrosome; it contains distinct layers and has no centrioles but is surrounded by electron dense nodules resembling PCM (Graf et al., 2000). The centrosome cycle is also different, the centrosome is cytoplasmic during interphase and embedded within the nuclear envelope during mitosis. Duplication only takes fifteen minutes and occurs exclusively in mitosis (Ueda et al., 1999). Antibodies have been raised to a variety of *D. discoideum* centrosome proteins but await further characterization (Graf et al., 1999). Other species used for centrosomal studies are the nematode worm *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster*. The centrosome structures themselves are very similar to those in mammalian cells, although *Drosophila* centrioles tend to be shorter than vertebrate centrioles.

Homologous functions are conserved between MTOCs in different species despite the variation in structure. In the African clawed toad, *Xenopus laevis*, fertilization of eggs is accompanied by conversion of the sperm basal body into the first zygotic centrosome. The zygotic centrosome is essential for cleavage and development of the embryo. MTOCs from other species can be added to *Xenopus* egg extracts to see if they can nucleate microtubules, or be injected into eggs to see if this can induce parthenogenesis (Tournier et al., 1999). Addition of a yeast SPB does not induce aster formation or parthenogenesis, whereas a *Drosophila* centrosome can form asters but not initiate parthenogenesis (Tournier et al., 1999). Therefore, *Drosophila* and *Xenopus* centrosomes share common nucleation methods but are not entirely interchangeable. Injection of human centrosomes into starfish oocytes can permit parthenogenetic development indicating a great degree of conservation between the centrosomes of these species (Picard et al., 1987). Human centrosomes can also induce parthenogenesis in *Xenopus* (Tournier et al., 1989). Plants do not contain centrosomes or SPBs at all and have developed alternative mechanisms for cell cycle progression and DNA segregation (Canaday et al., 2000; Wasteneys, 2002).
1.3 Microtubules

1.3.1 Microtubule structure and dynamics

Microtubules are dynamic macromolecular structures within cells that are essential for a diverse range of processes including formation of the interphase cytoskeleton and mitotic spindle, cell migration and intracellular trafficking (Alberts et al., 2002). The molecular structure of a microtubule comprises a hollow tube (25 nm in diameter) containing thirteen parallel protofilaments surrounding a central lumen. The protofilaments are comprised of tubulin heterodimers, each containing an α- and β-tubulin subunit which gives the overall structure a polarity due to assembly occurring in the same direction. The α-tubulin subunit contains one GTP molecule that is encased within the protein structure and never hydrolysed or exchanged. The β-tubulin subunit also contains one GTP molecule but has the capacity for hydrolysis producing GDP, which is exchangeable with the cytoplasmic GTP pool. The hydrolysis and availability of GTP has a major impact upon microtubule dynamics as β-tubulin containing GTP supports microtubule growth, while β-tubulin containing GDP supports microtubule shrinkage (Job et al., 2003). Because microtubules continuously elongate and depolymerize, a change in the rate of either growth or shrinkage, or shift from growth to shrinkage (catastrophe) or shrinkage to growth (rescue) will determine the length at any given moment (Alberts et al., 2002; Mitchison and Kirschner, 1984).

The β-tubulin-GTP is present upon the dynamic growing end of the microtubule, and is called the plus end, the less dynamic β-tubulin-GDP end is called the minus end. Generally, the plus end is located at the cell cortex/away from the MTOC while the minus end is in close proximity to the MTOC (Alberts et al., 2002). Microtubule stability can also be regulated by microtubule associated proteins (MAPs), some of which bind preferentially to either the plus or minus end (Dammernann et al., 2003; Mimori-Kiyosue and Tsukita, 2003). The growing plus ends capture mitotic chromosomes, cellular organelles and contact the cell cortex and associated protein complexes at the plus ends are responsible for this “search and capture” mechanism. Proteins known to associate with the plus ends include adenomatous polyposis coli, Tea1p, cytoplasmic linker proteins, LIS-1, dynein, dynactin, ch-TOG and EB1 (Mimori-Kiyosue and Tsukita, 2003; Schroer, 2001). The mechanisms by which these proteins confer stability and allow microtubules to attach to their targets are unknown. The focus of the remainder of this section however, is aimed
at events occurring at the minus end of microtubules as this is the site of microtubule nucleation and anchorage. Microtubule plus ends will not be discussed in any further detail.

1.3.2 Microtubule nucleation

The PCM contains the necessary elements for microtubule nucleation in centrosome containing cells and harbours a protein called γ-tubulin which is directly responsible for microtubule nucleation. γ-tubulin is conserved in all eukaryotes being present in centrosomes, SPBs and other MTOCs (Oakley, 2000; Oakley et al., 1990). In higher eukaryotes, γ-tubulin exists in a large protein complex (25-32S, ~2MDa) called the γ-tubulin ring complex (γ-TuRC), which consists of at least eight additional proteins and conforms to a ring like structure (Job et al., 2003; Martin et al., 1998; Moritz et al., 1995; Murphy et al., 1998; Zheng et al., 1995). γ-tubulin can only nucleate microtubules when integrated as a component of the γ-TuRC, the γ-TuRC is the most efficient microtubule nucleator in vivo (Hannak et al., 2002). A tetrameric complex (γ-tubulin small complex, γ-TuSC) consisting of Dgrip84 (homologue of S. cerevisiae Spc97p), Dgrip91 (homologue of S. cerevisiae Spc98) and two γ-tubulin molecules has been shown to be the minimal requirement for microtubule nucleation in Drosophila, but is twenty five times less efficient than the full γ-TuRC (Oegema et al., 1999). The γ-TuSC is an integral component of the γ-TuRC, has homologous proteins in all eukaryotes and does not exist alone in vivo except for Drosophila and S. cerevisiae (Knop and Schiebel, 1997; Martin et al., 1998; Oegema et al., 1999). Homologues of Spc97p-Spc98p have been characterized in other species and are called Dgrip84-Dgrip91 (Drosophila), Xgrip109 (Xenopus Spc98p) and GCP2-GCP3 (human), all can bind directly to γ-tubulin in vivo (Knop et al., 1997; Knop and Schiebel, 1997; Martin et al., 1998; Murphy et al., 1998; Oegema et al., 1999). The human γ-TuRC contains at least seven additional proteins to γ-tubulin (GCP1) which are called GCPs (γ-tubulin complex proteins); to date five GCPs have been characterized and all can bind to microtubules (GCP2-6) (Fava et al., 1999; Murphy et al., 2001; Murphy et al., 1998).

The mechanism by which γ-TuRCs nucleate microtubules is unknown, but two models have been suggested. As the γ-TuRC itself is the same diameter as a microtubule (25 nm) and forms a ring structure, the first “template” model proposes that the γ-TuRC may act as a template upon which the α/β-tubulin heterodimers assemble. It is proposed that thirteen
γ-tubulin molecules arranged in a ring bind the α/β-tubulin heterodimers; these in turn allow assembly of further heterodimers and an increase in microtubule length (Zheng et al., 1995). The second model called the "protofilament model" suggests linear filaments of α/β-tubulin heterodimers form and these filaments then assemble laterally while rotating around the γ-TuRC (Erickson and Stoffler, 1996). Current evidence favors the former template model (Keating and Borisy, 2000; Moritz et al., 2000; Wiese and Zheng, 2000).

During interphase, 80% of γ-tubulin is cytoplasmic, the remainder associates with the centrosome (Moudjou et al., 1996). At the G2/M transition, additional γ-tubulin is rapidly recruited to the centrosome, whereas from anaphase through to telophase there is a cytoplasmic redistribution of γ-tubulin (Dictenberg et al., 1998; Khodjakov and Rieder, 1999). The mechanism by which γ-tubulin is recruited to the centrosome is unknown, but both microtubule dependant and independent pathways have been proposed (Dammermann and Merdes, 2002; Dictenberg et al., 1998; Khodjakov and Rieder, 1999; Young et al., 2000). Salt-stripped centrosomes are devoid of nucleating activity but addition to a cell extract restores nucleating capability (Moritz et al., 1998; Schnakenberg et al., 1998). However, incubating only salt-stripped centrosomes with γ-TuRCs does not restore nucleating capability, indicating that the γ-TuRC alone is not sufficient for microtubule nucleation (Moritz et al., 1998). Cytoplasmic γ-TuRCs are devoid of nucleating activity and centrosome association is required to facilitate microtubule nucleation (Moudjou et al., 1996), implying that a docking site (anchor) at the centrosome must activate the γ-TuRC. Likewise, the yeast γ-TuSC (Spc97, Spc98 and two Tub4 molecules) is nucleation competent only when bound to the SPB; Spc97p and Spc98p anchor to the SPB by direct attachment to Spc110p (nucleoplasm-inner plaque) and Spc72p (cytoplasmic-outer plaque) (Figure 1.5A) (Knop and Schiebel, 1997; Knop and Schiebel, 1998; Nguyen et al., 1998).

A distant homologue of Spc110p exists in humans called kendrin/pericentrin B (Flory et al., 2000), which is an alternate splice variant of a centrosomal protein called pericentrin (Flory and Davis, 2003). Pericentrin is a conserved centrosomal protein found in the PCM which interacts with the γ-TuRC and is implicated in microtubule nucleation and organization (Dictenberg et al., 1998; Doxsey et al., 1994; Mogensen et al., 1997; Young et al., 2000). Kendrin complexed with another centrosomal protein CG-NAP (also called AKAP350/AKAP450) is capable of anchoring the γ-TuRC to the centrosome by direct association with GCP2 and GCP3. Antibodies to either kendrin or CG-NAP abolish
microtubule nucleation (Takahashi et al., 2002). Recently, a *Drosophila* protein called CP309 has been characterized that shows high similarity to CG-NAP and kendrin and facilitates a similar function by binding to the γ-TuRC and permitting microtubule nucleation (Kawaguchi and Zheng, 2004). Hence, γ-TuRC anchoring mechanisms may be highly conserved in all eukaryotes.

The centrosomal protein Nlp has been implicated in interphase microtubule nucleation as antibodies against Nlp reduced the rate of microtubule regrowth from centrosomes after microinjection into U2OS cells (Casenghi et al., 2003). GST-Nlp could pull down γ-tubulin and GCP4 from cell extracts implying that it may function as an additional component of the γ-TuRC anchor (Casenghi et al., 2003). A *Drosophila* protein called Asp is required for recruiting γ-TuRCs to the spindle pole during mitosis, but what γ-TuRC components it associates with is unknown (Avides and Glover, 1999; Saunders et al., 1997). Two other proteins called CEP135 and CPAP also interact with the γ-TuRC and inhibition of either hinders aster formation, but their exact roles have yet to be determined (Hung et al., 2000; Ohta et al., 2002). The simpler structure of the SPB combined with the biochemical data implicate a simple γ-TuRC anchoring mechanism in yeast. The more complex structure of the centrosome combined with larger γ-TuRCs and associating proteins suggests a more complex anchoring mechanism may exist in higher eukaryotes. Spc110p and kendrin are present at the SPB and centrosome, respectively, throughout the cell cycle, but Nlp is centrosomal during interphase only, suggesting that alternative mechanisms of γ-tubulin anchoring may exist between interphase and mitotic cells.

Centrosomes are absent in plant cells and some animal meiotic cells, yet microtubules and a bipolar spindle still form (Heald et al., 1996; Hyman and Karsenti, 1998). During mitosis and meiosis, chromosomes are capable of nucleating microtubules through the Ran-GTPase (Ohba et al., 1999; Wilde and Zheng, 1999). The guanine nucleotide exchange factor, RCC1, is associated with chromatin and maintains Ran in a GTP-bound state around the vicinity of chromosomes, thereby ensuring only local nucleation (Carazo-Salas et al., 1999). Ran-GTP appears to promote the release of "aster promoting activators" including TPX2 and NuMA, but exactly how these control microtubule nucleation is unknown (Gruss et al., 2001). Microtubule nucleation in plants occurs only at the nuclear surface (Mizuno, 1993; Stoppin et al., 1994). Plants also contain γ-tubulin and a homologue of Spc98p; both localize to the nuclear envelope and are essential for
microtubule nucleation (Erhardt et al., 2002; Liu et al., 1994). Hence, proteins involved in microtubule nucleation appear to be very well conserved in all eukaryotes.

1.3.3 Microtubule release and anchorage

Following nucleation, microtubules are frequently released from the centrosome (Keating and Borisy, 1999). They may remain in close proximity to the centrosome to establish an aster, or they may be released from the centrosome, translocated, then anchored at an apical site creating a pool of centrosomal and non-centrosomal microtubules (Keating et al., 1997; Mogensen, 1999; Mogensen et al., 1997). The mechanism controlling microtubule release from the centrosome is unknown but a protein called katanin has been implicated in this process (McNally et al., 1996; McNally and Vale, 1993). Katanin is an ATPase that can sever and disassemble microtubules from polymers into α/β-tubulin heterodimers (McNally and Vale, 1993). Katanin is localized at the centrosome during the entire cell cycle, although its localization is dependant upon microtubules which may themselves be severed (McNally et al., 1996; McNally et al., 2000). In *Xenopus* egg extracts, katanin is centrosomal during mitosis only, implying it may release the astral microtubules characteristic of mitotic onset (McNally et al., 2002).

The PCM associated with both centrioles is capable of microtubule nucleation but only the mother centriole appears to anchor microtubules. This is based upon the observation that microtubule minus ends associate with the tips of the appendages (Mogensen et al., 2000; Ou et al., 2002; Piel et al., 2000). However, few proteins have been implicated in interphase microtubule anchorage at centrosomal and non-centrosomal sites. One of the best characterized of these is ninein, a large coiled coil protein containing a putative EF-hand and GTP binding site (see Figure 4.2). Ninein is concentrated around the mother centriole appendages, the proximal ends (minus ends) of both centrioles and microtubule minus ends at apical microtubule arrays in epithelial cells (Bouckson-Castaing et al., 1996; Mogensen et al., 2000; Ou et al., 2002; Piel et al., 2000). Murine ninein has been reported to be centrosomal throughout mitosis and can associate with the spindle until metaphase (Bouckson-Castaing et al., 1996), although the abundance of murine ninein at the centrosome in mitosis maybe decreased (Mette Mogensen, personal communication). Indeed, human ninein is absent from the centrosome during prophase to anaphase and reaccumulates on the mother centriole during telophase, concurrent with appendage re-appearance (Chen et al., 2003; Ou et al., 2002). Ninein is recruited to the centrosome by
the satellite containing PCM-1 complexes, which in turn are recruited to the centrosome in an ATP-dependent manner by the motor protein dynein (Dammermann and Merdes, 2002; Kubo et al., 1999). Inhibition of PCM-1 causes a reduction in ninein assembly at the centrosome and leads to disruption of the microtubule radial organization but not microtubule nucleation, consistent with a role for ninein in anchorage but not nucleation (Dammermann and Merdes, 2002).

Ninein is also associated with released microtubules as they translocate to apical sites; here ninein may act to cap the minus ends stabilizing the microtubules until they have relocated (Mogensen et al., 2000). The apical sites contain ninein but not pericentrin or γ-tubulin, therefore ninein appears to anchor microtubules at both centrosomal and non-centrosomal sites (Mogensen et al., 1997; Mogensen et al., 2000). Overexpressing ninein increases the anchoring capacity of the centrosome and prevents microtubule release; in migrating cells this prevents locomotion by preventing microtubules reaching the lamellipodial regions (Abal et al., 2002). Microinjection of antibodies against ninein in interphase cells only disrupts the microtubule array, but microinjection into mitotic cells causes abnormal centrosomes to form upon mitotic exit implying that ninein is also involved in centrosome reorganization (Ou et al., 2002). The regulation of ninein is poorly understood although the centrosomal targeting domain has recently been identified (Chen et al., 2003). Ninein can be phosphorylated by PKA and Aurora A in vitro, suggesting that a mitotic kinase may displace ninein from the centrosome in a manner similar to C-Nap1 and Nlp (Casenghi et al., 2003; Chen et al., 2003; Mayor et al., 2002). Displacement of ninein may be a requirement for bipolar spindle formation in human systems by reorganizing the microtubule anchoring capabilities of the centrosome. The microtubule binding protein dynactin is also implicated in microtubule anchoring at centrosomes, but it is unclear whether this is mediated by a direct attachment to the centrosome, or cross-linking of released microtubules (Quintyne et al., 1999). Ran (which associates with the γ-TuRC anchor CG-NAP) also associates with microtubule minus ends and localizes to the PCM and appendages at centrosomes. In addition, Ran is also detected at apical non-centrosomal microtubule arrays suggesting it has a role in microtubule anchorage, but the molecular details remain elusive (Keryer et al., 2003).
1.3.4 Bipolar spindle formation

When present, the centrosome is the major site of microtubule nucleation and anchorage within the cell, but microtubule nucleation and anchorage alone are insufficient to establish a bipolar spindle. MAPs are essential in bipolar spindle formation, with a particularly important role played by plus end and minus end directed ATPase motor proteins. The major motor proteins involved are the plus and minus end directed kinesin related proteins and the minus end directed dynein/dynactin complex (Compton, 1998; Gaglio et al., 1996; Merdes and Cleveland, 1997; Walczak and Mitchison, 1996). Dynein/dynactin together with the structural protein NuMA form a complex and are believed to cross-link and tether the microtubules close to the centrosome thereby forming a compact spindle pole. Antibody disruption to any of these three components prevents a compact spindle pole from forming (Dionne et al., 1999; Gaglio and Dionne, 1997; Merdes et al., 1996; Quintyne et al., 1999). The minus end directed kinesins are also believed to crosslink microtubules in a manner similar to dynein/dynactin/NuMA (Compton, 1998; Overchkina and Wordeman, 2003; Walczak and Mitchison, 1996). Antibody inhibition of a Drosophila kinesin Ncd or Xenopus kinesin XCTK2 causes non-focused spindle poles (Matthies et al., 1996; Walczak et al., 1997). The kinesin plus end motor Eg5 is involved in spindle formation on two fronts: it can move centrosomes towards the spindle poles and may focus microtubules at chromosomes in a manner similar to the minus end directed motor proteins (Gaglio et al., 1996; Giet et al., 1999; Walczak and Mitchison, 1996).

During mitosis, the mitotic spindle is anchored to the centrosome, but the majority of spindle microtubules do not appear to be directly connected to the centrosome (Mastronarde et al., 1993; McDonald et al., 1992). Dynein, dynactin and NuMA are thought to play a major role in anchoring microtubules in the region of the spindle pole (Dionne et al., 1999; Merdes et al., 1996; Quintyne et al., 1999). This complex may bind directly to γ-TuRCs that remain capping the minus end of microtubules as γ-tubulin localizes to the spindle as well as poles during metaphase (Khodjakov and Rieder, 1999; Leguy et al., 2000; Wiese and Zheng, 2000; Zheng et al., 1995). Another group of proteins that may anchor and stabilize the spindle microtubules are the TACCs (Transforming Acidic Coiled Coil proteins). Characterization of TACCS began in Drosophila, which encodes one isoform (D-TACC) and was subsequently extended to humans which contain three (TACC1, 2 and 3) isoforms (Gergely et al., 2000a; Gergely et al., 2000b). D-TACC associates with the centrosome throughout embryogenesis, whereas TACC1 and TACC3
are centrosomal only during mitosis while TACC2 is centrosomal throughout the cell cycle. The centrosomal association of all TACCs is independent of microtubules (Gergely et al., 2000a; Gergely et al., 2000b). Functional analysis of TACCs suggests a role in microtubule anchorage and stabilization as the generation of mutants or antibody microinjection causes the spindle to be less dense, shorter and less stable (Gergely et al., 2003; Gergely et al., 2000a; Gergely et al., 2000b). A MAP identified in *Xenopus* called XMAP215 produces the same phenotype upon immunodepletion as inhibition of the TACCs; mutations in the *Drosophila* homologue of XMAP215 (Msps, Mini spindles) also give similar phenotypes. Centrosome preparations containing the human homologue of XMAP215 (ch-TOG) promote microtubule assembly (Charrasse et al., 1998; Cullen et al., 1999; Kinoshita et al., 2002). D-TACC/TACC can interact with microtubules by complexing with XMAP215/Msps/ch-TOG, explaining the identical phenotype (Gergely et al., 2003; Lee et al., 2001). TPX2 and Ran-GTP are also involved in spindle formation in addition to affecting chromosome mediated microtubule nucleation (see section 1.3.2). TPX2 can organize the spindle pole by activating Aurora A which is known to influence bipolar spindle formation by centrosome maturation and migration (see section 1.4.1) (Tsai et al., 2003; Wittman et al., 2000). Figure 1.6 summarises potential roles of some of the proteins discussed in bipolar spindle formation.

Acentrosomal systems such as oocytes from *Drosophila* and *Xenopus* can still form a bipolar spindle during meiosis (Heald et al., 1996; Matthies et al., 1996). Microtubule motor proteins are responsible for focusing the spindle at microtubule minus ends, while microtubule nucleation is initiated at the chromosomes and further promoted by γ-tubulin accumulating at the minus ends (as previously discussed).

### 1.4 Centrosome Regulation

#### 1.4.1 Regulatory enzymes at the centrosome

The centrosome is carefully regulated through the cell cycle with phosphorylation playing a major role (Fry et al., 2000; Hinchcliffe and Sluder, 2001; Lange, 2002)(Figure 1.7). During late telophase/early G1, the centrosome cycle is initiated by centriole disorientation, whereby the two centrioles lose their orthogonal orientation while remaining connected by the intercentriolar linkage. The SCF ubiquitin ligase is responsible for targeting proteins for proteasome mediated degradation, inhibiting the SCF
Figure 1.6 Model of centrosome mediated spindle attachment
The centrosome nucleates microtubules and can anchor them directly by γ-TuRC association. Alternatively, microtubules can be released with or without γ-TuRC still capping their minus end. Released microtubules may have two fates: firstly, the TACC/ch-TOG complex captures and anchors them at an alternative centrosomal site; secondly, they become cross-linked by dynein/dynactin/NuMA and/or kinesin minus-end directed motor proteins. A γ-tubulin containing complex (not necessarily a γ-TuRC) may cap and stabilize the unanchored microtubules. A stable cross-linked microtubule structure is thus formed called the spindle pole.
ubiquitin ligase in vertebrate cells prevents disorientation from occurring. This suggests that a protein connecting the two perpendicular centrioles must be degraded to allow disorientation (Freed et al., 1999). Overexpressing RanBP1 (an activator of GTP hydrolysis by Ran) in mammalian cells causes centrioles to disorientate prematurely in early mitosis, resulting in tri/tetrapolar spindles (Fiore et al., 2003). RanBP1 can only induce splitting in mitotic cells suggesting that Ran-GTP is required to prevent premature centriole disorientation. It is interesting to speculate that the target for SCF degradation is therefore a Ran GTPase exchange factor such as RCC1.

Centriole duplication begins at the G1/S transition with the appearance of a procentriole; the release of E2F transcription factors and CDK2/cyclinA/E are required for this (Hinchcliffe et al., 1999; Hinchcliffe and Sluder, 2002; Lacey et al., 1999; Meraldi et al., 1999; Tarapore et al., 2002). CDK2/cyclin E can phosphorylate a centrosomal component called Nucleophosmin/B23, promoting its dissociation from the centrosome. Nucleophosmin/B23 only associates with unduplicated centrosomes, and is absent at the centrosome from S phase until mitosis, concurrent with CDK2 inactivation (Okuda et al., 2000; Tokuyama et al., 2001). Nucleophosmin/B23 has therefore been proposed as a centrosome duplication “licensing factor”. Mono polar spindle (Mps1) is a protein kinase that is essential for an intact spindle checkpoint and also appears to play a role in centriole elongation (Fisk et al., 2003; Fisk and Winey, 2001; Stucke et al., 2002). Mps1 kinase is a substrate for CDK2 and its phosphorylation appears to stabilize Mps1 and facilitate centriole elongation. Substrates of Mps1 involved in this process are unknown (Fisk et al., 2003; Fisk and Winey, 2001). The current model is that CDK2 phosphorylation of Nucleophosmin/B23 is required to initiate centriole duplication and that the action of Mps1 is then required to drive elongation. A second kinase identified as a regulator of centriole elongation was identified in C. elegans, called ZYG-1 (O'Connell et al., 2001). ZYG-1 is hypothesized to be the C. elegans homologue of Mps1 (O'Connell, 2002).

Upregulation of CDK2 can cause an increase in centrosome number, presumably by inappropriate duplication initiation (Hinchcliffe and Sluder, 2002). p53−/− mice also display centrosome hyperamplification, potentially caused by premature activation of CDK2 (Fukasawa et al., 1996; Tarapore and Fukasawa, 2002). In Drosophila, the SCF ubiquitin ligase targets cyclin E for degradation so inhibiting the SCF ubiquitin ligase causes multiple centrosomes to form through maintaining high activity of CDK2 (Wojcik et al.,
Xenopus egg extracts require calcium/calmodulin-dependant kinase II for centriole duplication, implying that calcium fluctuations may also be involved in this process (Matsumoto and Mailer, 2002).

Duplicated centrosomes must be split apart in a process called centrosome disjunction to enable establishment of the bipolar spindle. The Nek2 kinase is a prime candidate in triggering centrosome disjunction as overexpression can split centrosomes prematurely (Fry et al., 1998b). An in vivo centrosomal substrate for Nek2 has been identified, C-Nap1, with properties that reflect a role in centrosome cohesion (Mayor et al., 2002; Mayor et al., 2000; Meraldi and Nigg, 2001). Additional to Nek2, overexpressing CDK2, PKA or Cdc14A phosphatase can also induce premature centrosome splitting (Kaiser et al., 2002; Lutz et al., 2001; Mailand et al., 2002; Meraldi and Nigg, 2001). The substrates of Cdc14A and CDK2 are unknown with respect to this phenotype, although PKA can apparently phosphorylate centrin (Lutz et al., 2001).

Centrosome maturation sees additional material recruited to the centrosome, which enables bipolar spindle formation. During the maturation process, centrosomes change in microtubule nucleating capacity as well as separate and exhibit poleward movement. Polo-like kinases (see section 1.5.1), PP4 and Aurora A are implicated in maturation (Duterte et al., 2002; Glover et al., 1998; Nigg, 1998; Sumiyoshi et al., 2002). PP4 depletion causes γ-tubulin and Plk1 to be mislocalized from the centrosome, subsequently causing the formation of a defective bipolar spindle (Sumiyoshi et al., 2002). Aurora A kinase activity is required to recruit γ-tubulin, CeGrip (C. elegans Spc98p), ZYG-9 and D-TACC, all of which are required for bipolar spindle formation (Duterte et al., 2002; Giet et al., 2002; Hannak et al., 2001). Plk1 phosphorylation of Asp is required to recruit γ-tubulin to the spindle pole in Drosophila (Avides et al., 2001). Centrosome separation and poleward movement also require both Aurora A and Plk1. The association of Eg5 with microtubules is dependant upon phosphorylation and Aurora A can phosphorylate Eg5 and permit centrosome separation (Giet et al., 1999). Polo-like kinases have also been implicated in centrosome separation as antibody microinjection causes the formation of monopolar spindles with duplicated, but unseparated, centrosomes (Lane and Nigg, 1996) (see section 1.5.1). No motor protein substrates involved in centrosome movement have yet been identified for the Polo-like kinases.
1.4.2 Features of the duplicating centrosome

Centriole duplication is first detected by electron microscopy with the emergence of a procentriole perpendicular to the mother. How the procentriole appears on the wall of the older centriole and then elongates is entirely unknown. Deletion and mutational studies have implicated centrin as an essential component of both centrioles in humans and *Chlamydomonas*. Acentriolar or monocentriolar MTOCs appear as a result of centrin interference leading to cytokinetic defects (Salisbury et al., 2002; Taillon et al., 1992). In addition to α- and β-tubulin, δ- and ε-tubulin are also required for centriole assembly in *Xenopus* and *Chlamydomonas*, both are components of the core centrioles (Chang et al., 2003; Dutcher et al., 2002; Dutcher and Trabuco, 1998). Homologues of δ- and ε-tubulin also exist within the human genome; δ-tubulin is associated with the centrioles while ε-tubulin is found in the PCM and appendages (Chan and Stearns, 2000; Chang et al., 2003). A centriolar component identified in *C. elegans*, called SAS-4, may regulate centrosome size (Kirkham et al., 2003). SAS-4 is stably incorporated into the centrioles and removing SAS-4 prevents the formation of a daughter centriole. Reducing SAS-4 (but not eliminating it) permits the daughter centriole to grow, but the length is restricted. The quantity of PCM recruited is proportional to the centriole size as, when SAS-4 levels are reduced, less PCM associates with the centrioles. SAS-4 may therefore act as a centriolar scaffold to which the PCM associates (Kirkham et al., 2003).

In most adult cell cycles, centriole duplication utilises the mother centriole as a template from which the daughter emanates. However, in some embryonic systems centrosomes appear *de novo* whereby they form from no pre-existing centriole (Beisson and Wright, 2003). Chinese hamster ovary (CHO) cells arrested at S phase allow multiple rounds of centriole duplication to occur. If centrosomes are destroyed by laser microsurgery in S phase arrested CHO cells, centrioles reform randomly by a *de novo* pathway. First, a PCM cloud appears (5-8 h), followed by the centrioles (~24 h) within the PCM (Khodjakov et al., 2002). The number of centrioles formed does not correlate with a doubling number, indicating that ablated centrosomes are unlikely to be acting as a template. Normally, a mechanism is responsible for controlling centrosome number so that reduplication does not occur. Cell fusion experiments show that duplicated centrosomes will not re-duplicate even in an environment supportive of duplication (Wong and Stearns, 2003). It is most likely therefore that, under physiological conditions, duplicated centrosomes are modified so that they cannot reduplicate until progression through mitosis has occurred.
Figure 1.7 Regulators of the centrosome duplication cycle
Known regulators of the centrosome cycle with their potential function. Phenotypes generated by protein overexpression, RNAi, gene knockout, dominant negative mutants and immunodepletions have revealed the function of the proteins listed above.
1.5 Polo-like kinases

An important family of enzymes involved in centrosome regulation and cell cycle progression are the Polo-like kinases. The forefather of this family is called polo kinase and was initially identified as a *Drosophila* mutant allele causing monopolar mitoses with metaphase chromosomes conforming to a circular arrangement (Llamazares et al., 1991; Sunkel and Glover, 1988). This phenotype is in agreement for a role in centrosome maturation and separation as briefly mentioned above. Polo-like kinases have been characterized in a range of species and are called Cdc5 (*S. cerevisiae*), Plol (*S. pombe*), Plcl (*C. elegans*), Plxl (*Xenopus laevis*) and Plkl (mammals) (Golsteyn et al., 1994; Kitada et al., 1993; Kumagai and Dunphy, 1996; Ohkura et al., 1995; Ouyang et al., 1999). In addition to centrosome maturation and separation, Polo-like kinases also influence other aspects of mitosis, which include mitotic entry, spindle formation, metaphase/anaphase transition and cytokinesis (Dai et al., 2002; Glover et al., 1998; Nigg, 1998).

1.5.1 Functions of Polo-like kinases

Polo-like kinases, which have peak activity in mitosis, typically comprise of a highly conserved N-terminal catalytic domain and a C-terminal polo box domain (both greater than 50% identity between species) separated by an unconserved region (Figure 1.8A). All Polo-like kinases influence aspects of mitosis but do not perform identical functions in all species (Figure 1.8B), however the localization to the MTOC during early mitosis is a common feature (Dai et al., 2002; Glover et al., 1998; Nigg, 1998). Polo-like kinases have been implicated in mitotic entry by indirectly regulating the kinase activity of CDK1 (Cdc2). CDK1 activity is required to initiate mitosis and must be dephosphorylated by the Cdc25 phosphatase to permit mitotic initiation (Ohi and Gould, 1999). Cdc25 can be phosphorylated and activated by Plxl *in vitro* and *in vivo*, integrating Plxl into the CDK1 amplification loop to drive mitotic initiation (Abrieu et al., 1998; Kumagai and Dunphy, 1996; Qian et al., 1998a; Qian et al., 2001). Antibody inhibition of Plkl in non-immortalised cell lines causes a G2 arrest suggesting a conserved function in human systems (Lane and Nigg, 1996). Plkl can also phosphorylate cyclin B1 (Jackman et al., 2003) and Myt1, a repressor of CDK1 activity, therefore implicating Plkl in multiple pathways required for mitotic initiation (Nakajima et al., 2003). Localization to the MTOC at the time of CDK1 activation may facilitate these pathways through local protein concentration (Fry and Hames, 2004).
As introduced earlier, Polo-like kinases are involved in centrosome maturation, characterized by an increase in centrosome size prior to mitosis. Antibody microinjection against human Plk1 or *Drosophila* polo results in centrosomes with a reduced quantity of PCM and MPM2 phospho-epitopes (Lane and Nigg, 1996; Logarinho and Sunkel, 1998). Polo-like kinases are also involved in bipolar spindle formation as antibody inhibition or temperature sensitive mutants all lead to a monoastral spindle, with duplicated but unseparated MTOCs (Lane and Nigg, 1996; Ohkura et al., 1995; Qian et al., 1998a; Schlid and Byers, 1980; Sunkel and Glover, 1988).

The mechanisms by which polo-like kinases stimulate centrosome maturation and bipolar spindle formation are elusive, but some potential substrates have been identified. *Drosophila* embryos that are deficient for *Abnormal spindle* (Asp) develop mitotic spindle poles with no defined foci and show an increase in metaphase arrest caused by activation of the spindle checkpoint (Donaldson et al., 2001). Asp is a microtubule binding protein which organizes and recruits γ-TuRCs within the PCM to form a focused mitotic spindle (Avides and Glover, 1999; Saunders et al., 1997). Asp function is dependent upon phosphorylation by polo (Avides et al., 2001) as polo mutant cells display identical phenotypes to those lacking Asp (Donaldson et al., 2001). In contrast, Nlp is a vertebrate centrosomal protein involved in interphase microtubule organization, and is displaced from the centrosome during mitosis by Plk1 (Casenghi et al., 2003). Overexpressing Nlp in mitotic cells causes abnormal spindle poles to form implying its removal is essential for the establishment of a bipolar spindle (Casenghi et al., 2003). Plx1 may regulate the severing activity of katanin during mitosis, implying that it assists in the breakdown of astral microtubules as well as mitotic spindle shortening ( McNally et al., 2002). Microtubule nucleation also occurs from the kinetochores of mitotic chromatin, and can form a bipolar spindle in the absence of centrosomes (see section 1.3.4). A microtubule destabilizing protein called Op18/stathmin localizes to the kinetochores and can bind α/β-tubulin, thereby preventing its polymerization. Op18 loses affinity for tubulin during mitosis as a result of phosphorylation, thereby allowing kinetochore mediated microtubule nucleation to occur (Budde et al., 2001). Plx1 can phosphorylate Op18 *in vitro* and is a strong candidate for mediating this aspect of bipolar spindle formation, due to its kinetochore association during mitosis (Budde et al., 2001).
Figure 1.8 Structure and functions of Polo-like kinases

(A) Comparative structure of the Polo-like kinases from various species. **Blue boxes**, catalytic domains, **green boxes**, polo box domains and **red bars**, degradation motifs (D-box, RxxL). Numbers denote amino acid length. (B) Summary of mitotic functions performed by specific Polo-like kinases in different species. ✓, evidence suggests a role, ✗, no evidence to imply a role, ?, unknown.
The progression from metaphase to anaphase during mitosis requires the anaphase promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase which targets specific proteins for proteasome mediated degradation. Phosphorylation of the APC/C and its activating subunit Cdc20 is one mechanism which regulates its activity (Kraft et al., 2003; Morgan, 1999; Page and Hieter, 1999). The Polo-like kinases are involved in direct regulation of the APC/C as well as mitotic progression from this point. The kinase activity of Cdc5 is required to activate the APC/C, cause Clb2p degradation (cyclin B) and promote chromosome movement towards the spindle poles (Charles et al., 1998; Cheng et al., 1998; Shirayama et al., 1998). Plk1 and Plol directly phosphorylate the APC/C and Cdc20 causing activation (Kotani et al., 1998; Kraft et al., 2003; May et al., 2002), while Plxl also regulates APC/C activity but in an unknown manner (Brassac et al., 2000; Descombes and Nigg, 1998). Polo-like kinases also affect anaphase progression by targeting cohesin proteins required to hold sister chromatids together pre-anaphase. Scc1 (cohesin) can be phosphorylated by Cdc5 and this phosphorylation causes separase to cleave Scc1 allowing sister chromatids to be pulled apart to the spindle poles (Alexandru et al., 2001). In vertebrates, it has not been shown whether polo-like kinases are required for cohesin cleavage. The bulk of cohesins disappear from the chromosomes during prophase leaving a small population adhering to the sister chromatids until anaphase. Plxl kinase activity is required to remove cohesins from sister chromatids during prophase, and phosphorylation may decrease the affinity of cohesins for chromatin (Sumara et al., 2002). During anaphase, microtubules become more dynamic in order to segregate DNA. Overexpressing a microtubule stabilizing protein called TCTP prevents anaphase and causes multinucleated cells to form, which induces apoptosis. Plk1 can phosphorylate and displace TCTP from microtubules allowing an increase in microtubule dynamics, thereby aiding anaphase progression after release from the spindle checkpoint (Yarm, 2002).

Polo-like kinases also have roles in mitotic exit and cytokinesis. Cdc5 is involved in a signal transduction pathway to initiate cytokinesis but the downstream targets are unknown (Geymonat et al., 2003; Park et al., 2003). Plol is also involved in a signal transduction cascade with unknown targets, however Plol must localize to the SPB to initiate cytokinesis and may associate with other factors thereby concentrating them at the SPB (Mulvihill and Hyams, 2002; Mulvihill et al., 1999; Ohkura et al., 1995). Overexpressing Plol in interphase causes the formation of multiple septa (Ohkura et al., 1995). Cytokinesis in multicellular organisms involves formation of a central spindle, a structure
not present in yeast. Polo and Plk1 become localized to the central spindle and require a kinesin-like motor protein, called Pavarotti or Mklp2 respectively, for this relocation. Deletion of either motor protein results in a phenotype identical to polo/Plk1 deletion with failed/abnormal cytokinesis (Adams et al., 1998; Carmena et al., 1998; Neef et al., 2003). Plk1 can phosphorylate Mklp2 and this is essential for Plk1 movement to the central spindle (Neef et al., 2003). Polo is thought to phosphorylate Pavarotti in an identical manner but this has yet to be shown in vivo (Carmena et al., 1998). The localization of polo/Plk1 to the central spindle may allow these kinases to phosphorylate yet undetermined substrates, signaling to the cell cortex when to constrict/assemble a contractile ring; polo/Pavarotti mutants fail to form a contractile ring while Plk1/Mklp2 mutants form a contractile ring which cannot constrict (Carmena et al., 1998; Neef et al., 2003).

1.5.2 Regulation of Polo-like kinases

The regulation of Plk1 is the best characterized for this family, but cell cycle abundance and localization are not identical across species. Polo and Plxl do not alter in abundance during the embryonic cell cycle, but the kinase is catalytically active only during mitosis; Plol also does not change in abundance during the cell cycle and is only active during mitosis (Descombes and Nigg, 1998; Llamazares et al., 1991; Mulvihill and Hyams, 2002; Qian et al., 1998a). Cdc5 and Plk1 display cell cycle regulated abundance with peak levels in mitosis (Charles et al., 1998; Cheng et al., 1998; Golsteyn et al., 1995; Golsteyn et al., 1994; Shirayama et al., 1998). In human cells, Plk1 abundance must be precisely regulated as overexpression causes mitotic delays leading to multinucleated cells (Mundt et al., 1997), while depletion causes mitotic defects and apoptosis (Liu and Erikson, 2002; Liu and Erikson, 2003). Plk1 abundance is primarily regulated by transcription that keeps protein levels low during G0 and G1 with a progressive increase towards G2/M (Uchiumi et al., 1997). E2F1 and E2F4 can bind to the Plk1 promoter and cause transcriptional repression during G1 and transcriptional activation in S and G2 (Ren et al., 2002). Inhibiting the centrosomal chaperone Hsp90 with geldanamycin causes centrosomal defects identical to Plk1 mutants (Lange et al., 2000). Hsp90 is therefore thought to be required to maintain the structure of Plk1 (de Carcer et al., 2001). All Polo-like kinases are phosphorylated when catalytically active (Dai et al., 2002; Glover et al., 1998; Nigg, 1998) and autophosphorylation and upstream kinases are dually responsible for activation of Plxl and Plk1 (Jang et al., 2002; Kelm et al., 2002; Qian et al., 1998b). The kinase xPlkk1
is a strong \textit{in vivo} candidate, while \textit{in vitro} data implicate CDK1, PKA and the MAPKs as potential upstream kinases (Kelm et al., 2002; Okano-Uchida et al., 2003; Qian et al., 1998b).

\textit{Plk1} is degraded at the end of mitosis by the proteasome (Ferris et al., 1998) and a D-box sequence in \textit{Plk1} was recently identified as the destruction motif recognized by the APC/C adaptor protein Cdh1 (Lindon and Pines, 2004). Cdc5 is also degraded by the APC/C complexed to the adaptor protein Cdh1 during late mitosis, but Cdc5 contains two destruction motifs within its N-terminal domain (Charles et al., 1998; Cheng et al., 1998; Shirayama et al., 1998). Plx1 and polo contain the D-box present in \textit{Plk1} (Lindon and Pines, 2004), but their abundance does not alter during the embryonic cell cycle as Cdh1 is absent (Lorca et al., 1998). Plx1 must be deactivated though as cells remain mitotic until the kinase activity is suppressed, implying that down regulation of kinase activity is a requirement for mitotic exit (Descombes and Nigg, 1998). Adult cells of \textit{Drosophila} and \textit{Xenopus} would be expected to show the same degradation pattern as \textit{Plk1}. \textit{Plk1} can also be deactivated (not degraded) in response to the DNA damage checkpoint at the G2/M transition (Smits et al., 2000).

The C-terminal domain of Polo-like kinases harbours two polo box motifs that together constitute the polo box domain (PBD). Deletion of the PBD has no effect upon kinase activity, but the enzyme no longer localizes to the centrosome and cannot target its mitotic substrates (Lee et al., 1998; Reynolds and Ohkura, 2002). The reason for this has recently been discovered. The PBD binds to a consensus motif consisting of a serine followed by a phosphorylated serine or threonine (S-pS or S-pT) (Elia et al., 2003a). The two polo boxes have identical folds consisting of a six-stranded $\beta$-sheet with an $\alpha$-helix, despite showing only 12% sequence identity. The phosphoserine/threonine binds at a site between the two polo boxes in a positively charged cleft (Cheng et al., 2003; Elia et al., 2003b). This implies that a priming kinase must phosphorylate a \textit{Plk1} substrate first in order for \textit{Plk1} to recognize it. As \textit{Plk1} is active during mitosis, CDK1 is thought to be a priming kinase. \textit{Plk1} may also be the priming kinase as in the case of Mklp2 (Neef et al., 2003). It is also possible that other mitotically active kinases such as Aurora A or Nek2 may be priming kinases for \textit{Plk1}. The consensus phosphorylation site for the \textit{Plk1} kinase domain has also been established being D/E-x-S/T (Kelm et al., 2002). This motif is present in Cdc25, cyclin B, Mklp2 and Nlp, and led to the discovery of Myt1 being a substrate for \textit{Plk1}.
Taken together, the PBD target sequence and consensus phosphorylation motif should make identification of other potential polo-like kinase substrates more straightforward.

1.5.3 Polo-like kinase 2 and 3

*Drosophila* and yeast contain only one polo-like kinase within their genomes, whereas *C. elegans, Xenopus*, mouse and human contain three different polo-like kinases, termed Plc/Plx/Plk1, 2 and 3 (Dai et al., 2002). *C. elegans* Plc1, 2 and 3 have been identified within the genome, but no biochemical functions have yet been ascertained (Chase et al., 2000; Ouyang et al., 1999). Most studies have been conducted on Plx/Plk1 as these kinases show highest sequence similarity to polo. In mammalian and *Xenopus* systems, Plx/Plk2 and 3 are less well defined but still contain an N-terminal catalytic domain and a PBD in the C-terminal domain (Duncan et al., 2001; Kauselmann et al., 1999). Plx2 and 3 can enhance maturation of *Xenopus* oocytes in a kinase dependent manner when overexpressed, but the *in vivo* substrates are unknown (Duncan et al., 2001). Plk2 (also called Snk) is most active at the G1/S transition when it can localize to the centrosome. Functional studies suggest that Plk2 may have a role in centrosome duplication (I. Hoffmann, Heidelberg, personal communication). Gene knockout of Plk2 leads to cells that have a delay in S phase entry (Ma et al., 2003a), whereas newborn mice are viable but show growth retardation (Ma et al., 2003b). The spindle checkpoint can activate Plk2 via the transcription factor p53 (Burns et al., 2003). Overexpression of Plk2 leads to apoptosis after 24 h (Burns et al., 2003; Ma et al., 2003b), suggesting that a failure of mitotic exit is the cause of apoptosis.

Plk3 (also called Fnk and Prk) is most similar to Plk2 but appears to complement some Plk1 functions. Overexpressing either Plk1 or Plk3 rescues lethality in yeast which are devoid of Cdc5 (Lee and Erikson, 1997; Ouyang et al., 1997). Plk3 abundance does not alter during the cell cycle, but becomes phosphorylated and active during late S/G2 (Bahassi et al., 2002; Ouyang et al., 1997). Cellular distribution indicates Plk3 localizes with the centrosome during the entire cell cycle as well as the spindle, midbody and cell cortex during mitosis. However, its centrosome localization is microtubule dependant and it does not localize at the kinetochores like Plk1 (Conn et al., 2000; Wang et al., 2002). Plk3 may function as a tumor suppressor gene as it becomes down regulated in lung carcinomas and overexpression causes cell proliferation to stop at G2/M as well as induce
apoptosis (Conn et al., 2000; Li et al., 1996; Wang et al., 2002; Xie et al., 2001). Plk3 becomes active in response to DNA damage or activation of the spindle checkpoint, with potential downstream targets being Chk2, p53 and Cdc25 (Bahassi et al., 2002; Xie et al., 2001). Plk3 phosphorylation activates p53 and Chk2 so that cell cycle arrest occurs, whereas phosphorylation of Cdc25 is at a site antagonistic to that of Plk1 and causes Cdc25 to become inactive (Bahassi et al., 2002; Xie et al., 2001). Therefore, Plk2 and Plk3 appear to function in proliferation of the cell cycle like Plk1, although Plk2 and Plk3 may be more involved in checkpoint control, whereas Plk1 is essential to ensure a viable mitotic progression. The appearance of Plk3 at multiple sites during mitosis would suggest that other potential functions for this protein await elucidation.

1.6 NIMA related kinases

1.6.1 NIMA

Another family of protein kinases involved in cell cycle control and centrosome regulation are the NIMA related kinases (O'Connell et al., 2003). The forefather of this kinase family is NIMA itself, initially identified from a temperature sensitive mutant in the filamentous fungus *Aspergillus nidulans*. The temperature sensitive mutant of nimA causes a G2 arrest with failure to progress into mitosis (hence the name, never in mitosis (nim)), upon restoration to the permissive temperature mitosis occurs and cell cycling resumes (Bergen et al., 1984; Oakley and Morris, 1983; Osmani et al., 1987). Another phenotype caused by the nimA mutant is SPBs which duplicate but fail to separate, consequently causing an absence of nucleoplasmic microtubules (Oakley and Morris, 1983). Overexpressing NIMA from any point in the cell cycle causes a premature mitotic induction to occur, detectable by chromosome condensation, nuclear envelope abnormalities and bipolar spindle formation; the phenotype is ultimately lethal (Osmani et al., 1988). Mitotic commitment in eukaryotes is driven principally by the activation of CDK1 (Ohi and Gould, 1999); however, *A. nidulans* requires both CDK1 and NIMA (Osmani et al., 1991), the only example to date where CDK1 activity alone is not sufficient for mitotic entry. As mitosis is closed in *Aspergillus*, NIMA may phosphorylate a nuclear transporter in order to allow CDK1 access to the nucleus (de Souza et al., 2003; Wu et al., 1998).

The abundance and activity of NIMA is cell cycle regulated to co-ordinate an accurate and viable mitosis. The NIMA protein contains an N-terminal catalytic domain and C-terminal
regulatory domain (Figure 1.9A). NIMA protein abundance is initially regulated by mRNA abundance, which increases from S through to G2 before the mRNA is degraded at the onset of mitosis (Osmani et al., 1987). This change in abundance correlates with kinase activity (Osmani et al., 1991). The regulatory domain contains two PEST motifs (Pu and Osmani, 1995) and the APC/C is the prime candidate for ubiquitin mediated destruction through recognition of these motifs in late mitosis (Ye et al., 1998). In addition to changes in protein abundance, the kinase activity of NIMA is also controlled by post-translational phosphorylation that is essential for a functional enzyme. NIMA autophosphorylation has been demonstrated (Lu et al., 1993), as well as in vitro phosphorylation by CDK1 (Ye et al., 1995). An essential nuclear localization sequence is also present in the regulatory domain (Pu and Osmani, 1995).

During mitosis NIMA localizes to the mitotic chromatin, mitotic spindle and SPB (de Souza et al., 2000); as kinase activity is required for mitotic progression it is likely that some substrates lie within these regions. Lu and colleagues used NIMA to screen a synthetic peptide library containing possible phosphorylation site motifs. Using this approach they identified an optimal phosphorylation motif for NIMA designated F-R-x-S/T (Lu et al., 1994). Using database searching to identify potential substrates for NIMA has proved unsuccessful and to date only one potential substrate has been identified in A. nidulans which is serine 10 on histone H3 (de Souza et al., 2000). Phosphorylation of this residue is a marker of condensed chromatin and provides evidence of how NIMA may induce chromatin condensation. However, as serine 10 on histone H3 has been shown to be phosphorylated by Aurora B in other systems, the role of NIMA in histone H3 phosphorylation has yet to be clarified in vivo (de Souza et al., 2000; Giet and Glover, 2001). Despite the lack of in vivo substrates, a recent NIMA interacting protein has been identified called TINA. TINA can colocalize with NIMA during G2 and appears to play a role in mitotic spindle formation (Osmani et al., 2003).

1.6.2 NIMA homologues in lower eukaryotes
As NIMA is essential for mitotic entry in A. nidulans, the identification of homologues in other species was keenly awaited. Overexpression of both wild type and mutant NIMA in diverse organisms provided evidence of the existence of NIMA-like kinases from yeast to vertebrates (Lu and Hunter, 1995). NIMA overexpression induced germinal vesicle breakdown (meiotic nuclear envelope breakdown) in Xenopus oocytes and premature
Figure 1.9 Structure of the NIMA kinases

(A) Structures of NIMA and related kinases in various species, taken from O’Connell et al. (2003). (B) Detailed structure of human Nek2 isoforms. Numbers associated with each isoform represent amino acids, LZ, leucine zipper domain, CC, coiled coil domain. Adapted from Fry (2002).
mitotic events in HeLa cells independently of CDK1, while the mutant NIMA caused a G2 arrest (Lu and Hunter, 1995). Related proteins to NIMA have now been identified in species ranging from yeast to humans, however, despite some homology within the catalytic domain, conservation is low outside of this region (Figure 1.9A)(O'Connell et al., 2003). The only known homologue that can complement an *Aspergillus nimA* mutant is *niml* from the fungus *Neurospora crassa*, suggesting a conserved function for this kinase in filamentous fungi (Pu et al., 1995).

The *S. cerevisiae* genome contains only one NIMA homologue called Kin3, which is a non-essential gene (Barton et al., 1992; Jones and Rosamond, 1990; Schweitzer and Philippsen, 1992). However, recent work suggests Kin3 might have a role in chromosome segregation by phosphorylating a kinetochore protein Hec1 (Chen et al., 2002). The *S. pombe* genome also contains one NIMA homologue called Fin1. Overexpressing Fin1 from any point in the cell cycle induces premature chromosome condensation whereas deletion causes a delay in G2 but no major cell cycle arrest. Deleting Fin1 shows the gene is not essential as viability is unaffected, but these yeast display an increase in chromosome segregation defects (Krien et al., 1998). Observing different structures within Δfin1 yeast reveals that they contain a disrupted nuclear envelope and microtubules which are more sensitive to depolymerizing drugs. Double mutant strains lacking Fin1 with various components of the spindle checkpoint exhibit a lethal phenotype (Krien et al., 2002). Yeast which contain a kinase deficient Fin1 form a defective mitotic spindle as only one SPB is capable of microtubule nucleation (Grallert and Hagan, 2002). These data would appear to support a function for Fin1 in bipolar spindle assembly. Chromosome condensation is unlikely to be a function of Fin1 because in its absence chromosome condensation occurs and premature condensation induced by Fin1 overexpression is independent of other factors required for normal chromosome condensation. Fin1 associates with the SPB during mitosis and can promote the affinity of Plo1 for the SPB, therefore Fin1 is a potential Plo1 priming kinase. Plo1 localization to the SPB has been implicated in the activation of CDK1 creating a positive feedback loop for mitotic entry; this could explain the delay in G2 progression in the absence of Fin1 (Grallert and Hagan, 2002). As Polo like kinases are also implicated in the formation of a bipolar spindle, this provides another possible mechanism by which Fin1 influences mitotic progression (see section 1.5.1).
Less related NIMA homologues have been identified in other lower eukaryotes including the protozoan Tetrahymena, bi-flagellate green algae Chlamydomonas reinhardtii and human malarial parasite Plasmodium falciparum. Tetrahymena NIMA (Nrk) mRNA displays a similar expression pattern to NIMA mRNA, but no further information exists (Wang et al., 1998). The Chlamydomonas NIMA (FA2) is involved in severing microtubules during deflagellation and creating a new flagella. Mutations in the FA2 gene cause a delay in the G2/M transition as well as a delay in flagella assembly post mitosis, highlighting possible defects in the basal body cycle (Mahjoub et al., 2002). The Plasmodium falciparum NIMA (PfNek1) may be part of a signal transduction pathway during early malarial infection; the biochemical details are unknown but it could be a potential therapeutic target (Dorin et al., 2001).

1.6.3 Nek2
The most extensively studied NIMA homologues are those that exist within vertebrate systems called NIMA related kinases (Neks). The Nek superfamily contains at least eleven members in humans (Figure 1.9A)(O’Connell et al., 2003) and ten in mice (Forrest et al., 2003), but only recently are biochemical functions being attributed to this growing family of kinases. Of all the vertebrate Neks, the best characterized to date is Nek2. This is because Nek2 shares highest similarity to NIMA from within the Nek superfamily (44% within the catalytic domain). Nek2 was initially identified by degenerate PCR from a human leukaemia cDNA library using oligonucleotides against conserved residues within kinase catalytic domains (Schultz and Nigg, 1993). Like NIMA, Nek2 protein abundance and activity increases from S phase through G2 but decreases after mitotic entry (Fry et al., 1995; Schultz et al., 1994). The Nek2 kinase consists of an N-terminal serine/threonine-directed catalytic domain followed by a C-terminal regulatory domain consisting of a leucine zipper, PP1 binding motif, two degradation motifs (KEN-box and D-box) and a coiled coil region (Figure 1.9B)(Fry, 2002). Subcellular localization studies showed that 90% of the Nek2 protein is cytoplasmic and 10% centrosomal (Fry et al., 1998b). Overexpressing the wild type kinase induced premature centriole splitting (mother and daughter centriole separated by >2 μm) from any point in the cell cycle, while overexpressing a catalytically inactive mutant (Nek2-K37R) did not. However, prolonged expression both Nek2 and Nek2-K37R led to a disintegration of centrosome structure with equal efficiency suggesting that centrosomal proteins were being titrated by the overexpressed Nek2 protein (Fry et al., 1998b).
Degenerate PCR and cDNA library screening identified a *Xenopus laevis* homologue of Nek2 (Fry et al., 2000b; Uto et al., 1999). X-Nek2 exists as two alternative splice variants which differ only at the 3' end. Alternative splicing results in a longer sequence containing degradation motifs (X-Nek2A, 48 kDa) and a shorter sequence in which they are absent (X-Nek2B, 44 kDa). Two splice variants have subsequently been shown to exist in the human genome and produce similar alternative 3' sequences as for *Xenopus* (Figure 1.9B)(Hames and Fry, 2002). X-Nek2A and X-Nek2B possibly have a role in meiosis due to a high expression level in the testis and ovary (Uto et al., 1999). However, upon fertilization only X-Nek2B can be detected in the developing embryo until the early neurula stage (stage 14); X-Nek2B protein abundance becomes undetectable after this and is replaced by X-Nek2A which persists into adulthood (Uto et al., 1999). Like Nek2A, X-Nek2B is also associated with centrosomes (Fry et al., 2000b; Uto and Sagata, 2000).

Fertilization begins with the penetration of an egg by a sperm and therefore the developing zygote contains both maternal and paternal molecules. A *Xenopus* egg is acentrosomal while sperm contain a centrosome-like structure called a basal body that is required for generation of the microtubules within the sperm flagellum. Fertilization is accompanied by entry of the basal body into the egg where upon it is converted into a centrosome competent for microtubule nucleation. Upon fertilization, proteins are recruited from the oocyte cytoplasm to the assembling zygotic centrosome; X-Nek2B is one protein which displays this behavior (Fry et al., 2000b; Uto and Sagata, 2000). X-Nek2B recruitment to the basal body is an early event and occurs independently of microtubules and other proteins. The recruitment of X-Nek2B is not dependant on kinase activity but X-Nek2B does become hyperphosphorylated upon recruitment to the basal body (Twomey et al., 2004). Immunodepleting X-Nek2B from *Xenopus* egg extracts causes a delay in γ-tubulin recruitment and aster formation, although a nucleating centrosome does eventually form (Fry et al., 2000b). Interfering with X-Nek2B in developing embryos by antibody microinjection or microinjection of mRNA coding for X-Nek2B-K37R causes lethality (Uto and Sagata, 2000). Embryos show abnormal cleavage as a result of a defective bipolar spindle. At the 16/32 cell stage blastomeres appear irregular in size and shape, due to asymmetrical cleavage. Examining the integrity of centrosomes in embryos injected with X-Nek2B-K37R mRNA revealed the appearance of multiple dot-like structures, each capable of aster formation, therefore a bipolar spindle could not be established (Uto and Sagata, 2000). These results suggest that X-Nek2B plays an important role in centrosome
assembly and maintenance during early development. Further evidence for a centrosome-specific role is that interfering with X-Nek2B during meiosis had no effect upon bipolar spindle formation or chromosome segregation, a process which is acentriolar in *Xenopus* (Uto and Sagata, 2000). Likewise, formation of acentrosomal asters in egg extracts supplemented with taxol did not require X-Nek2B (Twomey et al., 2004).

Nek2 homologues exist in other species but remain less well defined than in human and *Xenopus*. Murine Nek2 (mNek2) is highly expressed in both male and female germ cells (Arama et al., 1998; Di Agostino et al., 2003; Rhee and Wolgemuth, 1997; Tanaka et al., 1997). Overexpressing mNek2 in spermatocytes causes an increase in defective spermatogenic cells (apoptotic and enlarged) suggesting a role in the normal development of male germ cells, but the molecular details remain unknown (Rhee and Wolgemuth, 2002). mNek2 is also detectable (albeit at lower levels) in actively dividing tissues (Tanaka et al., 1997). Cell cycle distribution of mNek2 in transformed cell lines reveals that its behavior is very similar to human Nek2 with respect to abundance and centrosomal localization. However, it is also reported to be nuclear during S phase, associate with chromosomes during prophase and metaphase as well as localize to the midbody during cytokinesis. These localization patterns, that have not been seen in human cells, have been used to suggest a possible role in chromosome condensation and mitotic exit (Kim et al., 2002). Porcine Nek2 is also highly expressed in oocytes and associates with chromosomes during metaphase II, again suggesting a role in meiosis and chromosome condensation (Fujioka et al., 2000). A yeast two hybrid screen with Nek2 as the bait identified a protein called NIP1 which can be phosphorylated *in vivo* by Nek2. NIP1 is a Golgi protein and suggests that Nek2 may also regulate Golgi inheritance as well as centrosome inheritance at the onset of mitosis (Yoo et al., 2004). The first non-vertebrate Nek2 was identified in the slime mould *Dictyostelium discoideum* (DdNek2). DdNek2 localizes to the central plaque of the centrosome throughout the cell cycle (Graf, 2002). Overexpression of either DdNek2 or DdNek2-K33R (a kinase dead mutant) causes the formation of multiple ectopic MTOCs, which display microtubule nucleating capacities. Additional MTOCs are not lethal though in *D. discoideum* as mitosis is closed and only one centrosome becomes embedded within the nuclear envelope (Graf, 2002).
1.6.4 Nek2 regulation

Nek2 seems to be subject to transcriptional repression during G0 and G1, explaining why abundance is low during this period. The E2F transcription family is again the most likely candidate for Nek2A transcriptional repression. E2F4 can bind to the Nek2 promoter and recruit p107 and p130. These inhibit the formation of active transcription complexes thus maintaining low abundance of Nek2A in G0 and G1 (Ren et al., 2002). In S and G2, transcriptional repression is relieved allowing accumulation of Nek2 protein. Mitotic onset sees a rapid decrease in Nek2A abundance as a result of ubiquitin mediated protein degradation (Hames et al., 2001). The APC/C is an E3 ubiquitin ligase responsible for ubiquitylating Nek2A targeting it for destruction by the 26S proteasome. The D-box and KEN-box are the destruction motifs recognized by the APC/C adapter proteins Cdc20 and Cdh1, respectively. Current evidence suggests that D-box recognition occurs prior to metaphase causing rapid Nek2A degradation during early mitosis, whereas KEN-box recognition occurs after metaphase maintaining Nek2A degradation in late mitosis and G1. Deletion of both the KEN-box and D-box completely stabilizes Nek2A in mitosis (Hames et al., 2001).

Nek2 monomers are able to homodimerise with each other through an unusual leucine zipper domain (the only regulatory domain present in both Nek2A and Nek2B) forming a salt stable complex (Fry et al., 1999). Leucine zippers usually have heptad repeats (a-g)n, with a hydrophobic core consisting of the a and d residue, flanked by charged amino acids at position e and g (Branden and Tooze, 1999). The Nek2 leucine zipper contains a leucine at position d but is flanked by a positive charge at position g and a negative charge at position a (Fry et al., 1999). Homodimerisation is essential for the kinase activity of Nek2A possibly because it promotes transautophosphorylation.

The phosphorylation state of Nek2A is tightly regulated by protein phosphatase 1 (PP1). The catalytic subunit of PP1 can bind directly to Nek2A via a KVHF motif in the C-terminal domain of Nek2A and dephosphorylate it causing a reduction in Nek2A activity (Helps et al., 2000). If Nek2A activity is increased above PP1 activity, Nek2A can phosphorylate PP1 and inactivate it. Nek2A and PP1 therefore form a complex with properties of a double negative feedback bistable switch (Helps et al., 2000; Meraldi and Nigg, 2001). PP1 can be inhibited by association with a protein called Inhibitor-2 (Inh2). Inh2 is a natural protein that can bind to PP1 on its regulatory domain while PP1 is
complexed to Nek2A (Eto et al., 2002). Inh2 has also been localized to the centrosome and can form a ternary complex with PP1 and Nek2. Furthermore, overexpressing Inh2 can induce centriole separation as efficiently as overexpressing Nek2A, suggesting that PP1 regulation of Nek2A can be controlled by Inh2 (Eto et al., 2002). During mitotic entry, Inh2 becomes phosphorylated possibly by CDK1. This leads to increased binding to PP1, allowing Nek2 to become hyperactive (Leach et al., 2003; Puntoni and Villa-Moruzzi, 1995). Additional phosphorylation of Inh2 later in mitosis by CDK5 appears to repress Inh2 inhibition of PP1, allowing PP1 inhibition of Nek2 to occur (Figure 1.10A) (Tan et al., 2003). PP1 can be activated by dephosphorylation of threonine 320 in response to ionizing radiation (Guo et al., 2002). PP1 induced activity as a result of ionizing radiation could inhibit Nek2, therefore providing a scenario of how centrosome separation can be suppressed as a result of DNA damage (Larner et al., 2003). Nek2B cannot interact with PP1 or be degraded by the APC/C. Yet Nek2B is less abundant than Nek2A during the adult cell cycle, except for during mitosis when Nek2A is degraded. Nek2B abundance decreases during G1, probably due to E2F4-mediated transcriptional repression (Hames and Fry, 2002).

No evidence exists in human cells for a Nek2A kinase kinase, although mNek2 can be phosphorylated and activated in vitro by the MAPK pathway which is required for chromosome condensation in mouse spermatocytes (Di Agostino et al., 2002). A pathway for chromatin condensation driven by MAPK activation of Nek2 during spermatogenesis has recently been proposed. Nek2 can phosphorylate a chromatin associated protein called HMGA2 in vitro possibly causing HMGA2 to lose its affinity for chromatin at the G2/M transition (Figure 1.10B)(Di Agostino et al., 2003).

1.6.5 Substrates of Nek2
To elucidate the molecular mechanism by which Nek2A kinase activity might influence centriole separation, a yeast two hybrid screen using Nek2A as the bait was performed. This identified a novel centrosomal protein called C-Napl (Centrosomal Nek2 Associated Protein 1) (Fry et al., 1998a). The same protein was independently identified by screening an expression library from patients with autoimmune sera to centrosomes and called CEP 250 (Mack et al., 1998). C-Napl is a protein predicted to be 281 kDa, consisting of two extensive coiled coil regions separated by a potential hinge and with globular N- and C-terminal domains (Figure 1.11A). Immunoelectron microscopy revealed that Nek2 and C-
Figure 1.10 Nek2 regulation
(A) Model of Nek2A kinase activity in cycling human cells. During S phase, Nek2 activity is repressed by PP1 phosphatase activity. At mitotic onset PP1 is repressed by CDK1 phosphorylation of Inh2 allowing Nek2 to autophosphorylate and further repress PP1. During mitosis, Inh2 is phosphorylated further (CDK5 is a likely candidate) and can no longer repress PP1 allowing an increase in phosphatase activity against Nek2. In combination Nek2A is also degraded to inhibit its activity. (B) Model of Nek2 regulation in mouse spermatocytes. Erk1 stimulation begins a phosphorylation cascade targeting p90Rsk2 which then phosphorylates and activates Nek2. HMGA2 must be displaced from meiotic chromatin to allow chromosome condensation to occur; Nek2 may phosphorylate HMGA2 and facilitate MAPK induced chromatin condensation.
Nap1 are localized to the same region at the proximal end of both mother and daughter centrioles, although neither are detectable in the electron dense material connecting the two centrioles (Fry et al., 1998a). C-Nap1 is centrosomal in the presence or absence of microtubules during interphase, but decreases in abundance at the centrosome during prophase (centrosome separation) and reappears during telophase/G1 (Fry et al., 1998a; Mayor et al., 2000). Microinjection of a C-Nap1 antibody or overexpressing truncated forms of C-Nap1 also induced premature centriole splitting from any point in the cell cycle implying that C-Nap1, like Nek2A, has a role in centrosome cohesion (Mayor et al., 2000).

Centrosome cohesion is clearly regulated by protein phosphorylation and Nek2A is a prime candidate to facilitate this (Meraldi and Nigg, 2001). Nek2A can phosphorylate C-Nap1 upon its C-terminal domain in vitro (Fry et al., 1998a) and C-Nap1 becomes phosphorylated during mitosis in vivo (Mayor et al., 2002). Phosphorylation may alter either the structure or charge of C-Nap1 causing it to lose association with the proximal end of centrioles. Cytoplasmic C-Nap1 is not degraded and protein levels remain constant throughout the cell cycle (Figure 1.11B). Overexpressing C-Nap1 produces large centrosome associated aggregates, but these can be dispersed by cotransfection with active, but not inactive Nek2A (Mayor et al., 2002). More recently, it has been shown using a tetracycline-inducible stable cell line, that overexpression of Nek2A-K37R causes the accumulation of abnormal centrosomes characterized by additional material or supernumerary centrioles. During mitosis, these cells display monopolar spindles consistent with centrosomes failing to separate, while prolonged expression causes cells to become polyploid (Faragher and Fry, 2003). Together, these data support a model in which Nek2A and C-Nap1 regulate centrosome separation, a necessary step in bipolar spindle formation.

Apart from Nek2 itself, PP1, HMGA2 and C-Nap1, Nek2 has also been reported to phosphorylate a kinetochore associated protein called Heel (highly expressed in cancer) (Chen et al., 2002). Heel phosphorylation is essential for accurate chromosome segregation during mitosis and an interaction was detected between Nek2A and Heel1 in the yeast two hybrid system (Chen et al., 1997b), and through co-immunoprecipitation at the G2/M transition (Chen et al., 2002). Microinjection of antibodies against Heel1 causes severe chromosome segregation defects (Chen et al., 1997a) as it a component of the spindle checkpoint and functions by recruiting Mad1/Mad2 to the kinetochore (Martin-Lluesma et al., 2002). Yeast cells lacking Heel1 display high chromosome segregation
Figure 1.11 C-Nap1 structure and regulation

(A) C-Nap1 protein structure with major domains identified. The red regions represent the N and C-terminal globular domains, the yellow central region is the potential hinge separating the coiled coil domains. Numbers represent amino acid position. (B) C-Nap1 localization through the cell cycle. C-Nap1 appears at the proximal ends of both mother and daughter centriole during late mitosis/early G1 when the centrioles become connected by the intercentriolar linker. During the G2/M transition, Nek2A phosphorylates C-Nap1 and causes its dissociation from the parent centrioles. Cytoplasmic C-Nap1 does not appear to be degraded, and presumably an unknown phosphatase permits C-Nap1 to reassociate with the centrosome during late mitosis/early G1.
defects and cannot be rescued by addition of a mutant Hec1 which is insensitive to Nek2A phosphorylation. The same outcome occurs using yeast Hec1 with the NIMA homologue Kin3 (Chen et al., 2002). Nek2 though has not been localized to kinetochores in adult human cells so the relevance of this interaction remains to be proven.

1.6.6 Other Neks

Based on similarities in the catalytic domain, genomic and biochemical approaches have identified eleven Neks in the human genome (O'Connell et al., 2003) and ten in the completed mouse genome (Forrest et al., 2003). However, with the exception of Nek2 little is known about the molecular functions of the other Neks (Figure 1.9A). mNek1 is expressed in greatest abundance in neurons and germ cells, suggesting a role in neural development or meiosis (Arama et al., 1998; Letwin et al., 1992). Unusually, Nek1 was reported to display dual phosphorylation upon serine/threonine and tyrosine residues (Letwin et al., 1992). A mutant copy of Nek1 in a laboratory strain of mouse was causative of a polycystic kidney disease (PKD) with the appearance of renal enlargement and fluid filled cysts (Upadhya et al., 2000). Yeast two hybrid analysis using Nek1 as the bait identified an interaction with other proteins involved in PKD such as KIF3A and tuberin; proteins involved in double strand DNA break repair at the G2/M transition were also identified (Surpili et al., 2003). mNek3 is expressed in mitotically active tissues but an increased abundance is detected in G0 cells as compared to cycling cells; its functions remain unknown (Chen et al., 1999; Tanaka and Nigg, 1999). mNek4/STK2 is expressed in all cell types with highest abundance in the testis; again, the mitotic or meiotic functions are unknown (Chen et al., 1999; Hayashi et al., 1999). Overexpression of neither mNek3 nor mNek4/STK2 disrupts cell cycle progression in cultured cells.

Nek6 and Nek7 share a high identity (87%) and consist of simply a catalytic domain (Kandli et al., 2000). Initial studies suggested roles for Nek6/7 in activation of S6K (Belham et al., 2001), but subsequent studies revealed that Nek6/7 did not appear to be involved in the physiological activation of S6K (Lizcano et al., 2002). In contrast, overexpressing a kinase deficient Nek6 or depletion by RNAi caused a cell cycle arrest at the metaphase/anaphase transition with 50% cells becoming apoptotic (Yin et al., 2003). Importantly, then, Nek6 (and Nek7) may have a role in mitotic progression. Antibody inhibition of another Nek, Nercc1/Nek9, caused abnormalities in bipolar spindle formation followed by segregation defects with cells either arresting at prometaphase or proceeding
to become aneuploid (Roig et al., 2002). Nercc1/Nek9 can bind, phosphorylate and activate Nek6 and Nek7 in vivo (Belham et al., 2003). This provides an exciting insight into a possible Nek signal transduction cascade where Nercc1/Nek9 can phosphorylate Nek6/Nek7 and affect mitotic progression. The downstream targets of Nek6/Nek7 await elucidation. Another potential substrate of Nercc1/Nek9 is Bicd2, a protein involved in microtubule cytoskeletal formation and maintenance (Holland et al., 2002). A function in chromatin organization has also been suggested for Nercc1/Nek9. Nercc1/Nek9 can interact with FACT, a chromatin structure modulator that is required for interphase progression and Nercc1/Nek9 becomes phosphorylated when complexed to FACT (Tan and Lee, 2003). Nek11 shows an increase in abundance from S phase through to the G2/M transition and preliminary data suggest a possible involvement in the checkpoint response to DNA replication errors and genotoxic stress (Noguchi et al., 2002).

MLK3 (mixed lineage kinase 3) shares similarity with NIMA within the C-terminal regulatory domain but not catalytic domain. MLK3 is a MAPKKK in the JNK pathway but localizes to centrosomes during interphase, prophase and telophase. Overexpression of MLK3 causes interphase microtubules to be dispersed but has no effect upon the mitotic spindle, raising the possibility that MLK3 may play a role in targeting microtubule destabilizing motors (Swenson et al., 2003).

In summary, no one mammalian Nek appears to be the direct homologue of NIMA. However, functions associated with NIMA during fungal cell cycle progression may have been divided up between several Neks with most current interest being focused on Nek2, Nek6/Nek7 and Nercc1/Nek9.

**1.7 Centrosomes and cancer**

The centrosome plays a dominant role in the nucleation of microtubules and hence dictates the bipolarity of the spindle (Heald et al., 1996). Abnormal centrosome numbers or activity could therefore lead to aneuploidy and chromosome instability. Aneuploidy is a condition where a cell has more or less chromosomes than its usual genetic content (+/- 2n in G1), while chromosome instability reflects a constant rate of change of chromosome content. Generally, if cells lose or gain chromosomes they die by apoptosis, but if this pathway is compromised then aneuploid cells may survive. Chromosome loss could remove genes required for basic survival (housekeeping genes) but also lead to loss of
heterozygosity of tumour suppressor genes leading to loss of control over cell proliferation. Alternatively, chromosome gain could increase the copy number of critical growth promoting oncogenes. The notion that centrosomes may contribute to chromosomal instability and aneuploidy was first suggested by Theodor Boveri over a century ago (Boveri, 1914), but only now are potential molecular mechanisms for this phenotype being dissected. Aneuploidy and chromosomal instability are common hallmarks of nearly all tumors and in parallel additional centrosomes (supernumary centrosomes) have also been detected in many mammalian tumors (Figure 1.12). The presence of supernumary centrosomes in tumors suggests they may contribute to chromosomal instability, but whether they are a cause or consequence of cellular transformation is still an open and much debated question in this field (Brinkley, 2001; Brinkley and Geopfert, 1998; D'Assoro et al., 2002; Duensing and Munger, 2001; Nigg, 2002; Pihan and Doxsey, 1999; Pihan et al., 1998; Salisbury et al., 1999).

A cell containing multiple centrosomes could cause a multipolar mitosis resulting in aneuploid, unviable progeny, therefore additional centrosomes would seem to be a disadvantage to cells. Adaptation must therefore occur so that viability is maintained in most cells with the favored model being the coalescence of centrosomes which results in multiple centrosomes incorporated into two spindle poles (e.g. three centrosomes at each spindle pole in a hexacentrosomal cell) (Brinkley, 2001). Centrosomes in cancer cells display an abnormal morphology characterized by combinations of the following: an increase in number or volume, excess PCM, supernumary centrioles, inappropriate phosphorylation of centrosomal proteins and unusually large microtubule arrays (Lingle et al., 1998). The mechanism by which centrosome amplification occurs is unknown, but regulators of the centrosome cycle are an obvious link (Figure 1.7). Deregulation of p53 is a common occurrence in many tumors, and in mouse fibroblasts which are p53−/− over 30% cells contain more than two centrosomes (Fukasawa et al., 1996). Aurora A is frequently overexpressed in human cancers often as a result of gene amplification (Bischoff et al., 1998; Sen et al., 1997). Overexpressing Aurora A in mouse and human cells can cause transformation suggesting Aurora A is an oncogene (Bischoff et al., 1998; Zhou et al., 1998). Aurora A may transform cells by causing a failed cytokinesis, as overexpressing Aurora A causes 25% of cells to be tetraploid and contain more than one centrosome. The additional DNA and centrosomes are most likely a result of duplicated centrosomes and DNA failing to be separated as abscission fails. Overexpressing Aurora A in p53−/− cells
Figure 1.12 Supernumerary centrosomes in cancer cells

(A) HBL100 immortalized breast epithelial cell line, 0% supernumerary centrosomes. Cells were stained with antibodies against α-tubulin (green) and centrosomes with antibodies against Nek2 (red); DNA was stained with Hoechst 33258. (B) MDA-MB 468 breast cancer-derived cell line, 5% supernumerary centrosomes. Cells were stained with antibodies against α-tubulin (green) and centrosomes with antibodies against Nek2 (red); DNA was stained with Hoechst 33258. (C) U2OS osteosarcoma cell line, 1% supernumerary centrosomes. Cell is in prophase and centrosomes are stained with antibodies against γ-tubulin; DNA was stained with Hoechst 33258. (D) U2OS osteosarcoma cell line, 1% supernumerary centrosomes. Cell is in metaphase and stained with antibodies against α-tubulin (green) and centrosomes are stained with antibodies against γ-tubulin (red); DNA was stained with Hoechst 33258. Scale bar, 10 μm. Images A and B were kindly taken by D. Hayward and image D kindly taken by S. Wattam (Department of Biochemistry, University of Leicester, UK).
causes 80% to become tetraploid, which is most likely to be the result of no ploidy monitoring system (Meraldi et al., 2002).

Upregulation of Plk1 has also been shown in a variety of human cancers (Holtrich et al., 1994). Overexpressing Plk1 in mammalian cells can cause transformation, while injecting Plk1 transformed NIH 3T3 cells into nude mice causes tumors to develop (Smith et al., 1997). As discussed, Plk1 controls many aspects of mitosis, and transformation may result from aborted mitosis and cytokinesis in a mechanism similar to Aurora A (Meraldi et al., 2002). Nek2 has yet to be confirmed as a transforming oncogene, but studies have shown that its mRNA levels are upregulated in childhood osteosarcomas and diffuse large B-cell lymphomas (de Vos et al., 2003; Wai et al., 2002). Work conducted in our group has shown that Nek2 protein is upregulated in a wide range of cancer cell lines and in primary breast tumors (D. Hayward and A. M. Fry, unpublished data).

The TACC (transforming acidic coiled coil) proteins obtained their name as overexpressing TACC1 in mouse fibroblasts is capable of causing transformation (Still et al., 1999a). TACC1 and TACC3 are upregulated in various human cancers and probably influence transformation by forming abnormal bipolar spindles (Raff, 2002; Still et al., 1999a; Still et al., 1999b). Surprisingly, TACC2 may rather function as a tumor suppressor gene as it is down regulated in various tumors (Chen et al., 2000; Raff, 2002). The TACCs are not enzymatic centrosome regulators but centrosome associated proteins involved in spindle formation. It is likely that other centrosome/spindle-associated proteins may provide novel mechanisms of how centrosomes may participate in genetic instability and aneuploidy. A more complete understanding of how centrosomes are regulated and the molecules involved could highlight potential therapeutic targets for the prevention of tumors in the future.
1.8 Research project aims and objectives

The initial aim of this project was to investigate the function and regulation of centrosomal substrates of the Nek2 kinase. Nek2 is proposed to regulate centrosome disjunction at the G2/M transition and one substrate implicated in this process is C-Nap1. However, it is likely that in addition to C-Nap1, Nek2 has further unidentified mitotic/centrosomal substrates. The specific objectives could therefore be summarized as:

1. To isolate a *Xenopus laevis* homologue of C-Nap1 which could be used to study the role of C-Nap1 in centrosome regulation and spindle formation in cell free extracts of *Xenopus* eggs. cDNA library screening, degenerate PCR and database screening were applied with the aim of isolating a full-length cDNA.

2. To identify novel Nek2 substrates using a yeast two hybrid interaction screen. A catalytically inactive version of Nek2 was used as the bait on the premise that this may interact more tightly with substrates.

3. To characterize the properties of a new centrosomal substrate of Nek2 called Nlp. Antibodies would be generated to Nlp, which would allow characterization of its localization and expression through the cell cycle in embryonic and adult cells.

4. To assess the regulation of Nlp by Nek2 mediated phosphorylation using both *Xenopus* and human cells. Plk1 has previously been shown to displace Nlp from the interphase centrosome, therefore cotransfection experiments using both Plk1 and Nek2 were applied to study the co-ordinated regulation of Nlp.
CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

2.1.1 Suppliers and manufacturers
All chemicals purchased were of molecular, analytical or pure grade (>99 %) and bought from BDH (Poole, UK), Fisher (Loughborough, UK), Melford (Chelsworth, UK) or Sigma (Poole, UK) unless otherwise stated below.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amersham (Little Chalfont, UK)</td>
<td>dNTPs, ECL Western blotting reagents, Streptavidin Texas Red, nylon transfer membrane</td>
</tr>
<tr>
<td>Beckton Dickinson (Le Pont de Cloix, France)</td>
<td>Peptone</td>
</tr>
<tr>
<td>Bio 101 (Carlsbad, Canada)</td>
<td>Yeast nitrogen base, CSM selective media</td>
</tr>
<tr>
<td>Biorad (York, UK)</td>
<td>Protein A beads, purification columns, affigel beads, BCA kit</td>
</tr>
<tr>
<td>BOC (Guildford, UK)</td>
<td>Dry ice</td>
</tr>
<tr>
<td>Calbiochem (Nottingham, UK)</td>
<td>Chloramphenicol, Hoechst 33258</td>
</tr>
<tr>
<td>Flowgen (Ashby de-la Zouch, UK)</td>
<td>Protogel 30% acrylamide</td>
</tr>
<tr>
<td>Fluka (Gillingham, UK)</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Fuji Photo Film (Tokyo, Japan)</td>
<td>Super RX X-ray film</td>
</tr>
<tr>
<td>Helena Biosciences (Sunderland, UK)</td>
<td>Agarose</td>
</tr>
<tr>
<td>Invitrogen/Gibco BRL</td>
<td>Ethidium bromide, penicillin/streptomycin, Leibovitz L-15 medium, foetal calf serum, DMEM medium, tetracycline, RPMI medium, lipofectamine</td>
</tr>
<tr>
<td>MBI Fermentas (Sunderland, UK)</td>
<td>Restriction endonucleases</td>
</tr>
<tr>
<td>New England Biolabs (Hitchin, UK)</td>
<td>Restriction endonucleases, amylose resin</td>
</tr>
<tr>
<td>Oxoid (Basingstoke, UK)</td>
<td>Tryptone, yeast extract, agar</td>
</tr>
<tr>
<td>Pierce (Tattenhall, UK)</td>
<td>Dialysis membrane</td>
</tr>
<tr>
<td>Promega (Southampton, UK)</td>
<td>In vitro translation kit, reverse transcriptase</td>
</tr>
<tr>
<td>Premiere Beverages (Spalding, UK)</td>
<td>Marvel dried powder milk</td>
</tr>
<tr>
<td>Qiagen (Crawley, UK)</td>
<td>Mini prep spin kit, midi/maxi filter kit, gel</td>
</tr>
<tr>
<td>Extraction kit, nucleotide removal kit, PCR purification kit, nickel agarose beads</td>
<td>Roche (Lewes, UK)</td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td></td>
<td>Schleicher and Schuell (Dassel, Germany)</td>
</tr>
<tr>
<td></td>
<td>Thermohybad (Egelsbach, Germany)</td>
</tr>
<tr>
<td></td>
<td>Whatttman International (Maidstone, UK)</td>
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### 2.1.2 Radioisotopes

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Specific Activity</th>
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</tr>
</thead>
<tbody>
<tr>
<td>$[\gamma-^{32}P]dCTP$</td>
<td>311 TBq/mmol</td>
<td>Perkin Elmer</td>
</tr>
<tr>
<td>$[\gamma-^{32}P]ATP$</td>
<td>167 TBq/mmol</td>
<td>ICN</td>
</tr>
<tr>
<td>$[^{35}S]$ methionine</td>
<td>43.5 TBq/mmol</td>
<td>NEN Life Science products</td>
</tr>
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### 2.1.3 Vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>Application</th>
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</tr>
</thead>
<tbody>
<tr>
<td>pACT II</td>
<td>Yeast two hybrid</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGAD</td>
<td>Yeast two hybrid</td>
<td>(James et al., 1996)</td>
</tr>
<tr>
<td>pGBDU</td>
<td>Yeast two hybrid</td>
<td>(James et al., 1996)</td>
</tr>
<tr>
<td>pBluescript</td>
<td>Cloning</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET32</td>
<td>Bacterial protein expression</td>
<td>Novagen</td>
</tr>
<tr>
<td>pMAL</td>
<td>Bacterial protein expression</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pEGFP with T7 promoter</td>
<td>Eukaryotic protein expression</td>
<td>Gift from K. Tanaka</td>
</tr>
<tr>
<td>pRcCMV</td>
<td>Eukaryotic protein expression</td>
<td>Invitrogen</td>
</tr>
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</table>
## 2.1.4 Antibodies

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Working concentration</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>Anti C-Nap 1 polyclonal</td>
<td>1 µg/ml</td>
<td>(Fry et al., 1998a)</td>
</tr>
<tr>
<td>Anti cyclin B1 (<em>Xenopus</em>) monoclonal</td>
<td>2 µg/ml</td>
<td>Gift from H. Yamano, South Mimms, UK</td>
</tr>
<tr>
<td>Anti Eg2 (Aurora A <em>Xenopus</em>) monoclonal</td>
<td>1/500</td>
<td>Gift from E. Nigg, Martinsreid, Germany</td>
</tr>
<tr>
<td>Anti GFP polyclonal</td>
<td>1/5000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti His monoclonal</td>
<td>1/3000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti GT335 monoclonal</td>
<td>1/100</td>
<td>Gift from B. Edde, Montpellier, France</td>
</tr>
<tr>
<td>Anti Myc monoclonal</td>
<td>1/2000</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Anti Nek2 (human) monoclonal</td>
<td>1.0 µg/ml</td>
<td>Transduction Laboratories</td>
</tr>
<tr>
<td>Anti Nek2 (<em>Xenopus</em>) polyclonal</td>
<td>1.9 µg/ml</td>
<td>(Fry et al., 2000b)</td>
</tr>
<tr>
<td>Anti Nlp (human) polyclonal</td>
<td>1.0 µg/ml</td>
<td>(Casenghi et al., 2003)</td>
</tr>
<tr>
<td>Anti Nlp (<em>Xenopus</em>) polyclonal</td>
<td>2.5 µg/ml</td>
<td>This work</td>
</tr>
<tr>
<td>Anti α-tubulin monoclonal</td>
<td>1/2000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti γ-tubulin monoclonal</td>
<td>1/2000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti γ-tubulin polyclonal</td>
<td>1/2000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Secondary Antibodies</td>
<td>Working concentration</td>
<td>Supplier</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Anti mouse alkaline phosphatase conjugate</td>
<td>1/7500</td>
<td>Promega</td>
</tr>
<tr>
<td>Anti rabbit alkaline phosphatase conjugate</td>
<td>1/7500</td>
<td>Promega</td>
</tr>
<tr>
<td>Anti mouse horseradish peroxidase conjugate</td>
<td>1/2000</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti rabbit horseradish peroxidase conjugate</td>
<td>1/2000</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti mouse biotin conjugate</td>
<td>1/100</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti rabbit biotin conjugate</td>
<td>1/100</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti mouse Alexa 488 nm</td>
<td>1/200</td>
<td>Molecular probes</td>
</tr>
<tr>
<td>Anti rabbit Alexa 488 nm</td>
<td>1/200</td>
<td>Molecular probes</td>
</tr>
<tr>
<td>Anti mouse Alexa 594 nm</td>
<td>1/200</td>
<td>Molecular probes</td>
</tr>
<tr>
<td>Anti mouse Alexa 594 nm</td>
<td>1/200</td>
<td>Molecular probes</td>
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</table>

2.1.5 Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>$\Phi$80d lacZΔM15, recA1, endA1, gyrA96, thi-1, hsd R17(rK-mK')</td>
</tr>
<tr>
<td>E. coli</td>
<td>supE44, relA1, deoR, Δ[lacZYA-argF] U169</td>
</tr>
<tr>
<td>Rosetta (DE3)</td>
<td>FompT hsdS₈ (r₈,m₈) gal dcm (DE3) pRARE (argU, argW, ileX, gluT, leuW, proL)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>SOLRᵀᴹ strainᵃ</td>
<td>e14' (McrA') $\Delta$(mcrCB-hsdSMR-mrr) 171sbcC recB recJ uvrC umuC</td>
</tr>
<tr>
<td>E. coli</td>
<td>::Tn5 (Kan') lac gyrA96 relA1 thi-1 endA1 λ' [F'proAB lac⁺ ZΔM15]</td>
</tr>
<tr>
<td></td>
<td>Su' (non suppressing)</td>
</tr>
<tr>
<td>XL1-Blue MRF'</td>
<td>$\Delta$(mrcA) 183Δ (mcrCB-hsdSMR-mrr) 173endA1 supE44 thi-1 recA1</td>
</tr>
<tr>
<td>E. coli</td>
<td>gyrA926 relA1 lac [F'proAB lac⁺ ZΔM15 Tn10 (Tet')]</td>
</tr>
</tbody>
</table>

2.1.6 Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJ69-4A</td>
<td>MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, GAL4Δ gal80Δ,</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>LYS::GAL1-HIS3, GAL2-ADE2met2::GAL7-LacZ</td>
</tr>
</tbody>
</table>

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2.1.7 Standard buffers and solutions

All solutions were prepared using deionised, distilled purified water (Elgastat optima 2 water purifier) and filtered where necessary (0.22 μm filter). Sterilisation of solutions and glassware was performed by autoclaving at 15 pounds/inch² for 20 min. All pH adjustments were performed at 25°C.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Laemmli buffer</td>
<td>104 mM Tris pH 6.8, 3.3% (w/v) SDS, 8.3% (v/v) β-mercaptoethanol, 16.7% (v/v) glycerol, 0.016% (v/v) bromophenol blue</td>
</tr>
<tr>
<td>PBS</td>
<td>137 mM NaCl, 26.8 mM KCl, 2.7 mM Na₂HPO₄, 1.4 mM KH₂PO₄</td>
</tr>
<tr>
<td>TBE</td>
<td>89 mM Tris, 89 mM boric acid, 1 mM EDTA pH 8.0</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris pH 8.0, 1 mM EDTA</td>
</tr>
<tr>
<td>20X SSPE</td>
<td>3 M NaCl, 0.2 M NaH₂PO₄.H₂O, 22 mM EDTA, pH 7.4</td>
</tr>
<tr>
<td>20X SSC</td>
<td>3 M NaCl, 0.34 M sodium citrate, pH 7.0</td>
</tr>
</tbody>
</table>

2.2 Bacterial methods

2.2.1 Growth and storage of bacteria

One colony of bacteria was selected and grown in Luria Bertani (LB-NaCl 10 g/l, Tryptone 10 g/l, yeast extract 5 g/l, adjust to pH 7.0 then autoclaved) overnight (O/N), shaking at 250 rpm at 37°C. Where appropriate a selective antibiotic (ampicillin 100 μg/ml, chloramphenicol 34 μg/ml, kanamycin 50 μg/ml, tetracycline 12.5 μg/ml) was supplemented. For plated cultures, agar was added to LB at 2% (w/v) prior to autoclaving and grown at 37°C O/N. Stocks of bacteria were prepared by taking 200 μl of O/N culture added to 800 μl sterile glycerol, vortexing and storing at -80°C.

2.2.2 Competent bacterial cell production

DH5α was streaked onto LB-agar and incubated at 37°C O/N, one colony was then picked and grown in 2 ml of LB O/N. 1 ml of the overnight culture was then added to 500 ml of LB supplemented with 10 mM MgCl₂ and shaken at 100 rpm at room temperature (RT) until the optical density at 600 nm (OD₆₀₀) was between 0.3-0.6 (~18 h). The culture was then rapidly cooled in an ice slurry and spun down (2500 g, 10 min, 0°C). The pellet was
resuspended in 150 ml of ice-cold transformation buffer (15 mM CaCl₂, 250 mM KCl, 10 mM PIPES pH 6.7, 55 mM MnCl₂) and spun down as before. The pellet was gently resuspended in 40 ml of ice-cold transformation buffer, and 3 ml of DMSO added with gentle swirling. Aliquots of 500 µl were immediately frozen in liquid nitrogen and stored at -80°C.

2.2.3 Transforming competent bacteria
Competent cells were defrosted on ice while 100 ng of DNA was added to cold tubes on ice. 100 µl of competent bacteria was added to the DNA and gently mixed by tapping before incubating on ice for 30 min. The sample was then heat shocked at 42°C for 1 min, returned to the ice and 500 µl of RT LB was added. The bacteria were incubated at 37°C for 1 h, pelleted (10000 g, 10 s, RT) and resuspended in 75 µl LB before being plated.

2.3 Yeast methods

2.3.1 Growth and storage of yeast
One yeast colony was selected and grown in 10 ml of YPD (peptone 20 g/l, yeast nitrogen base 10 g/l, glucose 20 g/l, adjust to pH 6.5-7.0 autoclave) with O/N shaking at 250 rpm at 30°C. Where appropriate nutritional deficient media (SD-yeast nitrogen base 10 g/l, glucose 20 g/l, x g/l deficient amino acid mix according to the manufacturer’s instructions) could also be used. For plated cultures, agar was added to YPD or SD at 2.4% (w/v) prior to autoclaving and grown at 30°C for 3-5 days. Stocks of yeast were prepared by taking 200 µl of O/N culture added to 800 µl sterile glycerol, vortexing and storing at -80°C.

2.3.2 Transforming yeast cells
One yeast colony was selected and grown in YPD or the appropriate SD O/N at 250 rpm at 30°C. The broth was then diluted to OD₆₀₀ in 50 ml of YPD and grown as before for 4 h. The cells were then pelleted (3000 g, 10 min, RT) and resuspended in 40 ml of TE and pelleted as before. The cells were resuspended in 2 ml of 0.1 M lithium acetate (LiAc) and incubated at RT for 10 min. 1 µg plasmid DNA was mixed with 100 µg of single stranded DNA (Herring sperm), 100 µl of yeast suspension was added and mixed well. 700 µl of 0.1 M LiAC/40% polyethylene glycol/TE was added to the yeast suspension, vortexed, incubated at 30°C for 30 min and 80 µl of dimethyl sulfoxide (DMSO) added. The yeast
suspension was heat shocked at 42°C for 7 min, pelleted (10000 g, 10 s, RT), resuspended in 1 ml of TE, pelleted again, resuspended in 75 μl of TE and plated.

2.3.3 Making yeast lysates
2.5 ml of O/N culture was pelleted (3000 g, 10 min, RT), resuspended in 1 ml of 0.25 M NaOH/1% β-mercaptoethanol, and incubated on ice for 20 min; 160 μl of 50% Tricarboxylic acid (TCA) was added after 10 min. The suspension was pelleted (10000 g, 10 min, RT), resuspended in 1 ml of ice-cold acetone by vortexing and pelleted as before. The protein pellet was air dried and resuspended in 300 μl of 3X Laemmli buffer.

2.3.4 Selection for interacting proteins by nutritional deficiency
Gal4 fusion protein constructs were transformed into yeast and grown on the appropriate SD media plates. Colonies were streaked onto SD-HIS plates supplemented with a range of 3-aminotriazole (3-AT) concentrations (1 mM-30 mM) to determine the concentration required to prevent growth in the absence of an interaction. An interaction between proteins was selected by growth on SD-HIS-ADE plates supplemented with the required concentration of 3-AT.

2.3.5 Assaying interaction strength by the LacZ gene-ONPG assay
One yeast colony was selected and grown in the appropriate media until the OD600=1.0-1.2. 2 ml of the culture was pelleted (10000 g, 1 min, RT) and resuspended in 1 ml of Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 40 mM β-mercaptoethanol, adjust to pH 7.0). From a pasteur pipette 3 drops of chloroform and 2 drops of 0.1% SDS were added and the suspension incubated for 2 min at 30°C. o-nitrophenyl-β-D-galactoside (ONPG) was added to the suspension at a final concentration of 0.67 μg/ml and incubated for 8 min at 30°C, the reaction was stopped by adding 500 μl of 1 M Na2CO3 and mixing. The suspension was pelleted as above to remove debris and quantitatively measured at an OD420 using Z-buffer as the blank.
2.4 Molecular biology techniques

2.4.1 Restriction digests
Typically 2 μg DNA was mixed with the appropriate 10X reaction buffer (supplied by the manufacturer) and the appropriate quantity of water added followed by 10 units (U) of restriction endonuclease. The reaction was incubated for 2 h at 37°C (or required temperature for specific restriction endonucleases) and stopped, using the nucleotide removal kit (if required). Double digestions used either both restriction endonucleases in one reaction or a sequential reaction was performed on the purified DNA using the appropriate restriction endonuclease and buffer. The nucleotide removal kit was used as per the manufacturer’s instructions and resulted in purified DNA being eluted in 30 μl of 10 mM Tris pH 8.5.

2.4.2 Alkaline phosphatase treatment
Dephosphorylation of DNA was performed directly on digested DNA by addition of 10X calf intestinal alkaline phosphatase (CIP) buffer with the appropriate quantity of water followed by 1 U of CIP. Reaction was incubated for 30 min at 37°C and purified (if required) using the nucleotide removal kit.

2.4.3 DNA ligation
A standard reaction used CIP treated vector with insert DNA. These were mixed together at a molar ratio of 1:3 with the addition of 1 μl of ligase buffer and water to a total of 9 μl, followed by 1 μl of T4 DNA ligase. Reactions were incubated at 14°C O/N followed by a bacterial transformation, with 4 μl of the ligation mix and plating on the appropriate antibiotic.

2.4.4 DNA agarose gel electrophoresis
To prepare a gel, agarose (between 0.25 g and 1.0 g dependant upon the DNA size to be examined) was mixed with 50 ml TBE and microwaved at full power for 1 min, followed by the addition of ethidium bromide at a final concentration of 0.2 μg/ml. Liquid agarose was poured into a DNA gel tank with the addition of a comb (comb size dependant upon the volume of DNA to be analyzed) and allowed to stand for 30 min at RT; once set the gel was covered in TBE and the comb removed. DNA was loaded by mixing with loading dye (50% glycerol, 0.1 M EDTA, 0.3% bromophenol blue) at a ratio of 5:1, respectively, and
pipetted into the wells (2 μl 1 Kb markers were added to one well). The gel was run at 80 V for 45 min-1 h.

2.4.5 Purifying DNA from agarose gels
Gels were placed upon a UV transilluminator and the respective DNA band(s) removed using a clean scalpel; the excised DNA was placed into a pre-weighed tube. The Gel Extraction Kit (Qiagen) was then used following the manufacturer’s instructions resulting in DNA being eluted into 30 μl of 10 mM Tris pH 8.5.

2.4.6 Oligonucleotide (primer) design and synthesis
Oligonucleotide sequences typically were 21-30 bp long with a GC content between 40 and 60% to give an annealing temperature between 45 and 55°C. Non-matching nucleotides comprised of restriction enzyme sites, additional bases to complement the vector reading frame or mutations for site directed mutagenesis. Oligonucleotides were synthesised by Thermohybaid and resuspended in a stated volume of water to give a concentration of 100 μM.

2.4.7 Polymerase chain reaction (PCR)
To screen cloning plasmids for DNA inserts a standard PCR reaction was performed using Taq T4 polymerase. Each reaction contained 0.1-0.5 μg DNA (or a bacterial colony), 1 mM of each primer, 10X reaction buffer, 0.4 mM dNTPs and the required amount of water followed by 1 U Taq polymerase. Various annealing temperatures and extension times were used based on the primer sequence and DNA length to be amplified. The table below shows a standard reaction using T7 and T3 primers for amplifying a DNA sequence of 3000 bp.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature-Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (X1)</td>
<td>94°C-5 min</td>
<td>Denature DNA</td>
</tr>
<tr>
<td>2 (X25)</td>
<td>94°C-30 sec</td>
<td>Denature DNA</td>
</tr>
<tr>
<td></td>
<td>50°C-30 sec</td>
<td>Primer annealing temperature</td>
</tr>
<tr>
<td></td>
<td>72°C-3 min</td>
<td>Extension time</td>
</tr>
<tr>
<td>3 (X1)</td>
<td>72°C-7 min</td>
<td>Clean up DNA ends</td>
</tr>
<tr>
<td></td>
<td>4°C-∞</td>
<td>Store DNA</td>
</tr>
</tbody>
</table>
For PCR cloning another approach was used to minimise errors caused by Taq T4 polymerase. Reactions contained more DNA, had fewer cycles and used a Taq polymerase with proof reading activity (Expand high fidelity). Reactions were performed according to the manufacturer's instructions but used 1 μg template DNA and were performed in duplicate to produce a viable quantity of DNA. The table below shows a standard reaction using cloning primers for amplifying a DNA sequence of 3000 bp.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature-Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (X1)</td>
<td>94°C-5 min</td>
<td>Denature DNA</td>
</tr>
<tr>
<td>2 (X10)</td>
<td>94°C-15 sec</td>
<td>Denature DNA</td>
</tr>
<tr>
<td></td>
<td>50°C-30 sec</td>
<td>Primer annealing temperature</td>
</tr>
<tr>
<td></td>
<td>68°C-3 min</td>
<td>Extension time</td>
</tr>
<tr>
<td>3 (X1)</td>
<td>72°C-7 min</td>
<td>Clean up DNA ends</td>
</tr>
<tr>
<td></td>
<td>4°C-∞</td>
<td>Store DNA</td>
</tr>
</tbody>
</table>

After the PCR reaction DNA was analyzed directly by agarose gel electrophoresis or purified using the PCR purification kit resulting in DNA being eluted in 30 μl of 10 mM Tris pH 8.5.

2.4.8 Plasmid preparation I (small scale)
One bacterial colony was selected and grown O/N in 5 ml LB supplemented with the appropriate antibiotic, the culture was spun down (3000 g, 10 min, RT) and supernatant aspirated. Plasmid isolation used the Mini Prep Spin Kit (Qiagen) resulting in 5-10 μg plasmid DNA being eluted in 50 μl of 10 mM Tris pH 8.5.

2.4.9 Plasmid preparation II (large scale)
One bacterial colony was selected and grown for 8 h in 2 ml of LB supplemented with the appropriate antibiotic. The starter culture was then diluted 1/500 in 100 ml of LB with the appropriate antibiotic and grown O/N. The culture was spun down (6000 g, 15 min, 4°C) and supernatant decanted. Plasmid isolation used the Qiafilter Maxi Prep Kit (Qiagen) resulting in 500 μg-1 mg plasmid DNA being resuspended in 200 μl of 10 mM Tris pH 8.5. The DNA was then diluted to give a concentration of 1 μg/μl.
2.4.10 Quantifying DNA concentration
To calculate the DNA concentration, dilutions of the plasmid were either run on a gel next to known quantities of DNA and then estimated, or measured using UV light at an OD$_{260}$ in quartz cuvettes. DNA concentration using UV light was calculated using the equation below,

$$A_{260} \times \text{Dilution factor} \times 40 = \text{DNA concentration (µg/ml)}$$

2.4.11 DNA Sequencing
Samples were sent at the required dilution to Lark Technologies (Saffron Walden, UK) for sequencing. Required primers were either sent with samples or synthesised by Lark Technologies.

2.4.12 cDNA library screening
Library plating and plaque lifts were performed according to the manufacturer's instructions (Stratagene) using two *Xenopus laevis* embryonic cDNA libraries, stage 10.5 (gift from Dr. V. Allen, Manchester) and stage 42 (gift from Prof. H. Woodland, Warwick). Hybridisation probes were labelled using a Random Primed Labelling Kit with $^{32}$P-dCTP according to the manufacturer's instructions (Roche), then purified using the Nucleotide Removal Kit (Qiagen) with elution into 200 µl of 10 mM Tris pH 8.5. Prehybridisation buffer (0.1 M PIPES pH 7.0, 0.8 M NaCl, 200 µg/ml herring sperm DNA, 0.1% N-laurylsarcosine, 5X Denhardt's (50X-10 g/l Ficoll, 10 g/l polyvinylpyrrolidone, 10 g/l BSA), 50% de-ionised formamide) was boiled for 10 min then quenched on ice and transfer membranes soaked within. Transfer membranes had a piece of Whatman 3MM paper sandwiched between them and were pre-hybridised for 2 h at 30°C with rotation. Pre-hybridisation buffer was removed and replaced with hybridisation buffer (0.1 M PIPES pH 7.0, 0.8 M NaCl, 200 µg/ml herring sperm DNA, 0.1% N-laurylsarcosine, 2X Denhardt's, 50% de-ionised formamide, 10% dextrane sulphate, 200 µl labelled probe) which was boiled for 10 min and quenched on ice prior to addition to the transfer membranes, then incubated O/N at 30°C with rotation. Hybridisation buffer was removed and transfer membranes washed 3X 20 min in 2X SSPE/0.1% SDS with agitation at RT. Transfer membranes were exposed to X-ray film until a signal was detectable (typically O/N). Re-alignment of X-ray film with the plated library allowed for plugging positive plaques by using sterile cut pipette tips to transfer plaques into 500 µl SM buffer (0.1 M NaCl, 8 mM MgSO$_4$, 50 mM Tris pH 7.5, 0.01% gelatine) containing 10 µl chloroform,
which were shaken for 2 h at 37°C and vortexed. For single plaque isolation the plugged phage were diluted 1/250 and the process repeated using the probes from the first screen. If no single plaques could be isolated from the second screen, then a third screen was performed. To convert the positive isolated plaques into plasmids, *in vivo* excision was performed according to the manufacturer's instructions (Stratagene) and bacterial colonies were mini-prepped and the cDNA inserts sequenced.

2.4.13 Southern blotting

DNA to be tested was separated by agarose gel electrophoresis and assembled into a Southern blot stack. The gel was placed upon 3MM Whatman paper that could absorb 0.4 M NaOH from below. Placed above the gel was a Hybond-N transfer membrane, followed by 3X3 layers of Whatman 3MM paper, a stack of tissue paper and a full 1 litre bottle to sandwich the stack. DNA transfer occurred O/N at RT. The membrane was then washed 3X 10 min in 2X SSC. The DNA probe was prepared as described for cDNA library screening and was hybridised directly with the transfer membrane at 30°C with agitation. The membrane was washed 3X 10 min in 2X SSC/0.1% SDS at RT and exposed to X-ray film.

2.4.14 Computer analysis of sequences

The software Gene Jockey II was used for restriction site mapping, translation of reading frames and sequence alignment. Various web-based resources were also used for sequence analysis and are listed below.

<table>
<thead>
<tr>
<th>Website</th>
<th>URL</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entrez</td>
<td><a href="http://www.ncbi.nlm.nih/entrez">www.ncbi.nlm.nih/entrez</a></td>
<td>Obtaining DNA and protein sequences</td>
</tr>
<tr>
<td>NCBI BLAST</td>
<td><a href="http://www.ncbi.nlm.nih.gov/blast">www.ncbi.nlm.nih.gov/blast</a></td>
<td>Screening databases with either DNA or protein sequences</td>
</tr>
<tr>
<td>ExPASy</td>
<td>ca.expasy.org</td>
<td>Protein analysis</td>
</tr>
<tr>
<td>Coils</td>
<td><a href="http://www.ch.embnet.org/software/COILS_form.html">www.ch.embnet.org/software/COILS_form.html</a></td>
<td>Predicting coiled coil regions within protein sequences</td>
</tr>
<tr>
<td>Motif Scan</td>
<td>hits.isb-sib.ch/cgi-bin/PFSCAN?</td>
<td>Predicting protein motifs within protein sequences</td>
</tr>
</tbody>
</table>
2.5 Protein techniques

2.5.1 SDS-PAGE I-pouring protein gels

A standard protein gel was poured at varying acrylamide percentages, depending on the size of the protein in question. The table below shows the components of the lower separating gel and the respective protein size that can be analyzed.

<table>
<thead>
<tr>
<th>Component</th>
<th>15%</th>
<th>12%</th>
<th>10%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Protogel (Acrylamide)</td>
<td>3.0 ml</td>
<td>2.4 ml</td>
<td>2.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Water</td>
<td>1.5 ml</td>
<td>2.1 ml</td>
<td>2.5 ml</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Lower Tris (1.5 M Tris pH 8.8, 0.4% SDS)</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>75 µl</td>
<td>75 µl</td>
<td>75 µl</td>
<td>75 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Protein size (kDa)</td>
<td>15-40</td>
<td>20-70</td>
<td>40-100</td>
<td>120-250</td>
</tr>
</tbody>
</table>

The gel casting apparatus was assembled and 3.5 ml separating gel was pipetted into the cast followed by addition of water-saturated butanol to ensure an even gel interface. Once set, the butanol was removed and 3 ml upper stacking gel (0.65 ml 30% protogel, 3 ml water, 1.25 ml Upper Tris (0.5 M Tris pH 6.8, 0.4% SDS), 75 µl 10% ammonium sulphate, 5 µl TEMED) added followed by the appropriate loading comb. For larger gels all components were increased proportionally to fill the cast. Once set, the comb was removed and wells washed with water.

2.5.2 SDS-PAGE II-Loading and running protein gels

5X Laemmli buffer was added to samples to give a final concentration of 1X. Samples were boiled for 10 min and the appropriate quantity loaded into the wells. Marker proteins were added to one well for identifying protein size (Invitrogen, MBI Fermentas). Wells were equalised by the addition of 1X Laemmli buffer followed by pouring running buffer (0.1% SDS, 0.3% Tris, 1.44% glycine) over the cast to connect the electrodes. To separate the proteins, 180 V was applied until the Laemmli buffer ran off the gel (45 min-1 h), the cast was disassembled and the gel was now ready for further analysis.
2.5.3 Staining protein gels
To visualise protein content, the gel was soaked in Coomassie Blue stain (0.25% Coomassie Brilliant Blue, 40% IMS, 10% acetic acid) for 5 min followed by numerous washes in destain (25% IMS, 7.5% acetic acid) until the proteins were clearly visible. Gels were finally dried onto Whatman 3MM under vacuum at 80°C for long term storage.

2.5.4 Western blotting
SDS-PAGE gels along with nitrocellulose transfer membrane and chromatography paper were soaked in Western blotting buffer (25 mM Tris, 192 mM glycine, 10% methanol) and layered into the semi dry blotter (Hoefer Semiphor, Amersham) in the following order: 3 layers Whatman 3MM paper, 1 membrane, 1 gel, 3 layers Whatman 3MM paper. A current of 1 mA/cm² membrane was applied for 1 h. To visualise protein transfer, the membrane was soaked in Ponceau S (0.5% Ponceau S, 1% acetic acid) and destained using water until the markers were visible. The membrane was blocked by 3X 10 min washes in Marvel-Tween (5% Marvel, 1X PBS, 0.1% Tween 20) then probed using a primary antibody (see section 2.1.2) in a suitable volume of Marvel Tween (probing times vary for each antibody, typically 45 min was standard). The membrane was washed by 3X 10 min washes in PBS-Tween (1X PBS, 0.1% Tween 20) then probed using a secondary antibody (see section 2.1.2) as before for 45 min, then washed as before. For developing blots probed with alkaline phosphatase conjugated secondary antibodies, the membrane was soaked in AP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris pH 9.5) for 10 min and then replaced by 10 ml AP buffer supplemented with BCIP and NBT at a final concentration of 0.16 mg/ml and 0.33 mg/ml, respectively. Colour was allowed to develop until the protein of interest was detectable, then stopped by washing as before. For developing blots probed with HRP-conjugated secondary antibodies, the membrane was soaked in PBS and developed with ECL reagents (Amersham) according to the manufacturer's instructions. Exposure times to X-ray film were between 2 and 30 min before film development.

2.5.5 Immunoprecipitations
10 µl of protein A beads were spun down and washed 3X in 1 ml of PBS. To reduce non-specific binding, beads were blocked by incubation with 1 ml of PBS supplemented with 3% BSA (or 50 µl anti-sera in 1 ml PBS) on a rotating wheel for 1 h at 4°C, then washed as before. The appropriate antibody was added to the cell extract and incubated on ice for
1 h, combined with the blocked beads and placed on a rotating wheel for a further 1 h at 4°C, then washed as before. Samples were analyzed by Western blotting.

2.5.6 *In vitro* translation (IVT)
Standard IVTs were performed according to the manufacturer’s instructions (Promega) with the addition of the appropriate RNA polymerase (T7, T3, SP6). Translated proteins were either labelled by addition of \(^{35}\)S-methionine or unlabelled by addition of standard methionine. IVT products were analyzed by SDS-PAGE with Coomassie Blue staining and exposure to X-ray film or Western blotting.

2.5.7 Pulldown assays
To test for *in vitro* protein-protein interactions, one protein was either immunoprecipitated or attached to beads via a purification tag fusion construct (see section 2.6), while the other was an IVT product. Standard reactions composed of 50 μl beads (protein attached), 10 μl of IVT reaction mix and 1 ml of PBS; all were mixed together and rotated on a wheel O/N at 4°C, washed 3X in 1 ml of PBS and analyzed by SDS-PAGE.

2.5.8 TCA precipitation
TCA was added to samples at a final concentration of 20% and incubated on ice for 1 h. Samples were pelleted (20000 g, 15 min, 4°C), washed in 70% ethanol (-20°C), spun (20000 g, 5 min, 4°C), washed in 80% acetone (-20°C) and spun again. 20 μl of 5X Laemmli buffer was added and samples analyzed by SDS-PAGE.

2.5.9 Quantifying protein concentration
To calculate purified protein concentration, fractions were separated by SDS-PAGE alongside known amounts of BSA protein. Alternatively, the Bicinchoninic acid (BCA) assay was used following the manufacturer’s instructions (Pierce). Samples were measured in duplicate and the mean used to estimate protein concentration from a standard curve of BSA concentrations.
2.6 Protein expression in \textit{E. coli}

2.6.1 Expression of protein in bacteria
Protein encoding cDNA fragments were subcloned into prokaryotic expression vectors and transformed into Rosetta DE3 \textit{E. coli}. Four single colonies were selected and grown O/N in 1.5 ml of LB supplemented with the appropriate antibiotic. 500 µl of the O/N culture was inoculated into 10 ml of pre-warmed LB then grown at 37°C until the OD$_{600}$ was between 0.5-0.7 (1-2 h). For large-scale expression the procedure was identical except a 20 ml culture was grown O/N, and all 20 ml was inoculated into 1 litre of LB. Protein expression was induced by adding IPTG to the required final concentration (1 mM for pET32 constructs, 0.4 mM for pMAL constructs), and the culture grown for a further 5 h. Protein expression was determined by pelleting 1 ml of uninduced and induced culture followed by resuspension in 50 µl and 100 µl of 5X Laemmli buffer, respectively. 20 µl was analyzed by Coomassie Blue staining of SDS-polyacrylamide gels.

2.6.2 Preparation of bacterial cell lysates
One expressing colony was grown O/N in 1.5 ml of LB supplemented with the appropriate antibiotic. Bacteria were lysed according to the manufacturer’s instructions (NEB) and, following sonication, the crude lysate was centrifuged (9000 g, 20 min, 4°C) producing an insoluble pellet and supernatant. The pellet was resuspended in 5 ml Column buffer (20mM Tris pH 7.4, 200 mM NaCl, 1 mM EDTA) and 20 µl of the supernatant and pellet resuspension analyzed by Coomassie Blue staining of SDS-polyacrylamide gels.

2.6.3 Purification of pET32 constructs under native conditions
For producing native protein the manufacturer’s instructions (Qiaexpressionist 4\textsuperscript{th} edition) were followed. Purification was performed using a Ni$^{2+}$ agarose column. Purified proteins were dialysed (Slide a Lyzer 10,000 MWCO, Pierce UK) into 50 mM Hepes pH 7.4, quantified, and stored at -80°C.

2.6.4 Purification of pET32 constructs under denaturing conditions
For producing denatured protein the manufacturer’s instructions (Qiaexpressionist 4\textsuperscript{th} edition) were followed using 8 M urea lysis buffers until the protein elution phase. Purification was performed using a Ni$^{2+}$ agarose column. Protein washing used buffer B
(100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, pH 8.0) with elution using only buffer D (100 mM NaH₂PO₄, 10 mM Tris, 8 M urea, pH 5.9). Proteins were then stored at -80°C.

2.6.5 Purification of MBP fusion proteins
For purification of MBP-tagged proteins from pMAL fusion constructs, the manufacturer’s instructions (NEB) were followed until the elution phase. Purification was performed using an amylose resin. Proteins were eluted with 5X 5 ml elution buffer (20 mM Tris pH 7.5, 100 mM NaCl, 15 mM maltose) before dialysis (Slide a Lyzer 10,000 MWCO, Pierce UK) into 50 mM Hepes pH 7.4, quantified, and stored at -80°C.

2.7 Tissue culture techniques

2.7.1 Xenopus laevis A6 kidney and XTC cell culture maintenance
A6 and XTC cell lines were grown at 23°C in modified L-15 Leibovitz medium (60% Leibovitz medium, 40% water), supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively). Cells were grown on 10 cm cell culture plates in 10 ml modified L-15 until ~70% confluency, at which point they were split and replated. Splitting cells involved aspirating the media, washing in 10 ml CMFM (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 7.5 mM Tris pH 7.6) followed by aspiration, washing in 10 ml CMFM supplemented with 0.5 mM EDTA with 9 ml aspirated and then left for 5 min at RT. Cells were resuspended in 10 ml modified L-15 medium, plated at a 1/2 or 1/3 dilution with modified L-15 medium added to make a 10 ml suspension and gently swirled to evenly distribute the cells on a 10 cm plate. Cells were then incubated as before.

2.7.2 Human U2OS cell culture maintenance
U2OS cells were grown in DMEM (with 10% FCS, penicillin/streptomycin 50 IU/ml and 50 µg/ml, respectively) at 37°C in a 5% CO₂ atmosphere. Cells were grown on 10 cm cell culture plates in 10 ml DMEM until ~70% confluency, at which point they were split and replated. Splitting cells involved aspirating the media, washing in 10 ml PBS followed by aspiration, washing in 10 ml PBS supplemented with 0.5 mM EDTA with 9 ml aspirated and left at 37°C for 5 min. Cells were resuspended in 10 ml DMEM, plated at a 1/2-1/10 dilution with DMEM added to make a 10 ml suspension and gently swirled to evenly distribute the cells on a 10 cm plate. Cells were then incubated as before. To induce the
myc-His-Nek2A-K37R fusion protein, DMEM was supplemented with tetracycline at a final concentration of 1 μg/ml.

2.7.3 Transfection of cells
A6 cells were grown as above and split into 6 well plates each containing an acid-etched glass coverslip, then grown to 50-70% confluency. Transfection mixes were prepared by mixing 5 μg of DNA with 250 μl RPMI (1) and 5 μl of lipofectamine with 250 μl of RPMI (2). Solutions (1) and (2) were left for 5 min at RT, mixed together, and left for a further 30 min at RT. Cell media was aspirated and replaced with 2 ml modified L-15 medium, then all 500 μl of the DNA/lipofectamine/RPMI mixture was added to the cells dropwise. Cells were incubated for 6 h at 23°C, the transfection media replaced with fresh modified L-15 medium and cells grown for a further 16 or 24 h. The procedure was identical for U2OS cells except they were grown in DMEM and incubated at 37°C.

2.7.4 Preparation of cell lysates
Protein cell lysates were made from A6, XTC and U2OS cell lines using identical procedures. Cells were grown to 70-95% confluency, media aspirated, cells washed once in PBS and 1 ml of ice-cold NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris pH 8.0) added. Cells were rocked slowly on ice for 30 min, harvested and spun (10000 g, 15 min, 4°C) to remove debris. The supernatant was transferred to a clean tube, quantified by BCA assay and 300 μl of 5X Laemmli buffer added. Alternatively, cells were lifted from the plate using the splitting protocol, spun down (1000 g, 5 min, RT) washed in PBS and lysed as above.

2.7.5 A6 cell synchronisation
Cells were grown to ~70% confluency and synchronised by the addition of drugs or by serum starvation. A G0 population was made by aspirating media, washing 3X in PBS and adding serum free modified L-15 medium for 48 h. A G1/S population was made by adding hydroxyurea (HU) to modified L-15 medium at a final concentration of 1 mM followed by a 24 h incubation. A G1 population was made by a G1/S block, washing cells 3X in PBS with a further 16 h incubation in modified L-15 medium, whereas an S/G2 population was made by an 8 h release from the same block. A mitotic population was made by adding nocodazole to modified L-15 medium at a final concentration of 500 ng/ml followed by a 24 h incubation.
2.7.6 Flow cytometric analysis
Asynchronous or synchronised cells were lifted, spun down (1000 g, 5 min, RT) and fixed by resuspension in 1 ml of 70% ethanol (-20°C), cells could be stored under these conditions for several days. Fixed cells were spun down, washed in PBS and then resuspended in 1 ml of DNA stain solution (200 μg/ml RnaseA, 20 μg/ml propidium iodide) followed by incubating for 30 min at RT in the dark. Samples were then analyzed using a FACS machine (BD FACSCaliber, BD Biosciences) counting at least 4000 cells per sample.

2.7.7 Disruption of microtubules
To breakdown microtubules, nocodazole was added to modified L-15 medium at a final concentration of 6 μg/ml and cells incubated on ice for 30 min. For stabilisation of microtubules, taxol was added to modified L-15 medium at a final concentration of 5 μM and incubated for 4 h at 23°C.

2.7.8 Freezing cell lines
Cells were grown in the appropriate media until 90-95% confluency was reached, then lifted, spun down (1000 g, 5 min, RT), resuspended in PBS and spun down again. Cells were resuspended in 1 ml of the appropriate medium containing 10% DMSO and 20% FCS, then transferred to a cryo-tube. These were slowly frozen by incubation in a polystyrene box for 24 h at -20°C, then 24 h at -80°C and indefinite storage in liquid nitrogen.

2.8 Preparation of *Xenopus* tissue extracts

2.8.1 Organ and embryo isolation
A male toad was dissected and organs of interest removed using a scalpel and surgical scissors, ovaries were removed from a female toad in an identical procedure. A sample of each tissue was removed and stored at -80°C, whereas embryos were frozen in dry ice and then stored at -80°C.

2.8.2 Preparation of tissue extracts
Organ samples were thawed at RT, weighed, frozen in liquid nitrogen and ground up using a pestle and mortar with the required amount of Tri Reagent recommended by the
manufacturer (1 ml Tri reagent/100 mg tissue) (Sigma) and stored at -80°C. Five embryos were thawed at RT and ground up by suspension in 1 ml Tri reagent followed by vortexing and pipetting and stored at -80°C.

2.8.3 Isolating RNA and protein from cell and embryo extracts

Extracts were thawed at RT and RNA and protein were extracted according to the Tri reagent protocol (Sigma). Ultimately, RNA was resuspended in 200 µl of DEPC treated water, while protein was resuspended in 0.1% SDS at 1 ml/250 mg starting weight. RNA and protein samples were quantified by OD$_{260}$ and BCA assays, respectively, and stored at -80°C.

2.9 Indirect immunofluorescence microscopy

Cells were grown on acid treated glass coverslips to 50-70% confluency and transfected if necessary. Cells were either fixed in methanol (-20°C) and placed in a -20°C freezer for 5 min, or fixed in paraformaldehyde solution (3% paraformaldehyde, 2% sucrose, PBS) for 10 min, and permeabilised for a further 5 min in cold PBS supplemented with 0.5% Triton X-100. Following fixation, coverslips were washed 3X in PBS (4 min each) and then blocked by preincubation in PBS supplemented with 3% BSA for 10 min. Primary antibodies in PBS/3% BSA were added for 45 min. Coverslips were washed 3X in PBS (4 min each) before secondary antibodies/Hoechst 33258 (0.5 µg/ml) in PBS/3% BSA was added for 45 min in the dark. Coverslips were finally washed again and mounted on a drop of anti-fading mounting solution (90% glycerol/3% n-propyl gallate/100 mM Tris pH 9.0) on glass microscope slides. Coverslips were fixed in position with nail varnish and stored in the dark at 4°C. Cells were visualised using a Nikon TE300 inverted microscope and images captured by a Hamamatsu ORCA-ER camera and analyzed using Openlab 3.1.4 software. Quantitative centrosome imaging was performed at non-saturating exposure conditions using Openlab 3.1.4 software with a fixed region of interest (1 µm$^2$) and background readings deducted.
2.10 Antibody generation and purification

2.10.1 Pre-screening rabbit sera
Rabbits were selected for immunization by screening pre-immune sera by Western Blot analysis and immunofluorescence microscopy in A6 cells; four rabbit sera were screened for each protein fragment. For Western Blot analysis, 50 µg of asynchronous A6 cell lysate was separated by SDS-PAGE and probed using rabbit sera at a dilution of 1/250. Immunofluorescence microscopy was performed using γ-tubulin monoclonal antibodies to detect centrosomes, Hoechst 33258 and rabbit sera at a dilution of 1/250. Immunization candidates were selected on the basis of serum not recognising bands between 150-200 kDa on Western blots, and no centrosome reactivity by microscopy. Two rabbits were immunized per expressed protein fragment with 5 or 7 injections of 100 µg protein. Rabbits 1679 and 1681 were immunized with PET32:X-Nlp262-552; rabbits 1683 and 1685 with PET32:X-Nlp174-282; and rabbits 685 and 688 with PET32:X-Nlp800-1397. Bleeds were taken one week after each injection according to the programme run by Cambridge Research Biochemicals (Cleveland, UK).

2.10.2 Testing antisera reactivity
Rabbit sera were tested by Western blot and immunofluorescence microscopy as described in 2.10.1. The MBP:Nlp262-552 fusion protein was also used for Western Blot analysis to directly determine reactivity of bleeds from rabbits injected with pET32:Nlp262-552. Immunized rabbits which showed the strongest reactivity (1679 and 1681) had a further two immunizations to maximize the response.

2.10.3 Affinity purification of antibodies
Antibody affinity purification was performed using the MBP:Nlp262-552 fusion protein covalently attached to agarose beads (Affi-gel 15). 40 mg of MBP:X-Nlp262-552 was coupled to 5 ml of beads in 0.1 M Hepes pH 7.5. According to the manufacturer's instructions, 10 ml rabbit sera (bleed 5 and 6) was then purified over a 5 ml column of beads. Purified antibody was dialysed into PBS containing 0.02% NaN₃, quantified and stored at -80°C. Affinity purified antibodies were tested by Western Blot analysis and immunofluorescence microscopy as above. For a competition assay, 375 ng of antibody was pre-incubated with 2 µg of MBP:X-Nlp262-552 in PBS supplemented with 1% BSA and added directly to A6 cells grown on a coverslip.
2.11 Miscellaneous techniques

2.11.1 In vitro kinase assays

In vitro Nek2 phosphorylation assays were performed in a 50 μl reaction consisting of 5 μl of purified kinase for human Nek2A (purified from Sf9 insect cells; (Fry and Nigg, 1997)) or 5 μl of kinase bound to sepharose beads for *Xenopus* Nek2B (immunoprecipitated from an Sf9 insect cell lysate; (Twomey et al., 2004)), 5 μl of substrate (from a 5 mg/ml stock) and 40 μl of kinase buffer (50 mM Hepes pH 7.4, 5 mM MnCl₂, 5 mM β-glycerophosphate, 5 mM NaF, 4 μM ATP, 1 mM DTT, 10 μCi [³²P]-γ-ATP). In vitro Plxl (purified from Sf9 insect cells; (Descombes and Nigg, 1998)) phosphorylation assays were performed in a 50 μl reaction consisting of 0.5 μl of purified kinase, 5 μl of substrate (from a 5 mg/ml stock), and 44.5 μl of kinase buffer (20 mM Hepes pH 7.7, 15 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 5 mM NaF, 1 mM DTT, 100 μM ATP, 10 μCi [³²P]-γ-ATP). Reactions were performed for 30 min at 30°C for human kinases or 23°C for *Xenopus* kinases and stopped by addition of 50 μl of 3X Laemmli buffer. 20 μl of each sample was analyzed by SDS-PAGE and exposed to X-ray film. All reactions were performed in parallel using both active and inactive enzymes, with casein as a control substrate.

2.11.2 Glycerol gradient centrifugation

A 10 ml 15-50% glycerol gradient in CSF-XB (100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 50 mM sucrose, 10 mM Hepes pH 7.7, 5 mM EGTA) was poured using a gradient maker with mixing chamber into a polyethylene-tetrephlalate thin walled tube (Sorvall). One microgram of high-speed CSF egg extract was loaded onto a glycerol cushion with 200 μg of each marker protein (carbonic anhydrase (29 kDa), BSA (66 kDa), liver alcohol dehydrogenase (80 kDa), yeast alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (450 kDa)) loaded onto another. Samples were spun (36000 rpm, 24 h, 4°C in a TH-641 swing out rotor, Sorvall) and 400 μl samples collected from the top of the gradient before TCA precipitation (2.5.8) and analyzed by SDS-PAGE and Western blotting.

2.11.3 Sperm recruitment assays

1 μl *Xenopus laevis* sperm (3000/μl, prepared by S. Wattam) was incubated in 25 μl CSF-XB, *Xenopus* CSF egg extract or *Xenopus* interphase egg extract at 22°C for 30 min. These were supplemented with 1 μg/ml nocodazole to prevent microtubule aster formation.
All reactions were then diluted by addition of 500 µl ice-cold spindle buffer (10 mM PIPES pH 6.8, 0.3 M sucrose, 0.1 M NaCl, 3 mM MgCl₂), loaded onto a 25% glycerol cushion in spindle buffer and spun (3300 rpm, 20 min, 4°C in an HB4 swing out rotor, Sorvall) onto coverslips. The glycerol cushion was aspirated up to the last 1 cm above the coverslip. The interface was washed with 100 µl of 1% Triton X-100 which was then aspirated. The coverslips were lifted out of the tubes and fixed in methanol (-20°C) for immunofluorescence microscopy, as described previously.
CHAPTER 3

IDENTIFICATION OF A *XENOPUS LAEVIS* PROTEIN
WITH HOMOLOGY TO C-NAP1
3.1 Introduction

Previous work had led to the hypothesis that the centrosomal kinase Nek2 regulates centriole cohesion (Fry et al., 1998b). To identify Nek2 substrates involved in this process, a yeast two hybrid screen was performed using Nek2 as the bait. This yielded a 281 kDa protein called C-Nap1, which consists of two coiled coil domains separated by a hinge, with globular domains at both the N and C termini (Figure 1.11A). C-Nap1 is localized to the proximal ends of both centrioles during interphase but diminishes during mitosis (Fry et al., 1998a). C-Nap1 is an in vivo substrate of Nek2, and one hypothesis states that phosphorylation by Nek2 displaces C-Nap1 from centriolar ends to promote centrosome splitting (Mayor et al., 2002). However, the precise localization patterns suggest that C-Nap1 is unlikely to be the sole component of the intercentriolar linkage (Fry et al., 1998a). We therefore decided it would be informative to isolate a *Xenopus laevis* homologue of C-Nap1 that would enable us to perform functional studies in *Xenopus* egg extracts.

The *Xenopus laevis* cell free system has proven to be a very useful tool for analyzing centrosome assembly and bipolar spindle formation (Archer and Solomon, 1994). Demembranated sperm possess a pair of basal bodies that, when added to an egg extract recruit additional proteins which leads to conversion into a functional centrosome. In mitotic egg extracts, these centrosomes can then nucleate microtubule asters which can assemble bipolar spindles around the sperm chromatin. Specific proteins can be removed by immunodepletion from *Xenopus* egg extracts and recombinant proteins added back. In this way, the functional importance of these proteins can be addressed.

This chapter describes the identification of a *Xenopus laevis* homologue of the centrosomal protein rootletin. A variety of approaches were used to search for a *Xenopus laevis* homologue of the human centrosomal protein C-Nap1. Classical cDNA library screening and degenerate PCR were unsuccessful. However, by database screening an EST with high homology to C-Nap1 was identified and sequenced. Full-length sequencing revealed that the EST had highest homology to murine Rootletin, which in turn shares significant homology to C-Nap1.
3.2 Results

3.2.1 Nek2 complexes in egg cytoplasm

In human cells Nek2 has been shown to form homodimers and interact with PP1c (Fry et al., 1999; Helps et al., 2000). Furthermore, Nek2 can interact with the core centrosomal protein C-Nap1 (Fry et al., 1998a). Before searching for C-Nap1 in *Xenopus laevis*, we therefore decided to determine the size of Nek2 complexes in egg cytoplasm. To date, two splice variants of Nek2 have been identified in *Xenopus*, termed X-Nek2A (48 kDa) and X-Nek2B (44 kDa). X-Nek2A is expressed in spermatogenesis and later stages of embryogenesis, while X-Nek2B is expressed in oocytes, eggs and early development (Uto et al., 1999). X-Nek2B has been shown to be required for assembly of the first zygotic centrosome as well as for centrosome maintenance in early embryos (Fry et al., 2000b; Uto and Sagata, 2000). However, X-Nek2B lacks the binding site for PP1. To assess X-Nek2B complex size in eggs, high-speed supernatants of CSF-arrested egg extracts were spun through a 15-50% glycerol gradient with marker proteins (Figure 3.1 A). Fractions were collected from the top of the gradient, TCA precipitated and analyzed by Western blotting. The predominate complex ranges from approximately 60 kDa to 100 kDa, which correlates with an X-Nek2B dimer, smaller complexes also exist which are likely to be monomeric X-Nek2B. A larger complex of up to 200 kDa also exists, which is presumed to contain X-Nek2B and an unidentified protein(s) (Figure 3.1A&B). However, this large complex is unlikely to contain C-Nap1 as the size would be expected to be at least 360 kDa. Equivalent glycerol gradient analysis in the presence of 1M NaCl led to loss of the larger complex indicating that this interaction is salt sensitive. X-Nek2B dimers were still present consistent with previous reports that Nek2 dimerization is stable even in high salt (Fry et al., 1999). Based on this data, it was therefore not clear whether a *Xenopus* homologue of C-Nap1 was expressed in eggs or not.

3.2.2 Attempts to isolate a *Xenopus* homologue of C-Nap1

i) Low stringency cDNA library screening with human C-Nap1 probes

In a first attempt to isolate a *Xenopus laevis* cDNA with similarity to C-Nap1, four probes were constructed to various regions within the human C-Nap1 cDNA. The four probes represented the following regions corresponding to the human C-Nap1 cDNA: 1-445 (N-terminal domain), 2040-2938 (1st coiled coil), 2938-3836 (1st coiled coil) and 7213-7972 (C-terminal domain). Radiolabelled probes were produced using a random primed
Figure 3.1 Analysis of X-Nek2B complexes in *Xenopus* egg extracts

(A) 1 mg CSF extract (high speed supernatant) was spun through a 15-50% glycerol gradient. Fractions were collected from the top of the gradient, TCA precipitated and analyzed by Western blotting using anti-X-Nek2 (R81) antibodies. (B) 1 mg CSF egg extract was spun through a 15-50% glycerol gradient in the presence of 1 M NaCl. Samples were collected and analyzed as in (A). Marker proteins were separated in parallel and their relative positions in the gradients are indicated (kDa). E, 50 μg CSF egg extract loaded directly on the gels.
labelling kit and used to screen a *Xenopus laevis* early gastrula library (stage 10.5) (gift from Dr. V. Allen, Manchester, UK) and tadpole library (stage 42) (gift from Dr. P. Craig, Arizona). A low stringency (30°C) was used on the basis that the human cDNA sequences might be quite divergent to the *Xenopus* C-Napl cDNA. Previously, similar low stringency cDNA library screening had proved successful for the isolation of X-Nek2 using human Nek2 cDNA probes (Fry et al., 2000b). However, despite isolating a number of positive clones, no cDNA sequences were isolated from either library bearing significant similarity to C-Napl (data not shown).

ii) Degenerate PCR
Database searches using the human C-Napl cDNA identified ESTs with high similarity from the pig and cow genome. The overlapping sequences were used to design degenerate PCR primers that could be used to amplify DNA sequences from the *Xenopus* cDNA libraries. However, despite using decreasing annealing temperatures, again no cDNA sequences of significant similarity to C-Napl were identified (data not shown).

iii) *Xenopus* EST database screening
Advances in genome sequencing have led to new methods for identifying homologous genes in different species. Information currently available in genome databases can give a quick window to their existence. Database searches were employed routinely to screen for *Xenopus* ESTs with potential sequence similarity to human C-Napl. Two sequences were used to perform database searches. The first used the cDNA sequence of C-Napl to identify any ESTs with a similar nucleotide sequence (blastn). In the second, the protein sequence of C-Napl was used to identify any translated ESTs with a homologous protein sequence (tblastn). Using this second approach, an EST was identified with high similarity to C-Napl (Dec. 2001, Figure 3.2A). The alignment was based upon the 5' and 3' sequences of the EST alone. The EST (XL077c05) was obtained from the National Institute for Basic Biology (NIBB), Japan, and fully sequenced in both directions. The EST encodes an open reading frame of 1348 amino acids (55% the length of C-Napl), the natural AUG is not apparent but a STOP codon is present followed by a poly A signal. The highest similarity (55%) is between the first 250 amino acids of the EST and amino acids 150-400 of C-Napl, while the last 250 amino acids of the EST show a similarity of only 15% against amino acids 1050-1300 of C-Napl. The entire EST has a 23% similarity when compared to C-Napl. Based on the sequence data presented here we have identified
Figure 3.2 Analysis of *Xenopus laevis* ESTs with homology to human C-Nap1

(A) `tblastn` database search using human C-Nap1 protein sequence to search for related *Xenopus* ESTs (Feb. 2002). The highest match (pink) represents the XL077c05 EST.

(B) Predicted coiled coil regions of C-Nap1 (upper panel) and XL077 (lower panel) using the highest stringency window width of 28 in the `coils` software (Lupas 1996), and aligned based upon the `tblastn` result.
a *Xenopus* EST with significant protein sequence similarity to C-Napl that contains an open reading frame with a STOP codon, but lacks the 5' initiation codon. Coiled coil analysis was performed with the coils software (Lupas, 1996) using the highest stringency window of 28, C-Napl was also analyzed in an identical manner and aligned based upon the tblastn result (Figure 3.2B). The coiled coil prediction shows that a non-coiled region exists at the 5' and 3' ends of XL077c05, both of which are flanked by coiled regions, the remainder of XL077c05 is almost completely coiled coil. The structural similarities at the 5' and 3' ends of XL077c05 are the primary regions for alignment, which correspond to the N-terminal domain (amino acids 1-400) and potential hinge region of C-Napl.

To determine whether human C-Napl is the closest match to this EST, a database search using the full-length translated protein sequence of XL077c05 was undertaken. C-Napl was identified as a protein with high similarity, however it is not the highest matching mammalian sequence retrieved. The highest matching sequence is a mouse protein called rootletin (52% similarity over the entire EST length, published July 2002). Rootletin is a component of the ciliary rootlet originating from the basal body and is detectable between the centrioles using immunofluorescence microscopy (Yang et al., 2002). A human sequence (KIAA0445) has second highest similarity (51%) and would appear to be the human homologue of rootletin. Hence, it appears that this EST most likely represents the *Xenopus* homologue of rootletin (Figure 3.3&3.4A). C-Napl and rootletin are both structurally related proteins consisting of extensive coiled coil regions (Figure 3.4B), a common feature of many centrosome proteins (Salisbury, 2003).
Figure 3.3 Translated sequence of XL077c05
Open reading frame of the full-length XL077c05 EST. Motif scan identifies no specific protein domains (see Materials and methods).
Figure 3.4 Comparison of vertebrate rootletin and C-Nap1 proteins

(A) Representation of proteins with high homology to XL077. Alignment is based upon overall sequence identity to XL077. (B) Comparison of the incomplete X-Rootletin protein structure with homologous proteins from mouse (M) and humans (Hs). Shaded boxes represent coiled coil regions predicted by a window width of 28 using the COILS software (Lupas 1996). Numbers represent amino acids.
3.3 Discussion

Identification of a Xenopus homologue of C-Nap1 would open the door to a number of useful cell-free functional assays. The importance of C-Nap1 in centrosome assembly and spindle formation could be assayed using immunodepletion and reconstitution approaches. The expression pattern of C-Nap1 during Xenopus embryogenesis would also give important information on the mechanisms regulating centrosome cohesion in early development. However, to date a bona fide homologue of C-Nap1 in Xenopus laevis remains to be identified.

Analysis of X-Nek2B complexes in eggs revealed that Nek2 most likely exists predominately as monomers and dimers. X-Nek2B retains the leucine zipper motif shown to promote homodimerization of human Nek2A (Fry et al., 1999). A small fraction of Nek2B also exists as salt-sensitive complexes of approximately 200 kDa. If X-C-Napl is of similar structure and size to C-Napl, a complex of at least 360 kDa would be predicted but was not detected. It is possible that this complex could exist albeit at a very low abundance making detection impossible by Western blotting. It is possible also that C-Nap1 is not in a stable complex with soluble Nek2 in egg cytoplasm and that the proteins only interact when at the centrosome. A third possibility is that C-Nap1, like Nek2A, is not expressed in eggs.

For cDNA library and degenerate PCR screening two libraries were therefore tested representing early and late stage embryogenesis. However, both screening techniques failed to identify any DNA sequence that could be part of X-C-Napl. Again, C-Nap1 protein may not be expressed until adulthood or more likely, the sequences are not sufficiently similar to allow these techniques to work. The cDNA library screening used a low stringency temperature (30°C) and positive plaques were isolated (data not shown). However, these sequences turned out to show no significant similarity to human C-Nap1. A more stringent annealing temperature would be needed to eliminate this background binding. The degenerate PCR approach also provided no similar sequences at the temperatures used. We propose that if a Xenopus laevis homologue of C-Nap1 does exist the DNA sequence is sufficiently divergent and that DNA screening methods are unlikely to be successful.
The amount of sequence information from the *Xenopus laevis* genome is very limited compared to other species (just over 650,000 ESTs, Feb. 2004), restricting the data available. EST database searching, however, revealed a clone with significant similarity to C-Napl. Full-length sequencing of the EST though revealed that the most homologous protein is another centrosomal protein called rootletin. Rootletin and C-Napl both contain lengthy regions of coiled coil and localize to the centrosome (Fry et al., 1998a; Yang et al., 2002). Genome searching reveals that both humans and mice contain a homologue of C-Napl and rootletin, whereas *Drosophila* and *Xenopus* appear to only contain rootletin. C-Napl may only exist in mammals, whereas rootletin may facilitate the functions of C-Napl in non-mammalian systems. As both C-Napl and rootletin are highly coiled coil little selection pressure is applied to these sequences, therefore, C-Napl may contain a divergent sequence in non-mammalian systems or rootletin may be the bona-fide homologue. Rootletin is a 220 kDa centrosomal protein which localizes between the centrioles of the basal body and forms the structure of the ciliary rootlet (Yang et al., 2002). Rootletin may therefore also be a component of the intercentriolar linkage and contribute to centriole cohesion. Interestingly, C-Napl localizes to the proximal end of the centrosome suggesting a common domain may exist within each protein to cause this localization. It will be of particular interest to establish whether rootletin can interact with C-Napl or be phosphorylated by Nek2.
CHAPTER 4

XENOPUS LAEVIS NLP: INTERACTION WITH NEK2 AND ANTIBODY GENERATION
4.1 Introduction

A yeast two hybrid screen using the *Xenopus laevis* Polo-like kinase, Plxl, as a bait identified a *Xenopus laevis* partial cDNA fragment that was highly related to a human clone, KIAA0980 (Casenghi et al., 2003). This previously uncharacterized human clone was renamed Nlp due to its high similarity to ninein. Ninein is a previously characterized protein involved in microtubule anchorage at the mother centriole, as well as at apical sites in epithelial cells (Mogensen et al., 2000). Characterization of human Nlp revealed that it was a novel centrosomal protein that is capable of binding the γ-TuRC (Casenghi et al., 2003). Nlp is centrosome associated during interphase but disappears from the centrosome during mitosis, in a manner analogous to another Nek2 substrate C-Napl (Casenghi et al., 2003; Fry et al., 1998a; Mayor et al., 2002). Overexpression of active human Plk1 led to displacement of Nlp from the centrosome in U2OS cells (Casenghi et al., 2003). Recent evidence suggests Plk1 requires a priming kinase for the polo box domain to bind to its substrate (Cheng et al., 2003; Elia et al., 2003a; Elia et al., 2003b), therefore Nlp may be phosphorylated by another centrosomal kinase, e.g. Nek2.

We were therefore interested to test whether the centrosomal kinase Nek2 might also regulate Nlp. Using the yeast two hybrid interaction assay, we found a strong and specific interaction between X-Nek2 and the partial fragment of X-Nlp. ESTs were then isolated that encompassed the full-length *Xenopus* Nlp cDNA and a variety of antibodies were raised to distinct regions of X-Nlp. These molecular tools would provide the basis for further characterization of X-Nlp *in vitro* and *in vivo*. 
4.2 Results

4.2.1 Identification of an interaction between X-Nek2A and X-Nlp

The yeast two hybrid system is a technique that can allow protein-protein interactions to be detected. The system works by the transactivation of reporter genes in response to specific protein-protein interactions that lead to reconstitution of the two functional domains of a transcription factor. Typically two fusion proteins are generated, one fused to the Gal4 DNA binding domain (DBD) and the other to the Gal4 activation domain (AD); an interaction brings these two domains into close proximity and activates the reporter genes (Fields and Song, 1989). An *S. cerevisiae* strain called PJ69-4A has been genetically engineered to contain three different reporter genes (histidine, adenine and β-galactosidase) under the control of the Gal4 promoter (James et al., 1996). Expression of the reporter genes histidine and adenine allow growth on nutritional deficient media, while β-galactosidase expression can be measured in a quantitative colourimetric assay.

The catalytically-inactive full-length Nek2 kinase, X-Nek2A-K37R was fused to the DBD in order to test for an interaction with AD-X-Nlp. The catalytically inactive mutant was used because the wild-type enzyme was lethal upon transformation into yeast. Furthermore, the “dead” kinase may bind more tightly to its substrates due to a non-productive catalytic interaction. The X-Nlp fragment was the same construct isolated from the original screen using Plxl as the bait and was kindly provided by Prof. E. Nigg (Matinsried, Germany). X-Nek2A-K37R and X-Nlp were not able to activate the reporter genes either alone or when co-transformed with other control proteins. However, co-transformation of DBD-X-Nek2A-K37R with AD-X-Nlp resulted in yeast growing on nutritional deficient media indicating an interaction (Figure 4.1A). The strength of interaction was compared with a known positive control interaction between SNF1 and SNF4 (yeast transcriptional regulators) (Fields and Song, 1989). The growth rates between SNF1/SNF4 and X-Nek2A-K37R/X-Nlp were identical. To quantitate the interaction, the same yeast strains were analyzed using an ONPG assay, which determines the activity of β-galactosidase using ONPG as the substrate. An interaction produces ONP (yellow in colour), which can be quantified at 420 nm using a spectrophotometer. This assay indicated a 2-10 fold higher activity for X-Nek2A-K37R and X-Nlp than the negative controls and only slightly lower activity than the SNF1/SNF4 pair (Figure 4.1B). This was despite Western blotting showing that approximately 70% of the expressed DBD-X-
### Table 1: Yeast Transformants

<table>
<thead>
<tr>
<th>Gal4 DBD fusion</th>
<th>Gal4 AD fusion</th>
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</tr>
<tr>
<td>X-Nek2A-K37R</td>
<td>X-Nek2A-K37R</td>
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</table>

#### Figure 4.1 X-Nek2A can interact with X-Nlp in yeast

**A** Yeast two hybrid interaction analyses by growth on media lacking histidine and adenine. Yeast transformants are designated with the left column showing fusions to the Gal4 DNA binding domain (DBD), and right column showing fusions to the Gal4 activation domain (AD). On the right hand side of the table are photographs of the appropriate yeast colonies after 5 days growth. SNF1 and SNF4 are used as a positive control. Lamin and Mak5 (a novel, unpublished centrosomal protein, kindly provided by Dr. D. Stott, University of Warwick, UK) are negative controls. 

**B** Quantitative protein-protein interaction measurements indicating the level of expression of the lacZ reporter gene in an ONPG colorimetric assay, experiments were performed 3 times and error bars show s.d. The average result of the positive control (SNF1/SNF4) has been set as 1 with remaining data compared to this.
Nek2A-K37R protein was cleaved in vivo in yeast (data not shown). DBD-X-Nek2A-K37R alone partially activates the β-galactosidase reporter but fails to grow on nutritional deficient media highlighting the advantage of using more than one reporter gene.

4.2.2 Isolation of a full-length cDNA for X-Nlp

Using a combination of the Hs-Nlp protein sequence and the X-Nlp partial DNA sequence, Genbank non-mouse and non-human EST databases (available at http://www.ncbi.nlm.nih.gov/BLAST/) were screened in an attempt to isolate additional *Xenopus laevis* ESTs. The aim was to generate a full-length X-Nlp cDNA that would allow characterization of the protein function in *Xenopus* cells and egg extracts. Four ESTs from the *Xenopus laevis* genome were identified, ordered from NIBB and MRC Geneservice and sequenced. Sequencing of XL082 and IC 6630669 showed a perfect match to the original yeast two hybrid fragment. XL082 and IC 6630669 overlap with XL076 and are identical in sequence at this point, however IC 6630669 contains an additional open reading frame of 440 amino acids suggesting the existence of splice variants. Full-length sequencing of XL076 showed that it also contained the same sequence and natural STOP as XL088, therefore all ESTs represent different fragments of X-Nlp and combine to provide two open reading frames: one that is only 15 amino acids longer than Hs-Nlp (X-Nlp1) and one that is 455 amino acids longer than Hs-Nlp (X-Nlp2).

Comparison of human ninein with human and *Xenopus* Nlp reveals a number of conserved features (Figure 4.2). Hs-Nlp, X-Nlp and ninein each contain a predicted calcium binding EF-hand at their extreme N-termini. Downstream Hs-Nlp and X-Nlp have a second EF-hand, while ninein has a potential GTP binding site. The N-terminal half of Hs-Nlp (amino acids 1-691) shares 37% similarity with the equivalent domain in ninein, while the N-terminal half of X-Nlp1 (amino acids 1-699) shares 60% similarity with the N-terminal half of Hs-Nlp. The downstream-coiled coils do not share any obvious sequence similarity, however the extreme C-terminal (last 80 amino acids) of X-Nlp shares 86% similarity with the equivalent domain in Hs-Nlp. The major structural difference between X-Nlp and Hs-Nlp is the presence of more continuous coiled coil domain in the C-terminal half of X-Nlp. Ninein is expressed as at least two N-terminal splice variants in human cells, causing one isoform to lack the EF-hand. This may also be the case for the rat Nlp isoform which shares 45% similarity with Hs-Nlp (amino acids 1-1109 of rat-Nlp
Figure 4.2 Protein structure comparison of Human Ninein and Human, Rat and Xenopus Nlp

Hs-Ninein (human), Hs-Nlp (human), X-Nlp (Xenopus) and R-Nlp (rat) proteins are shown with respective amino acid length. EF hands (green bars) and the GTP binding site (blue box) were determined using a motif scan software (hits.isb-sib.ch/cgi-bin/PFSCAN?) and coiled coil regions (black boxes) determined with high stringency with a window width of 28 (Lupus 1996). Plk1 phosphorylation sites used for the A8 mutant (T22, S87, S88, T161, S349, S498, S670, S686) (Casenghi et al., 2003) are indicated above the Hs-Nlp protein (asterisks) while those conserved at identical positions +/- 1 residue are indicated above the X-Nlp protein (asterisks). The fragment isolated from the Plk1 yeast two hybrid screen is shown (Y2H), along with ESTs obtained from the National Institute of Basic Biology, Okazaki, Japan (XL082, XL088 and XL076) and the Medical Research Council (MRC) Geneservice, Cambridge, UK (I.C. 6630669). Note that I.C. 6630669 has additional sequence not present in XL082 that most likely represents an alternative spliced product. Dotted red lines represent the insertion present in X-Nlp2.
amino acids 273-1382 of Hs-Nlp). The predominant ninein splice variant according to Hong et al. (2000) is shown (Figure 4.2).

4.2.3 Expression of X-Nlp fragments in bacteria
To generate antibodies against X-Nlp, three different fragments of X-Nlp1 representing amino acid 174-282, 262-552 and 800-1397, were expressed in *E. coli*. Due to low levels of expression in standard BL21 *E. coli* (data not shown), the constructs were transformed into Rosetta DE3 *E. coli* which encodes rare usage codons. X-Nlp cDNA fragments were fused to the pET32 tag, which contains a thioredoxin, S and Histidine N-terminal tag (Figure 4.3A&B). PET32:X-Nlp174-282 was expressed and purified under native conditions using the His-tag (Figure 4.4A), while PET32:X-Nlp262-552 and PET32:X-Nlp800-1397 were expressed and purified under denaturing conditions using the His-tag (Figure 4.4B&C). Sufficient quantities (~10 mg) of each fusion protein was produced for the immunization programme (data not shown).

4.2.4 Characterization of antibodies raised to X-Nlp
Many rabbits have sera that cross-react with centrosomes prior to immunization. Before selecting rabbits, sera from ten animals were therefore pre-screened by Western Blotting and immunofluorescence microscopy at a dilution of 1/250. Based on the results of these analyses (data not shown), two rabbits were selected for each antigen that did not recognize centrosomes. Following immunization, all rabbits had four cycles of immunization and bleeding, except for rabbits 1679 and 1681 which had two further boosts of PET32:X-Nlp262-552. Rabbit bleeds were assessed for reactivity by Western blotting using asynchronous *Xenopus* A6 kidney cell lysates (Figure 4.5) and immunofluorescence microscopy of methanol fixed A6 cells (Figure 4.6). On Western blots, immune sera from all rabbits recognized two discrete protein bands of identical size, one approximately 150 kDa and the other approximately 200 kDa (as indicated by arrows in Figure 4.5) that were not detected by pre-immune sera. The predicted molecular weight for X-Nlp1 is 162 kDa while X-Nlp2 is 213 kDa. Rabbits 1679 and 1681 (raised to PET32:X-Nlp262-552) were also capable of recognizing an MBP:X-Nlp262-552 protein by Western blotting, confirming that antibody generation to the antigen was successful (data not shown).

For immunofluorescence microscopy, centrosomes were identified in A6 cells using monoclonal antibodies against γ-tubulin. Co-staining with the rabbit antisera revealed that
Figure 4.3 X-Nlp protein fusion constructs used for antibody production

(A) Protein domains of the fusion tag in the pET32 vector. Fusion proteins can be purified via thioredoxin, His or S tag. Numbers denote amino acid length. (B) Fusion constructs of X-Nlp fragments with the PET32 tag. (C) Fusion construct of an X-Nlp fragment fused to maltose binding protein (MBP). Numbers in B and C denote the amino acid fragments of X-Nlp that were used based on the full-length sequence shown in Figure 4.2 with construct titles written below.
Figure 4.4 Expression and elution profiles for the three PET32-X-Nlp constructs
Induction in BL21 E. Coli (Rosetta) was optimised for each construct and expressed proteins analyzed by Coomassie Blue staining of SDS-polyacrylamide gels. (A) PET32:X-Nlp_{174-282}, (B) PET32:X-Nlp_{262-552} and (C) PET32:X-Nlp_{800-1397}. PET32:X-Nlp_{77-282} was purified under native conditions, while the remaining two were purified under denaturing conditions. -, uninduced bacteria, +, induced bacteria, Lys, lysate after sonication, FT, flow-through from nickel column, W, washes and E, elutions required. M.wt markers (kDa) are indicated on the left of each panel.
Figure 4.5 Western blot analysis of A6 cell lysates using anti-X-Nlp antisera

Rabbit bleeds were taken according to the immunization programme and analyzed by Western blotting at 1/250 dilution against A6 cell lysates. (A) Rabbits 1679 and 1681 immunized with PET32:X-Nlp\textsubscript{262-552}. (B) Rabbits 1683 and 1685 immunized with PET32:X-Nlp\textsubscript{174-282}. (C) Rabbits 685 and 688 immunized with PET32:X-Nlp\textsubscript{1397-2000}. PI, pre-immune sera, and rabbit bleeds are numbered. Arrows identify common bands produced by antisera. M.wt markers (kDa) are indicated on the left of each panel.
each could detect centrosomes after immunization (Figure 4.6). All rabbits showed a progressive increase in specificity for the centrosome with subsequent bleeds. Apart from recognizing centrosomes, all antisera also gave weak staining of the cytoplasm (Figure 4.6).

4.2.5 Affinity purification of antibodies raised against PET32:X-Nlp262-552

To generate a fusion protein for antibody purification, the X-Nlp262-552 fragment was subcloned into the pMALc2G bacterial expression vector, which contains an N-terminal maltose binding protein (MBP) tag (Figure 4.3C). MBP:X-Nlp262-552 was then expressed in Rosetta DE3 *E. coli* and purified using amylose resin (Figure 4.7A&B). Purified MBP:X-Nlp262-552 was covalently bound to affigel beads for use as a scaffold for the affinity purification (Figure 4.7C). 5 ml of both bleed 5 and bleed 6 from rabbit 1679 immunized with PET32:X-Nlp262-552 was passed over the affinity column. After the removal of nonspecific antibodies using wash buffers supplemented with 500 mM NaCl, anti-X-Nlp antibodies were eluted under acidic conditions and detected by the presence of the IgG heavy chain using Coomassie Blue staining (Figure 4.7D). Purified antibodies from elutions G1-G5 were concentrated using a centrifugal membrane resulting in 5 ml anti-X-Nlp antibodies at a concentration of 250 µg/ml.

4.2.6 Characterization of affinity-purified anti-X-Nlp antibodies

To test the specificity of the affinity-purified antibodies, Western blotting and immunofluorescence microscopy were performed. By Western blotting, the purified antibody detected a single band in A6 cell lysates (Figure 4.8A), which corresponded to the dominant band (approximately 200 kDa) detected by all rabbit sera after immunization (Figure 4.5). Overexposing the affinity purified antibody failed to detect the lower molecular weight protein (~150 kDa), while pre-incubating the final bleed sera with a competing antigen removed detection of the upper band (~200 kDa) but not the lower band. An IVT of X-Nlp1 is also a different size to the lower species suggesting that this protein is an unrelated protein recognized by antibodies raised to the PET32 tag (Figure 4.8B). Immunofluorescence microscopy in methanol fixed A6 cells using antibodies against γ-tubulin and the affinity-purified antibody revealed highly specific centrosomal staining (Figure 4.8C). Pre-incubating 375 ng of affinity-purified antibody with 2 µg MBP-X-Nlp262-552 prior to A6 cell staining, led to complete abolition of centrosome detection (Figure 4.8C). To determine whether the affinity-purified antibodies could also
detect X-Nlp at the centrosome under aldehyde based fixation protocols, A6 cells were fixed using paraformaldehyde and stained as before. X-Nlp was still detected at the centrosome under these conditions (Figure 4.8D). Serial dilutions of the affinity-purified antibody showed that a working concentration of 2.5 μg/ml allowed detection of a single band by Western blotting and specific centrosome staining in A6 cells with minimal background (data not shown). The data presented here and in chapter 5 provides strong evidence that we have raised and purified an antibody that is specific to X-Nlp.
Figure 4.6 A&B characterization of X-Nlp antibodies by IF microscopy

Rabbit sera raised against PET32:X-Nlp\textsubscript{262-552} were tested in A6 cells to determine centrosome recognition. Centrosomes were stained with antibodies against \( \gamma \)-tubulin (red) and rabbit sera at a dilution of 1/250 (green); DNA was stained with Hoechst 33258 (blue). Scale bar, 10 \( \mu \text{m} \).
Figure 4.6 C&D characterization of X-Nlp antibodies by IF microscopy
Rabbit sera raised against PET32:X-Nlp\textsubscript{174-282} were tested in A6 cells to determine centrosome recognition. Centrosomes were stained with antibodies against $\gamma$-tubulin (red) and rabbit sera at a dilution of 1/250 (green); DNA was stained with Hoechst 33258 (blue). Scale bar, 10 $\mu$m.
Rabbit sera raised against PET32:X-Nlp<sub>800-1397</sub> were tested in A6 cells to determine centrosome recognition. Centrosomes were stained with antibodies against γ-tubulin (red) and rabbit sera at a dilution of 1/250 (green); DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm.
Figure 4.7 Affinity purification of anti-X-Nlp antibodies from rabbit 1679

(A) Solubility test for MBP:X-Nlp_{262-552} by Coomassie Blue staining. -, uninduced bacteria, +, induced bacteria, S, soluble protein from the cell lysate and I, insoluble protein from the pellet. (B) Expression and elution profile of soluble MBP:X-Nlp_{262-552} by Coomassie Blue staining of SDS polyacrylamide gels. -, uninduced bacteria, +, induced bacteria, Lys, lysate, W, wash and E, elutions. (C) Coomassie Blue staining showing depletion of MBP:X-Nlp_{262-552} after covalent binding to agarose beads. Pre, 10 µl supernatant prior to bead addition, Post, 10 µl supernatant after O/N incubation with agarose beads. (D) Affinity purification profile of anti-X-Nlp antibodies analyzed by Coomassie Blue staining. S, rabbit sera, W, washes, G, glycine pH 2.5 antibody elutions. M.wt markers (kDa) are indicated on the left of each panel. RSA, rabbit serum albumin.
Figure 4.8 Affinity purified antibodies detect X-Nlp by Western blotting and IF microscopy

(A) Western blot analysis of A6 cell lysate using pre-immune serum (PI) at a dilution of 1/250, final bleed serum (FB) at a dilution of 1/250 and affinity purified antibody (A) at a working dilution of 2.5 µg/ml. M.wt markers (kDa) are indicated on the left. (B) Western blot analysis of A6 cell lysate using the affinity purified antibody at a concentration of 10 µg/ml (A), and the final bleed serum pre-incubated with competing antigen at a dilution of 1/250 (C). The right hand lane shows a Western blot of X-Nlp1 generated by IVT with the affinity purified antibody (IVT). (C) IF microscopy of A6 cells fixed in cold methanol. Centrosomes are stained with antibodies against γ-tubulin (red) and X-Nlp antisera (green); DNA is stained with Hoechst 33258 (blue). Sera was either pre-immune (top), affinity purified (middle) or affinity purified pre-incubated with the competing antigen (bottom). (D) Immunofluorescence microscopy of A6 cells fixed with 3% paraformaldehyde. Centrosomes are co-stained with γ-tubulin (red) and X-Nlp (green); DNA is stained with Hoechst 33258 (blue). Scale bar, 10 µm.
4.3 Discussion

Plk1 is a protein kinase involved in multiple aspects of mitosis including centrosome maturation and bipolar spindle formation (Dai et al., 2002; Glover et al., 1998; Nigg, 1998). Substrates of Plk1 involved in these processes are likely to include the microtubule depolymerizing protein stathmin/Opl8 (Budde et al., 2001), the microtubule binding protein Asp (Avides et al., 2001) and the γ-TuRC binding protein Nlp (Casenghi et al., 2003). Nlp is not involved in generation of the mitotic spindle but its displacement from the mitotic centrosome by Plk1 mediated phosphorylation may be a requirement to enable spindle formation (Casenghi et al., 2003). Plk1 mediated phosphorylation of substrates is stimulated by interaction of Plk1 via the polo box domain (PBD) with a previously phosphorylated site (Cheng et al., 2003; Elia et al., 2003a; Elia et al., 2003b). This model therefore proposes that a priming phosphorylation event is required for recognition of substrates by Plk1. Nek2A displays optimal activity at the G2/M transition concurrent with Plk1 and is also localized to the centrosome. It therefore makes an ideal candidate as a priming kinase for Plk1 directed activity.

Fission yeast Plo1 is cytoplasmic during interphase but becomes recruited to the SPB during mitosis (Mulvihill et al., 1999). However, overexpression of the NIMA homologue Fin1 during interphase can initiate premature recruitment of Plo1 to the SPB (Grallert and Hagan, 2002). A direct interaction between Plo1 and Fin1 has not been observed and overexpression of Fin1 has no effect upon Plo1 activity suggesting that Fin1 does not phosphorylate and activate Plo1 directly (Grallert and Hagan, 2002). Therefore, the kinase activity of Fin1 may phosphorylate a site on an SPB component that is required for recognition by the Plo1 PBD. Nlp can be phosphorylated and displaced from the centrosome by the kinase activity of Plk1, while overexpression of a mutant Nlp lacking 8 Plk1 phosphorylation sites (NlpΔ8) causes aberrant mitotic spindles to form (Casenghi et al., 2003). Overexpression of kinase dead Nek2A also causes abnormal mitotic spindles to form (Faragher and Fry, 2003). There is thus a strong similarity between the phenotypes caused by manipulation of Nlp, Plk1 and Nek2A. Here, we show that an interaction can occur between X-Nek2 and X-Nlp. This led us to generate a variety of antibodies to X-Nlp that would enable us to characterize this relationship further.
The initial identification of X-Nlp was from a yeast two hybrid screen using Plxl as the bait. Using the same assay, we show here that X-Nlp can also interact with X-Nek2A. The DNA sequence of the X-Nlp fragment and protein sequence of Hs-Nlp allowed us to isolate overlapping clones that encode a full-length X-Nlp by EST database screening. The additional open reading frame in IC 6630669 also suggests that X-Nlp exists as two splice variants. Sequence comparison between X-Nlp, Hs-Nlp and ninein revealed similarities within the non-coiled coil N-terminal regions, but little conservation in the C-terminal coiled coil domains. X-Nlp, Hs-Nlp and ninein all contain a predicted EF-hand at their extreme N-termini, X-Nlp and Hs-Nlp contain a second predicted EF-hand while ninein has a potential GTP binding site. The EF-hands are likely to be a feature of the structural integrity of all three proteins, whereas the significance of ninein GTP has not been addressed. Interestingly, despite the similarity within the N-terminal domain, ninein is not a substrate of Plkl (Casenghi et al., 2003). Although limited, current evidence suggests ninein is involved in microtubule anchorage, whereas Nlp appears to regulate nucleation (Casenghi et al., 2003; Dammermann and Merdes, 2002; Mogensen et al., 2000).

While screening for X-Nlp ESTs, other homologous sequences were identified, but the sequences were not identical to X-Nlp. Further analysis showed that these ESTs were more identical to ninein than Nlp, and presumably are ESTs representing fragments of X-ninein. The highest similarity between the human and Xenopus Nlp homologues is located within the N-terminal domain. This domain is required for the binding of Nlp to the γ-TuRC, as well as localizing Nlp to the centrosome (Casenghi et al., 2003), although fine mapping of the exact binding region(s) has not been done. Five of the eight putative Plkl phosphorylation sites (used in the Δ8 mutant) are conserved in X-Nlp, although other potential Plxl phosphorylation sites do exist within the N-terminal domain. The C-terminal domains exhibit much greater diversity, with the exception of the last 80 amino acids. The Hs-Nlp C-terminal domain contains a long non-coiled coil region followed by a coiled coil domain, while the entire X-Nlp C-terminal domain is predicted to form a coiled coil, the significance of this unknown. The Hs-Nlp C-terminal domain has no role in the interaction between Nlp and the γ-TuRC, but appears to be required for Nlp aggregation (Casenghi et al., 2003). The large coiled coil may well perform a structural role and/or associate with other centrosomal components.
With the full sequence of X-Nlp available, three different protein fragments were selected for antibody production. All six rabbits immunized yielded sera that recognized identical proteins by Western blotting and centrosomes by immunofluorescence microscopy. After affinity purification, one of the antibodies detected a single protein band of approximately 200 kDa. The predicted molecular weight of X-Nlp1 is 162 kDa while X-Nlp2 is 213 kDa, suggesting that the antibody is detecting only X-Nlp2. A lower molecular weight product of approximately 150 kDa was no longer detectable after purification, even upon overexposure with the affinity-purified antibody. For each immunogen, the protein fragment was fused to the PET32 tag; hence the lower molecular weight band may well represent a protein recognized by antibodies raised to the PET32 tag. Affinity purification was carried out with an MBP tagged protein explaining why these antibodies were lost upon purification. As only X-Nlp2 is detected after affinity purification, this suggests that this isoform is much more abundant in A6 cells than X-Nlp1. However, it cannot be ruled out that other splice variants of X-Nlp or other closely related isoforms of X-Nlp exist in *Xenopus*. Hs-ninein exists as two splice variants called ninein and ninein-Lm, which show differential tissue expression patterns (Hong et al., 2000). Purified X-Nlp antibodies detect the centrosome with minimal cytoplasmic background in A6 cells and the competition assay confirmed the specificity of the antibody. To perform high-resolution immuno-EM we also tried a formaldehyde-based fixative. Under these conditions, X-Nlp could still be detected at centrosomes in A6 cells; therefore immuno-EM with this antibody may be a viable possibility for determining the precise sub-centrosomal location of X-Nlp.

The data presented in this chapter provides evidence of an interaction between the Nek2 kinase and the γ-TuRC binding protein Nlp. A full-length sequence for X-Nlp has been determined and antibodies have been raised to different regions of X-Nlp and purified. These recognize a single band on Western blots and centrosomes by immunofluorescence microscopy. We have thus achieved a primary goal in producing reagents specific to X-Nlp that will now enable us to characterize the properties and regulation of this protein in *Xenopus* cells.
CHAPTER 5

EXPRESSION AND LOCALIZATION ANALYSIS OF X-NLP
5.1 Introduction

Previous work on human Nlp had demonstrated that Nlp is a novel centrosomal protein capable of recruiting the γ-TuRC to the centrosome (Casenghi et al., 2003). Nlp was detected only at the centrosome during interphase and was absent during mitosis. However, it was not clear whether this was a result of epitope masking, protein degradation or displacement of Nlp from the centrosome. Microinjection of antibodies against human Nlp caused a reduction in interphasic microtubule polymerization whereas overexpression had the opposite effect. Microinjection of antibodies to Nlp had no effect on bipolar spindle formation in line with the observation that Nlp is not detected on spindle poles. In contrast overexpression of Nlp led to abnormal bipolar spindle formation (Casenghi et al., 2003). The observation described in the previous chapter that X-Nlp could interact with X-Nek2A raised the possibility that Nlp might be subjected to regulation by two centrosome kinases, Plk1 and Nek2. X-Nek2B is recruited to the first zygotic centrosome in a kinase independent manner while immunodepletion of X-Nek2B from Xenopus egg extracts causes a delay in the recruitment of γ-tubulin and aster assembly (Fry et al., 2000b; Twomey et al., 2004). Microinjection of antibodies against X-Nek2B into Xenopus oocytes causes a defective bipolar spindle to form and leads to abortive cleavage (Uto and Sagata, 2000). Therefore, we wished to examine whether X-Nlp is required for zygotic centrosome assembly or MTOC function.

This chapter describes work that analyses the properties of X-Nlp both in vitro and in vivo, using the previously characterized X-Nlp antibody. Experiments have been performed in adult Xenopus cultured cells, in extracts prepared from Xenopus eggs and Xenopus tissues. In particular, the antibody was used to address the cell cycle dependent expression and localization of Nlp in eggs, sperm, and adult cells.
5.2 Results

5.2.1 Expression analysis of X-Nlp in adult cell lines and egg extracts

X-Nlp (X-Nlp2, which will be referred to as X-Nlp from this point) has been shown to be present in A6 adult kidney cells by Western blotting and immunofluorescence microscopy. For comparison, Western blotting was performed on another transformed Xenopus cell line called Xenopus tissue carcass (XTC) derived from epidermal tissue (Smith and Tata, 1991). In parallel, Western blotting was also performed on low-speed cytoplasmic extracts prepared from CSF-arrested (metaphase II of meiosis) eggs and eggs triggered to enter interphase by calcium addition. Samples were equalized for protein content by BCA quantification and Coomassie Blue staining of SDS polyacrylamide gels. X-Nlp was detected equally in both A6 and XTC cell lines, but was absent in both types of egg extract (Figure 5.1A&B). Based on this data, X-Nlp protein is either not expressed or expressed at very low levels in eggs. X-Nlp1 was undetectable in all samples tested (data not shown).

It was possible that X-Nlp contributed to the zygotic centrosome but is paternally-derived with the sperm basal body as has been shown for centrin (Stearns and Kirschner, 1994). To investigate this possibility, demembranated Xenopus sperm were incubated in buffer alone, spun onto coverslips and analyzed by immunofluorescence microscopy. However, X-Nlp was undetectable at the basal body (Figure 5.2A-upper panel). Although it had not been possible to detect X-Nlp in egg extract, it was possible that X-Nlp might be recruited from the egg extract to the assembling zygotic centrosome whereupon it would become sufficiently concentrated to detect. Sperm were therefore incubated in either mitotic arrested egg extract or an interphase arrested egg extract. However, X-Nlp again proved to be undetectable on sperm basal bodies by immunofluorescence microscopy indicating that significant amounts of X-Nlp are not recruited to the zygotic centrosome (Figure 5.2A). As a positive, control X-Nek2B was shown to be recruited to the zygotic centrosome from mitotic egg extract as previously reported (Fry et al., 2000b; Twomey et al., 2004) (Figure 5.2B).

Analysis of X-Nlp in sperm and eggs thus provided no evidence that the protein is expressed during early development in Xenopus. As X-Nlp is present in adult transformed cells it was possible that X-Nlp only becomes actively transcribed/translated at some point during late embryogenesis. Xenopus embryos have been categorised into developmental
Figure 5.1 X-Nlp is absent in *Xenopus* egg extracts, but present in A6 and XTC cell lysates

(A) Western blot analysis of interphase (IE) and mitotic (ME) egg extract together with A6 and XTC cell lysates using purified X-Nlp (1679) antibodies. **(B)** Coomassie Blue stained gel of samples shown in (A) serving as a loading control. M.wt markers (kDa) are indicated on the left of each panel.
Figure 5.2 X-Nlp is not detected on sperm basal bodies before or after incubation in egg extracts

(A) *Xenopus* demembranated sperm were incubated in either CSF-XB buffer or *Xenopus* egg extracts as indicated before being spun onto coverslips. Immunofluorescence microscopy shows basal bodies stained with antibodies against α-tubulin (red) and X-Nlp (green); DNA is stained with Hoechst 33258 (blue). (B) Sperm were processed as in (A) and then stained with antibodies against X-Nek2B (green). Arrowheads denote basal bodies. Scale bar, 10 μm.
stages from fertilization to adulthood based upon major embryonic alterations in cellular
distribution. The Nieuwkoop and Faber scale (available at http://www.xenbase.org)
represents *Xenopus* development using a numerical reference, where fertilization is stage 1
and a fully developed tadpole stage 50. To determine whether X-Nlp became detectable
at the protein level at any point during embryogenesis, protein extracts were made from
embryos at different stages (gift from Prof. H. Woodland, Warwick, UK) and analyzed by
Western blotting. However, the procedure was inefficient at yielding sufficient quantities
of protein and X-Nlp was still not detected at any stage (data not shown).

To determine whether expression of X-Nlp in the adult toad is ubiquitous, major organs
were dissected before protein extracts were prepared and analyzed by Western blotting
(gift from Prof. H. Woodland, Warwick, UK). Protein content was equalized by the BCA
assay and confirmed by Coomassie Blue staining of an SDS-PAGE gel (Figure 5.3A). α-
tubulin staining was used to confirm protein loading, although this revealed variation
possibly caused by tissue specific α-tubulin expression (Figure 5.3B, upper panel). Using
purified antibodies, X-Nlp was only detected in skin, a rapidly dividing tissue, however the
abundance was significantly lower than in the two transformed cell lines (Figure 5.3B,
lower panel). Based on these results, X-Nlp appears to be in highest abundance in actively
dividing cells and is perhaps elevated in expression upon cell transformation. X-Nlp1 was
undetectable in all samples tested (data not shown).

### 5.2.2 Cell cycle localization of X-Nlp protein

In human U2OS cells Nlp is present on the interphase centrosome in a much higher
abundance than the mitotic centrosome (Casenghi et al., 2003). To determine if this
behavior was conserved in *Xenopus* cells, centrosomes in A6 cells were co-stained with
antibodies against γ-tubulin and X-Nlp; DNA staining was used to identify the stage of
mitotic progression (Figure 5.4). X-Nlp showed maximal abundance at the interphase
centrosome and was significantly reduced in abundance at spindle poles from prophase
through until anaphase. Upon anaphase exit, Nlp abundance began to increase at the
centrosome with this trend continuing into interphase (Figure 5.4). This observation shows
that the cell cycle-dependant centrosomal association of Nlp is well conserved between
humans and *Xenopus*, with loss of Nlp at the onset of mitosis, and reaccumulation upon
mitotic exit.
Figure 5.3 X-Nlp protein is only detected in actively dividing cells
(A) Coomassie Blue stained protein gel of extracts prepared from A6 and XTC cultured cells and various *Xenopus laevis* tissues. M.wt markers (kDa) are indicated on the left.
(B) Protein extracts were analyzed by Western blotting with antibodies against α-tubulin (upper panel) and X-Nlp (lower panel).
Figure 5.4 X-Nlp abundance at the centrosome is reduced during mitosis
Immunofluorescence microscopy was performed on methanol fixed A6 cells. Interphase cells and cells at different stages of mitosis were imaged using non-saturating exposure conditions. Centrosomes were co-stained with antibodies against γ-tubulin (red) and X-Nlp (green); DNA was stained with Hoechst 33258 (blue). Arrowheads denote centrosomes. Scale bar, 10 μm.
For a quantitative estimation of the change in abundance of X-Nlp at the centrosome, images of Nlp-stained centrosomes were captured under identical non-saturating imaging conditions. With the abundance of X-Nlp at interphase centrosomes being equalized to 100%, the amount of X-Nlp present at the centrosome in prophase declined to about 70%, and by anaphase to about 2%. Progression from anaphase to cytokinesis was accompanied by a slow increase from 2% to around 30% (Figure 5.5A). A direct comparison between an adjacent interphase and mitotic cell taken at an identical non-saturating exposure shows the extreme difference in X-Nlp abundance at the centrosome (Figure 5.5B). Similar patterns of cell cycle dependant association of X-Nlp with the centrosome were also obtained with antibodies raised to PET32:X-Nlp800-1397 (Figure 5.5C). Therefore the change in association of X-Nlp with the centrosome is highly unlikely to be a result of epitope masking. Identical results were also obtained in XTC cells (Figure 5.6). Data in this section provides strong evidence that X-Nlp localization at the centrosome is tightly regulated at the onset and exit from mitosis and that this is conserved between humans and *Xenopus*.

### 5.2.3 Cell cycle expression of X-Nlp protein

The abundance of X-Nlp, like human Nlp, at the centrosome decreases as cells enter mitosis and increases upon mitotic exit. However, it was not clear whether this was a result of protein displacement or degradation. To assess the total abundance of X-Nlp through the cell cycle, A6 cells were synchronized as described in the Materials and Methods (Figure 5.7A). Flow cytometric analysis was used to determine A6 cell DNA content to establish the proportion of cells in each phase of the cell cycle (Figure 5.7B). Quantitative cell counts of the flow cytometric data are shown in Figure 5.7C.

To biochemically confirm the degree of synchronization, cell lysates were analyzed by Western blotting for specific cell cycle markers (Figure 5.8). Cyclin B2 and aurora A/Eg2 are previously characterized proteins which increase in abundance from S phase through to mitosis, where they targeted for degradation by the APC/C at the onset of anaphase (Arlot-Bonnemains et al., 2001; Castro et al., 2002; Clute and Pines, 1999). Both proteins show the expected pattern of expression in the synchronized samples whereas α-tubulin, serving as a loading control, showed no abundance change during the cell cycle (Figure 5.8). Importantly, Western blotting with antibodies against X-Nlp showed that the total cellular abundance is unchanged through the cell cycle. However, a slight smear was apparent.
Figure 5.5 Quantitative analysis of X-Nlp abundance at the centrosome

(A) Quantitative analysis of X-Nlp abundance at the centrosome was performed by imaging cells at non-saturating exposure conditions and quantifying the mean pixel intensity in a 1 μm² box around the centrosome. Background values were removed and averages shown for 20 cells in each stage of the cell cycle, values for mitotic cells represent a sum of the abundance at the two spindle poles. Error bars show s.d. (B) Immunofluorescence microscopy of A6 cells showing a direct comparison between interphase and mitosis. Upper image is stained with antibodies against X-Nlp (green) and lower image (merge) stained with antibodies against γ-tubulin (red) and X-Nlp (green); DNA is stained with Hoechst 33258 (blue). (C) Immunofluorescence microscopy of A6 cells, stained with antibodies against γ-tubulin (red) and X-Nlp (either rabbit 685 or 688, green); DNA is stained with Hoechst 33258 (blue). Arrowheads denote centrosomes. Scale bar, 10 μm.
Figure 5.6 X-Nlp abundance at the centrosome is also reduced in mitotic XTC cells

Immunofluorescence microscopy was performed on methanol fixed XTC cells. Interphase cells and cells at different stages of mitosis were imaged at non-saturating exposure conditions. Centrosomes were stained with antibodies against γ-tubulin (red) and X-Nlp (green); DNA was stained with Hoechst 33258 (blue). Arrowheads denote centrosomes. Scale bar, 10 μm.
above the protein in mitotic extracts suggestive of phosphorylation (Figure 5.8). These results strongly suggest that Nlp is not degraded in a co-ordinated manner during the cell cycle, and that its disappearance from spindle poles is a result of protein displacement. X-Nlp1 was undetectable in all stages of the cell cycle (data not shown).

5.2.4 Specific localization of X-Nlp to the mother centriole

Many centrosomal proteins display a discrete sub-centrosomal localization that can be detected using either immunofluorescence microscopy or electron microscopy. Electron microscopy has proved an extremely useful technique for analyzing centrosome structure and depicting exactly where a protein resides. C-Nap1 was shown to localize to the proximal ends of both centrioles (Fry et al., 1998a) and ninein to the appendages (Mogensen et al., 2000) using immuno-EM. Some proteins may appear to be centrosomal but are not part of the core structure itself, instead they are localized within the vicinity of the centrosome as a result of microtubule association, e.g. Plk3 (Wang et al., 2002). To determine whether X-Nlp is a core centrosomal protein, A6 cells were stained for X-Nlp following depolymerization of the microtubule network. A6 cells were incubated at 4°C in media supplemented with 6 μg/ml nocodazole for 30 minutes before staining for microtubules with α-tubulin antibodies and X-Nlp. Despite complete depolymerization of microtubules, X-Nlp remained tightly centrosome associated (Figure 5.9, middle panel). Stabilizing microtubules by the addition of taxol to the media also had no observable effect upon X-Nlp distribution (Figure 5.9, lower panel). These observations suggest that X-Nlp is an integral component of the centrosome and that its association with the centrosome is not dependant on microtubules or microtubule dynamics. This result does not rule out the possibility though that X-Nlp is recruited to the centrosome via the microtubules; FRAP (fluorescent recovery after photobleaching) experiments would be required to determine the rate at which GFP-X-Nlp is recruited to the centrosome in the presence or absence of microtubules after photobleaching.

Centrosomes in *Xenopus* A6 cells appear to be more compact than human centrosomes when visualized by immunofluorescence microscopy and visualising two discrete centrioles is rare. However, careful analysis of centrosomes in A6 cells stained with antibodies against γ-tubulin and X-Nlp revealed that X-Nlp had a discrete sub-centrosomal localization with respect to localization of γ-tubulin (Figure 5.10A, a&b). Strikingly, when two discrete centrosomes were visible with γ-tubulin antibodies, X-Nlp was clearly
Figure 5.7 Cell cycle synchronisation of A6 cells

(A) Schematic diagram of the cell cycle, indicating treatments that can be used for synchronisation. (B) Flow cytometric analysis of A6 cells synchronised at different points of the cell cycle as described in the Materials and Methods. 1-3 represent the gated area for cells counted as G1, S or G2/M respectively, n and 2n show the relative DNA content per cell as a function of cell number. (C) Quantitative values representing the percentage of cells in each stage of the cell cycle as indicated by the flow cytometric graphs in (B). Graphs are based upon at least 4000 cells. A, asynchronous culture.
Figure 5.8 Total X-Nlp abundance is constant through the cell cycle
Western blot of synchronised A6 cell lysates using antibodies against X-Nlp, X-cyclin B2, Aurora A/Eg2 and α-tubulin. Lysates were made from identical populations to those used for flow cytometry shown in Figure 5.7.
associated with only one (Figure 5.10A, c-f). It was therefore important to determine whether Nlp was associated with the mother or daughter centriole. Electron microscopy can be used to distinguish the two centrioles due to the presence of appendages on the mother centriole. However, this approach is technically demanding and is a time consuming procedure. A simpler method to identify a maternal centriole is to co-stain with an antibody to a known maternal associated protein, e.g. ninein. However, unfortunately no such antibodies are available for *Xenopus* centrioles. Instead, we decided to look for growth of a primary cilium as the microtubules of the primary cilium are subtended by the mother centriole. Hence, the mother centriole is situated next to the primary cilium with the daughter centriole further out. Cell lines that require external growth signals to proliferate can be arrested in G0 by transferring them to serum free media. When cells enter G0, the centrioles move towards the cell surface and a primary cilium is generated from the mother centriole. The microtubules of the primary cilium contain polyglutamylated tubulin, which is also found within the core centrioles (Fouquet et al., 1994; Vorobjev and Chentsov, 1982). By arresting A6 cells in G0, the mother centriole can thus be detected by staining for the protruding cilium, with antibodies against polyglutamylated tubulin. GT335 is a monoclonal antibody that reacts with polyglutamylated tubulin; this was kindly provided by Dr. B. Edde (Montpellier, France) (Audebert et al., 1993). G0 arrested A6 cells were co-stained with both GT335 and \( \gamma \)-tubulin monoclonal antibodies, which were detected with the same secondary antibody. This reveals the two centrioles and the microtubule of the cilium (Figure 5.10B). Staining with antibodies to X-Nlp demonstrated that X-Nlp is specifically associated with the mother centriole only as it is exclusively detected on the centriole adjacent to the primary cilium (Figure 5.10B). This result puts Nlp in the same localization as ninein (Mogensen et al., 2000; Ou et al., 2002; Ou and Rattner, 2000). To dissect exactly where on the mother centriole X-Nlp localizes, immuno-EM will be necessary.
Figure 5.9 X-Nlp is a component of the core centrosome

Immunofluorescence microscopy of A6 cells that were either untreated, or incubated for 30 min with 6 μg/ml nocodazole at 4°C, or supplemented with 1 mM taxol as indicated. Microtubules were stained with antibodies against α-tubulin (red), while centrosomes were stained with antibodies against X-Nlp (green); DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm.
Figure 5.10 X-Nlp is specifically associated with the mother centriole
(A) Immunofluorescence microscopy of A6 cells stained with antibodies against γ-tubulin (red) and X-Nlp (green); DNA is stained with Hoechst 33258 (blue). b is an enlargement of the box shown in a and d-f are an enlargement of the box shown in c. Scale bars, 10 μm (a&c) and 1 μm (b, d, e and f). (B) Immunofluorescence microscopy of G0 arrested A6 cells; centrosomes are stained with antibodies against γ-tubulin (red), polyglutamylated tubulin (GT335, red), and X-Nlp (green); DNA is stained with Hoechst 33258 (blue). Arrowhead denotes mother centriole. Scale bar, 10 μm.
5.3 Discussion

The centrosome is the major site of microtubule nucleation within an animal cell, due to the high concentration of a protein complex called the γ-TuRC in the PCM (Khodjakov and Rieder, 1999; Moritz et al., 1995; Zheng et al., 1995). In yeast, the γ-TuRC is anchored to the SPB by Spc110p and Spc72p on the nuclear and cytoplasmic sides of the nuclear envelope, respectively (Knop and Schiebel, 1997; Knop and Schiebel, 1998; Nguyen et al., 1998). However, identifying mammalian proteins that anchor the γ-TuRC to the centrosome has proven more difficult. Nlp may be one candidate as it can interact with the γ-TuRC (Casenghi et al., 2003). Interfering with Nlp by the microinjection of antibodies caused a reduction in microtubule nucleation at the interphase centrosome as well as a reduction of previously focused microtubules. Conversely, overexpression of Nlp causes an increase in microtubule nucleation at the interphase centrosome and a broader focal point. Overexpressing Nlp in mitotic cells leads to defects in bipolar spindle formation with cells exhibiting multipolar and monopolar spindles (Casenghi et al., 2003). Therefore, Nlp clearly represents an important centrosomal protein in terms of microtubule regulation.

Having generated a highly specific antibody against *Xenopus* Nlp, we were now in a position to analyze Nlp expression and localization through the cell cycle in a different vertebrate model system. Western blotting with specific X-Nlp antibodies revealed that, whilst X-Nlp was easily detected in transformed cell lines, cytoplasmic extracts prepared from eggs induced to be in either a mitotic or interphase state showed no detectable X-Nlp. This meant that the protein was either in very low abundance or not expressed at this time of early development. Using sperm recruitment assays, X-Nlp could neither be detected on sperm basal bodies, nor was it recruited to the first zygotic centrosome after incubating sperm in egg extracts. These observations strongly imply that X-Nlp is not involved in centrosome function in early development. Early development cell cycles contain only S and M phase for rapid DNA replication and segregation, therefore an interphase microtubule aster will be rapidly turned over, hence no X-Nlp. X-Nek2B is known to be recruited to the assembling zygotic centrosome with protein levels decreasing dramatically during the mid-neurala stage (stage 18) (Fry et al., 2000b; Uto et al., 1999; Uto and Sagata, 2000). If Nlp is only expressed late in development it is unlikely that Nlp is a major substrate of the X-Nek2B splice variant. Interestingly, the original X-Nlp fragment was
isolated from a yeast two hybrid cDNA library prepared from an unfertilized *Xenopus* oocyte library (stage 0). Therefore, the mRNA for X-Nlp must exist but would appear to be translationally repressed (de Moor and Richter, 2001). The attempt to identify when translation of the X-Nlp mRNA began was inconclusive, perhaps due to a low concentration in embryo extracts. During *Xenopus* development, transcription begins at stage 8/9 (mid-blastula transition) when cell cycles change from developmental to somatic (Davidson, 1986; Kimelman et al., 1987). On the other hand, the switching of X-Nek2 isoforms from X-Nek2B to X-Nek2A occurs at the gastrula-neurala transition later in development. It is possible that X-Nlp becomes actively expressed at either of these points, however this was not detected in our experiment due to its low abundance.

As X-Nlp was not present in eggs or sperm, an immunodepletion approach in egg extracts was not practical to study Nlp function. Our studies therefore focused on the properties of X-Nlp in adult cells. With the exception of transformed tissue culture cell lines X-Nlp was only detected in the skin. Most cells within an adult organ are quiescent due to terminal differentiation, therefore non-proliferative. The skin is one of the few organs that is actively dividing. As X-Nlp is detected only in the skin (along with the transformed cell lines) its function may be restricted to actively dividing cells. X-Nlp was also not detected in the ovary or testis, therefore suggesting no specific role in meiosis. However, it should be noted that this experiment is limited by the quantity of the protein obtained from each organ/tissue. The two transformed cell lines represent the kidney (A6) and skin (XTC); as X-Nlp is only detectable in the skin and not the kidney it bolsters the argument for a role in actively dividing cells only.

Human Nlp has previously been shown to be absent from the mitotic centrosome (Casenghi et al., 2003). A similar result was observed here in *Xenopus* cell lines (A6 and XTC). The timing or extent of Nlp removal might be slightly different as the protein was almost undetectable in human prophase cells, whereas in *Xenopus* prophase cells 70% X-Nlp remained, but this dropped to 2% by anaphase. An unpredicted result was observed in A6 cells during mitosis with respect to the γ-tubulin abundance at the centrosome. In PtK₁ cells (rat kangaroo kidney epithelial cells), entry into mitosis is accompanied by recruitment of extra γ-tubulin to the spindle poles to enhance microtubule nucleation and promote assembly of the bipolar spindle (Khodjakov and Rieder, 1999). A6 cells however did not display this classic accumulation of γ-tubulin upon mitotic onset, although XTC
cells did. This may in part reflect epitope masking of γ-tubulin in a cell type specific manner. The loss of X-Nlp from the mitotic centrosome though is highly unlikely to be caused by epitope masking, as a second antibody raised to an alternative region produced the same result.

Nlp in human U2OS cells is a very low abundance protein and for this reason Casenghi et al. (2003) had not been able to determine whether there was a change in total abundance of Nlp through the cell cycle. Cell cycle synchronization experiments in A6 cells though showed that X-Nlp abundance does not alter through the cell cycle and X-Nlp disappearance from the centrosome is therefore not a result of global degradation. In support of this, sequence analysis of Hs-Nlp and X-Nlp shows that neither protein contains destruction motifs recognized by the mitotic E3 ubiquitin ligase, the APC/C. Furthermore, human Nlp is not degraded in a mitotic egg extract (M. Casenghi and E. Nigg, personal communication). The rapid reappearance of Nlp on the centrosome at the end of mitosis also argues against destruction. The most likely explanation is therefore that phosphorylation of Nlp by a mitotic kinase displaces it from the centrosome during mitosis, and dephosphorylation by an unknown phosphatase allows it to re-associate with the centrosome at mitotic exit. The slight smearing of X-Nlp, in the mitotic cell extract on Western blots would support this case. A similar scenario has been identified with another centrosomal protein C-Nap1 (Mayor et al., 2002). Interestingly, G0 arrested A6 cells showed an identical X-Nlp abundance when compared with cycling cells, but X-Nlp is not present in quiescent tissues. As X-Nlp does not contain any degradation motifs the protein is likely to be very stable, therefore a longer time period is likely to be required to degrade X-Nlp, hence detection in G0 cells that have only just entered quiescence.

Further localization analysis showed that X-Nlp is a core component of the centrosome and that it specifically associates with the mother centriole. As Nlp was reported to have a role in microtubule nucleation, it is surprising that it associates only with the mother centriole. The PCM surrounding both mother and daughter centriole can nucleate microtubules, but only the mother can anchor them (Piel et al., 2000). The mother centriole contains spoke-like appendages which are a site of microtubule anchorage and ninein is localized to these sites (Mogensen et al., 2000). Immunofluorescence microscopy of both ninein and Nlp show a barrel-like structure around the mother centriole (Casenghi et al., 2003; Mogensen et al., 2000; Ou et al., 2002; Ou and Rattner, 2000) which is also apparent for X-Nlp.
However, sub-centrosomal co-localization or a direct interaction between X-Nlp and ninein has yet to be established. So does Nlp have a role in microtubule anchorage, mother centriole-specific microtubule nucleation or both? Nlp can bind to the γ-TuRC and microinjection of antibodies against Nlp inhibits the rate of microtubule nucleation in interphase cells (Casenghi et al., 2003). Although this argues in favor of nucleation, the antibodies may also sterically hinder the anchorage of newly nucleated microtubules thus preventing generation of a radial aster. Overexpressing Nlp in interphase cells causes larger asters to form, which may be due to an increase in either nucleation or anchorage (Casenghi et al., 2003). Two populations of γ-tubulin are detectable at the centrosome, one which remains statically associated and the other dynamically exchanging with the cytoplasmic pool (Khodjakov and Rieder, 1999). It is possible that more permanently-associated γ-tubulin molecules are part of γ-TuRCs that become anchored by Nlp and/or ninein on the mother centriole. Thus, whilst we do not rule out a role in microtubule nucleation, we believe our data makes a persuasive case that the primary function of Nlp is in microtubule anchorage during interphase.
CHAPTER 6

REGULATION OF NLP BY NEK2A AND PLK1 KINASES
6.1 Introduction

The mitotic kinase Plk1 has a diverse range of cellular functions during mitosis, including mitotic entry, centrosome maturation, spindle formation, anaphase progression and cytokinesis (Dai et al., 2002; Glover et al., 1998; Nigg, 1998). The polo box domain (PBD) is required for Polo-like kinases to bind their in vivo substrates (Lee et al., 1998; Reynolds and Ohkura, 2002), but only in 2003 was the molecular mechanism governing this process identified (Elia et al., 2003a). The PBD binds to a motif consisting of a serine followed by a phosphorylated serine or threonine (S-pS or S-pT) (Cheng et al., 2003; Elia et al., 2003a; Elia et al., 2003b) which allows the catalytic domain to phosphorylate its substrate (consensus motif D/E-x-S/T) (Kelm et al., 2002; Nakajima et al., 2003). When Nigg and colleagues performed a yeast two hybrid screen using Plxl as a bait to identify further potential substrates, Nlp was isolated. Plk1 kinase can phosphorylate the Nlp N-terminal domain in vitro and in vivo and its activity is capable of displacing Nlp from the centrosome. Overexpressing an Nlp mutant lacking eight putative Plk1 phosphorylation sites (NlpΔ8) led to accumulation of Nlp on spindle poles and produced multipolar mitotic spindles that ultimately lead to missegregation of DNA (Casenghi et al., 2003).

Having shown in chapter 4 that Nlp can interact with Nek2, we set out to investigate whether Nek2 can regulate the association of Nlp with the centrosome in a similar manner to Plk1. We found that Nek2 can phosphorylate Nlp apparently on different sites from Plk1 and that this triggers Nlp displacement from the centrosome. Our data support a coordinate regulation of Nlp and Plk1 and raise the intriguing possibility that Nek2 acts as a priming kinase for Plk1.
6.2 Results

6.2.1 In vitro phosphorylation of X-Nlp by Nek2A
To determine whether Nlp is a substrate for Nek2, purified MBP:X-Nlp\textsubscript{262-552} was first used in an in vitro kinase assay as this fragment of Nlp overlapped with the sequence (amino acids 77-634) previously shown to interact with Nek2. This also contains five of the putative Plk1 phosphorylation sites. Kinase assays were performed using semi-purified Hs-Nek2A expressed in insect cells (Figure 6.1A)(Fry and Nigg, 1997), immunoprecipitated X-Nek2B from an insect cell lysate (Figure 6.1B)(Twomey et al., 2004) and purified Plx1 expressed in insect cells (Figure 6.1C)(Descombes and Nigg, 1998). α/β-casein served as the positive control and purified MBP alone the negative control. Both Hs-Nek2A and X-Nek2B could phosphorylate β-casein and MBP:X-Nlp\textsubscript{262-552}, but not MBP alone (Figure 6.1 A&B). Plx1 could phosphorylate α-casein and MBP:X-Nlp\textsubscript{262-552}, but not MBP alone (Figure 6.1C). Nlp is phosphorylated to a similar efficiency as β-casein by Nek2A, whereas Plx1 phosphorylation of Nlp was less efficient than α-casein. These results show that X-Nlp is a good in vitro substrate for Nek2, as well as Plx1.

6.2.2 In vivo phosphorylation of X-Nlp by Nek2A
As X-Nlp was a good in vitro substrate for Nek2, we wished to address whether this could be reproduced in vivo. Human Nek2A was transiently transfected into A6 cells and the effects upon the distribution of endogenous X-Nlp analyzed. The distribution of ectopic Nek2A was identical to previous studies showing Nek2A is centrosomal and cytoplasmic (Fry et al., 1998b). After 24 hours, overexpressed Nek2A led to displacement of X-Nlp from the centrosome in over 80% of A6 cells, whereas only 20% of centrosomes lacked Nlp when transfected with Nek2A-K37R (Figure 6.2B). This result clearly indicates that Nek2A triggers the displacement of X-Nlp from the interphase centrosome in a kinase dependant manner.

6.2.3 In vivo phosphorylation of human Nlp by Nek2A and Plk1
The in vivo phosphorylation experiments in Xenopus cells showed that Nek2A kinase activity can displace X-Nlp from an interphase centrosome. We have therefore identified a second mitotic kinase, in addition to Plk1, that can regulate Nlp localization. To confirm that Nek2 could also regulate Nlp in human cells, U2OS cells were transfected with
Figure 6.1 X-Nlp can be phosphorylated \textit{in vitro} by Nek2A, X-Nek2B and Plxl.
Purified MBP-X-Nlp\textsubscript{262-552} was subjected to \textit{in vitro} phosphorylation along with MBP and casein as indicated. Samples were separated by SDS-PAGE, stained with Coomassie Blue (lower panels) and exposed to autoradiography (top panels). (A) \textit{In vitro} phosphorylation using purified human Nek2A, WT, wild type kinase and KR, K37R kinase dead mutant. (B) \textit{In vitro} phosphorylation using immunoprecipitated X-Nek2B, WT, wild type kinase and KR, K37R kinase dead mutant. (C) \textit{In vitro} phosphorylation using purified Plxl, WT, wild type kinase and NA, N172A kinase dead mutant. M.wt markers (kDa) are indicated on the left of each panel.
Figure 6.2 Active Nek2A displaces X-Nlp from the centrosome in interphase A6 cells.

(A) A6 cells were transiently transfected with myc-Nek2A or myc-Nek2A-K/R and analyzed by immunofluorescence microscopy 24 h after transfection. Cells were co-stained with antibodies against the myc tag (red) and X-Nlp (green); DNA was stained with Hoechst 33258 (blue). Arrowheads denote centrosomes. Scale bar, 10 μm. (B) The presence or absence of X-Nlp at the centrosome was determined 24h after transfection. The graph indicates % cells with severely reduced or no staining of X-Nlp at the centrosome. 300 cells were counted from 3 independent experiments and error bars show s.d.
Nek2A, and Nlp distribution observed with an anti-human Nlp antibody (kind gift from Prof. E. Nigg, Matinsreid, Germany). Endogenous Nek2A stains the two centrioles while Nlp is predominantly mother centriole associated (Figure 6.3A, upper panel). As in Xenopus cells, overexpression of wild-type but not kinase inactive Nek2A resulted in Nlp displacement from the interphase centrosome in human cells (Figure 6.3, middle and lower panels). The same experiment was repeated by transfecting Plk1 into U2OS cells confirming published results that Plk1 overexpression can displace Nlp from the interphase centrosome in a kinase dependant manner (Casenghi et al., 2003)(Figure 6.3B). It is worth noting that the Plk1 used in this experiment has a mutation T210D, which makes it hyperactive.

Overexpression of either hyperactive Plk1 or wild-type Nek2A is independently sufficient to remove Nlp from the interphase centrosome. However, under normal circumstances both kinases may be required to trigger efficient displacement of Nlp. To address this question, co-transfection experiments were performed using a combination of active and inactive Nek2A and Plk1. Initially, co-transfection trials were performed to compare the relative levels of expression of each protein. Immunofluorescence microscopy revealed that of those U2OS cells expressing Plk1 less than 50% also expressed Nek2A, whereas over 95% of cells expressing Nek2A, were also expressing Plk1 (Figure 6.4A). Western blots of transfected cell extracts confirmed that Plk1 was expressed to higher levels than Nek2 in the cell population (Figure 6.4B). Importantly, co-transfected cells that showed intense Nek2 staining, always showed intense Plk1 staining. Therefore to detect co-transfected cells, only cells with an intense Nek2 signal were counted. The images shown in Figure 6.3 and 6.4A represent the intensity of Nek2A signal that was counted for studying the effects upon Nlp.

Transfection experiments after 24 hours showed that expressing either active kinase had a strong effect upon Nlp displacement from the interphase centrosome (over 80%), while expressing either kinase inactive mutant had a more modest effect on Nlp displacement (25-35%)(Figure 6.4B). Co-expressing Nek2A with Plk1-K82R did not alter the effectiveness of Nlp displacement caused by Nek2A. However, co-expression of Plk1 with Nek2A-K37R significantly reduced Nlp displacement caused by Plk1 (50%). Nek2A-K37R can disperse centrosomes in a time dependant manner (Fry et al., 1998b) and this is likely to explain in part the loss of Nlp from centrosomes with the kinase-dead
Figure 6.3 Active Nek2A and Plk1 can displace Nlp from the interphase centrosome in interphase U2OS cells

(A) U2OS cells were transiently transfected with myc-Nek2A or myc Nek2A-K/R and analyzed by immunofluorescence microscopy 24 h after transfection. Cells were co-stained with antibodies against Nek2 (red) and Nlp (green); DNA was stained with Hoechst 33258 (blue). (B) U2OS cells were transiently transfected with myc-Plk1-T/D (hyperactive mutant) or myc-Plk1-K/R (kinase-dead mutant) and analyzed by immunofluorescence microscopy 24 h after transfection. Cells were co-stained with antibodies against the myc tag (red) and Nlp (green); DNA was stained with Hoechst 33258 (blue). Arrowheads denote centrosomes. Scale bar, 10 μm.
Figure 6.4 Kinase dead Nek2A interferes with Plk1 stimulated Nlp displacement from the interphase centrosome

(A) U2OS cells were co-transfected with myc-Nek2A and myc-Plk1-T/D and analyzed by immunofluorescence microscopy 24 h after transfection to determine whether cells were expressing both proteins. Cells were co-stained with antibodies against Nek2 (red) and Plk1 (green); DNA was stained with Hoechst 33258 (blue). Scale bar, 30 μm. All cells expressing Nek2A were also expressing Plkl, but not vice-versa. (B) The presence or absence of X-Nlp at the centrosome was determined 24 h after transfection. The graph indicates percentage cells with severely reduced or no staining of X-Nlp at the centrosome; 300 cells were counted from 3 independent experiments and error bars show s.d. Singly transfected cells were co-stained with antibodies against the myc tag and Nlp, whilst co-transfected cells were stained with antibodies against Nek2 and Nlp. Cell lysates were analyzed by Western blotting using antibodies against the myc tag and α-tubulin. (C) Cells were treated as in (B), but analyzed 16 h after transfection.
mutant. To minimize this effect, a 16 hour expression time was used, which resulted in the catalytically-inactive Nek2A inducing displacement of Nlp from the centrosome in only 25% of cells. In combination with active Plk1, this level rose to only 38% (Figure 6.4C). These results suggest that both active Nek2A and Plk1 can displace Nlp from the centrosome, but that catalytically-inactive Nek2A markedly interferes with the ability of Plk1 to displace Nlp.

6.2.4 Kinase-inactive Nek2A interferes with displacement of endogenous Nlp from the mitotic centrosome

The Nek2 kinase shows highest activity at the G2/M transition, which coincides with the disappearance of endogenous Nlp from the centrosome. A stable U2OS Nek2A-K37R-myc-His cell line had previously been generated in our laboratory where Nek2A was under the control of a tetracycline-inducible promoter (Faragher and Fry, 2003). Addition of tetracycline to the cells induced expression of Nek2A-K37R-myc-His in all cells to a level approximately five times above endogenous Nek2A. We therefore decided to use this cell line to determine whether increasing Nek2A-K37R abundance would have a dominant negative effect upon Nlp displacement from the centrosome upon mitotic onset. To confirm Nek2A-K37R expression, induced and uninduced cells were stained with Nek2 antibodies (Figure 6.5A). Mitotic cells in prophase and metaphase were identified by DNA staining while γ-tubulin staining identified spindle pole location (Figure 6.5B). Quantitative measurements of Nlp intensity showed that the efficiency by which Nlp was removed from spindle poles was reduced when Nek2A-K37R was overexpressed. The average Nlp intensity is higher than for uninduced cells, which display a reduction from prophase to metaphase as expected. 25% of induced cells displayed readily detectable Nlp at the mitotic centrosome, as depicted in Figure 6.5B. Despite a wide range in the spread of the data (Figure 6.5C), the variation is significant with p<0.05. This result shows that overexpressing Nek2A-K37R causes significant interference with the complete displacement of Nlp from the spindle pole.

6.2.5 Nek2A can disperse recombinant Nlp assemblies in vivo

Overexpression of recombinant human Nlp causes the formation of large assemblies that associate with the centrosome. Plk1 can displace these large assemblies from the centrosome causing them to fragment into smaller particles that are no longer associated with the centrosome (Casenghi et al., 2003). To determine whether Nek2 could also
Figure 6.5 Nlp abundance increases at the mitotic centrosome in the presence of kinase dead Nek2A

(A) The U2OS myc-Nek2A-K/R-myc-His cell line was induced with tetracycline for 24 h and analyzed by immunofluorescence microscopy. Cells were co-stained with antibodies against Nek2 (red) and Nlp (green); DNA was stained with Hoechst 33258 (blue). Scale bar, 20 μm. (B) The U2OS induceable myc-Nek2A-K/R-myc-His cell line was induced for 24 h and mitotic cells analyzed by immunofluorescence microscopy, using antibodies against γ-tubulin (red) and Nlp (green); DNA was stained with Hoechst 33258 (blue). Arrowheads denote centrosomes. Scale bar, 10 μm. (C) The abundance of X-Nlp at the centrosome was determined 24 h after transfection in interphase (I), prophase (pro) and metaphase (met) cells. -, uninduced, +, induced. The graph indicates Nlp intensity (arbitrary units) determined by quantitative centrosome imaging as described in the Materials and methods. 20 cells were counted in each data set and error bars show s.d.
induce fragmentation of Nlp assemblies, myc-Nek2A and GFP-Nlp were co-transfected into U2OS cells. Nek2A led to a striking fragmentation of the large Nlp assemblies as well as co-localizing with the fragments. Nek2A-K37R was unable to reproduce this fragmentation but did still co-localize with the overexpressed Nlp protein (Figure 6.6A).

Based on the known phosphorylation consensus for Plk1 (D/E-x-S/T), a mutant Nlp lacking eight potential Plk1 phosphorylation sites was generated, called NlpΔ8. The NlpΔ8 mutant cannot be displaced from centrosomes when co-expressed with Plk1 (Casenghi et al., 2003). To determine whether Nek2 was capable of displacing the NlpΔ8 mutant, Nek2A and GFP-NlpΔ8 were co-transfected into U2OS cells. Nek2A was clearly able to fragment the NlpΔ8 aggregates, whereas Nek2A-K37R only co-localized with them as with wild-type Nlp (Figure 6.6B). Quantification of these data showed that fragmentation of GFP-Nlp occurred in 65% cells co-expressing Nek2A, while GFP-NlpΔ8 was fragmented in 57% cells co-expressing Nek2A (Figure 6.6C). To confirm that Nlp can be phosphorylated by Nek2A in vivo, transfected cell lysates were analyzed by Western blotting. When Nlp or NlpΔ8 were co-expressed with Nek2A a reduction in electrophoretic mobility was observed, a hallmark of protein phosphorylation; this did not occur when Nek2A-K37R was co-expressed with Nlp or NlpΔ8 (Figure 6.6D). Therefore, the fragmentation of Nlp and NlpΔ8 is most likely a result of Nek2A mediated phosphorylation. Furthermore, these sites appear to be quite distinct from those phosphorylated by Plk1, although it cannot be ruled out that Nek2A may phosphorylate some sites that are also targeted by Plk1.

The fragmentation of GFP-Nlp caused by Nek2A phosphorylation is very pronounced. To determine whether these small fragments were still associated with the centrosome, U2OS cells were transfected with Nek2A and GFP-Nlp while centrosomes were stained with anti-polyglutamylated tubulin antibodies which localize only to the core centrioles. Co-transfected cells were selected based upon GFP-Nlp fragmentation. Based on the lack of co-localization of GFP-Nlp with the centrioles, we conclude that the GFP-Nlp fragments are no longer centrosome associated in the presence of Nek2A, while GFP-Nlp is centrosome associated in the presence of Nek2A-K37R (Figure 6.7A). An identical result was obtained using the GFP-NlpΔ8 mutant in the presence of Nek2A and Nek2A-K37R (Figure 6.7B).
Figure 6.6 Nek2 can phosphorylate and displace recombinant Nlp from the centrosome in vivo

(A+B) U2OS cells were transiently transfected with myc-Nek2A or myc-Nek2A-K/R together with GFP-Nlp (A) or GFP-NlpΔ8 (B) and analyzed by immunofluorescence microscopy 24 h after transfection. Cells were stained with antibodies against Nek2 (red); the GFP signal is shown (green) and DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm. (C) The fragmentation of Nlp was determined 24 h after transfection. The graph indicates percentage cells showing Nlp fragmentation, 300 cells were counted from 3 independent experiments and error bars show s.d. (D) U2OS cells were transiently transfected with GFP-Nlp or GFP-NlpΔ8 with myc-Nek2A or myc-Nek2A-K/R and analyzed by Western blotting using antibodies against GFP. M.wt markers (kDa) are indicated on the left.
Figure 6.7 Nlp assemblies are no longer centrosome associated in the presence of active Nek2A

(A&B) U2OS cells were transiently transfected with myc-Nek2A or myc-Nek2A-K/R together with GFP-Nlp (A) or GFP-NlpΔ8 (B) and analyzed by immunofluorescence microscopy 24 h after transfection. Centrioles were stained with antibodies against polyglutamylated tubulin (GT335, red); the GFP signal is shown (green) and DNA was stained with Hoechst 33258 (blue). Images Ac' and Be' are enlargements of the squares in Ac and Be, respectively. Arrowheads denote centrosomes. Scale bar, 10 μm.
6.2.6 Overexpression of Nlp induces centriole splitting

Overexpression of Nlp during interphase causes an increase in microtubule nucleating activity at the centrosome as well as creating a more dispersed focal point for the microtubule network (Casenghi et al., 2003). While confirming endogenous Nek2 localizes with GFP-Nlp at the centrosome, it became apparent that GFP-Nlp overexpression was inducing the splitting of centrioles. In interphase cells, centrioles usually appear in close proximity being <2 μm apart (Figure 6.8A a-c). Active Nek2A overexpression has been shown to cause them to prematurely separate (Fry et al., 1998b; Meraldi and Nigg, 2002). Overexpression of GFP-Nlp also caused centriole splitting to occur as determined by co-staining with C-Nap1 antibodies. Splitting occurred in a dose dependant manner, as lower expression levels of GFP-Nlp did not induce splitting (data not shown). Figure 6.8A (d-f) shows an example of split centrioles embedded in the GFP-Nlp aggregate where the distance between the mother and daughter is greater than usual. Figure 6.8A (g-i) shows a more extreme split centriole phenotype with the GFP-Nlp aggregate surrounding only one centriole (presumably the mother), while the other is separated by more than 5 μm in the cytoplasm. The apparent weak co-localization of C-Nap1 antibodies with the large Nip assemblies may be an artifact due to bleedthrough from the GFP signal as C-Nap1 was reported not to interact with Nip (Casenghi et al., 2003). However, it is possible that the antibody may also bind non-specifically with these aggregates.

6.2.7 Nek2A activity does not perturb interaction of Nlp and the γ-TuRC

Nlp can interact with the γ-TuRC, therefore may have a role in anchoring the γ-TuRC to the interphase centrosome (Casenghi et al., 2003). Plk1 phosphorylation can disrupt this interaction and leads to loss of co-localization between Nlp and γ-tubulin. To determine whether phosphorylation of Nlp by Nek2A also interferes with this interaction, Nek2A and GFP-Nlp were co-transfected into U2OS cells. Co-transfected cells were selected based upon the GFP-Nlp fragmentation pattern. Endogenous γ-tubulin was stained to determine whether there was co-localization with Nlp fragments. Contrary to the results seen with Plk1, the phosphorylation of Nlp by Nek2A did not interfere with localization between γ-tubulin and Nlp (Figure 6.9A). To confirm that the γ-tubulin staining pattern was not caused by fluorescence bleedthrough from the intense GFP-Nlp signal, myc-Nek2A and GFP-Nlp were co-transfected and stained with the antibody to the myc-tag. At an identical exposure, red fluorescence is not seen in the green channel indicating that the γ-tubulin
Figure 6.8 Overexpression of Nlp causes premature centriole separation

(A) U2OS cells were transiently transfected with GFP-Nlp and analyzed by immunofluorescence microscopy 24 h after transfection. Centrioles were stained with antibodies against C-Nap1 (red); the GFP signal is shown (green) and DNA was stained with Hoechst 33258 (blue). d-f and g-i represent examples of split centrioles. Scale bar, 10 μm. (B) Splitting of centrioles was determined 24 h after transfection. The graph shows percentage cells with centrioles that were greater than 2 μm apart. 300 cells were counted from 3 independent experiments and error bars show s.d.
Figure 6.9 Nek2A kinase activity does not interfere with Nlp binding to γ-tubulin

(A) U2OS cells were transiently transfected with myc-Nek2A or myc-Nek2A-K/R with GFP-Nlp, and analyzed by immunofluorescence microscopy 24 h after transfection. Cells were stained with antibodies against γ-tubulin (red); the GFP signal is shown (green) and DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm. (B) U2OS cells were transiently transfected with myc-Nek2A-K/R and GFP-Nlp, and analyzed by immunofluorescence microscopy 24 h after transfection to confirm signals are not due to fluorescence bleedthrough. Cells were stained with antibodies against the myc tag (red); the GFP signal is shown (green) and DNA was stained with Hoechst 33258 (blue). Scale bar, 20 μm.
staining is representative of endogenous protein and not the GFP-Nlp signal (Figure 6.9B).
The results in this section show that phosphorylation of Nlp by Nek2A does not interfere
with the interaction between Nlp and the γ-TuRC.
6.3 Discussion

Entry into mitosis is accompanied by a dramatic change in microtubule organization. At the same time there are changes in both centrosomal protein composition and centrosomal protein phosphorylation (Blagden and Glover, 2003; Centonze and Borisy, 1990). The polo-like kinases are one example of several cell cycle regulated kinases that regulate centrosome organization at the G2/M transition (Lane and Nigg, 1996). Aurora A, Cdk1 and Nek2 are also active at this time in the cell cycle and localize at the centrosome. Data presented in earlier chapters indicates Nlp is implicated in microtubule anchoring at the interphase centrosome. Published data also shows that Nlp displacement from the centrosome during mitosis is possibly mediated by the kinase activity of Plk1 (Casenghi et al., 2003). Having shown though that Nek2 can also interact with Nlp, we decided to assess whether Nek2 might also regulate Nlp localization. Our data revealed that both Nek2 and Plk1 could target Nlp raising important questions about the nature of Nlp regulation in vivo.

The original fragment of X-Nlp encoding amino acids 77-634 isolated from the yeast two hybrid screen using Plxl as the bait could also interact with X-Nek2A. To determine whether a similar region could also be phosphorylated by Nek2 in vitro, kinase assays were performed with an MBP:X-Nlp\textsubscript{262-552} fusion protein. This fragment of X-Nlp could be phosphorylated by both Nek2A and X-Nek2B in vitro. Plxl could also phosphorylate this fragment of X-Nlp in vitro as expected, as two Plk1 phosphorylation sites are conserved within this fragment (S349 and S498). The number of Nek2 phosphorylation sites within the X-Nlp fragment is unknown, but recent data obtained in our laboratory suggest that at least two Nek2 phosphorylation sites are within this region (J. Baxter, personal communication). The level of phosphorylataion could be indicative of the number of sites present. Transfection studies also support the notion that Nlp is an in vivo substrate of Nek2. Co-expressing GFP-Nlp with active, but not kinase-dead Nek2A, led to a retardation in its gel migration indicative of phosphorylation. Furthermore, overexpressing human Nek2A could displace X-Nlp from the centrosome in over 80% of A6 cells, whereas Nek2A-K37R was four times less effective.

In human cells, it has been shown that high level overexpression of Nek2 for extended periods of time can lead to centrosome dispersal in a kinase-independent manner. It was
important to be certain therefore that the loss of Nlp was a specific response and not simply a result of centrosome disintegration. First of all, in Xenopus A6 cells overexpression of Nek2A for 24 hours led to <10% centrosome dispersal, yet Nlp was absent in >80% cells. The same experiment performed in human cells showed that overexpressing active Nek2A could also displace Nlp from the centrosome in >80% cells, whereas Nek2A-K37R could cause disappearance of Nlp in only approximately 35% cells. The latter result may well be due in part to centrosome dispersal. Using a shorter incubation time (24 h reduced to 16 h) reduced the effect of Nek2A-K37R from 35% to approximately 20% without compromising the effects of active Nek2A. Therefore, the partial displacement of Nlp from the centrosome by Nek2A-K37R is most likely a result of centrosome dispersal as previously reported, but active Nek2A clearly has a specific effect on Nlp localization.

As both Plkl and Nek2A could displace Nlp from the centrosome we wished to know whether this was occurring via independent pathways. By co-transfecting live and dead kinases, we showed that kinase dead Plkl did not hinder the efficiency with which Nek2A could displace Nlp from the centrosome, however, kinase dead Nek2A dramatically reduced the efficiency by which Plkl mediated displacement of Nlp from the centrosome (90% reduced to less than 40%). Therefore, Nek2A-K37R has a dominant negative influence upon Plkl kinase activity directed against Nlp. Nek2A-K37R could mask Plkl phosphorylation by binding to the substrate and not releasing it. Nek2A-K37R could bind to a region that consists of the polo box binding domain and mask this site, thereby preventing the PBD from binding. Alternatively, Nek2A-K37R could bind to a region that consists of the Plkl phosphorylation motif (D/E-x-S/T), thereby the PBD would bind but the catalytic domain would fail to identify its consensus. The mechanism by which polo boxes recognize their substrates suggests that a priming phosphorylation event must occur, and that the S-pS/S-pT must be exposed in order to be detected (Elia et al., 2003a; Elia et al., 2003b; Neef et al., 2003). Another intriguing explanation for our data is therefore that Nek2A is a potential candidate for priming Plkl substrates.

If Nek2A was a priming kinase it would phosphorylate Nlp at a site that facilitates binding of the PBD and stimulate Plkl mediated phosphorylation. Nek2A-K37R would compete with endogenous Nek2 and interfere with Nlp phosphorylation thereby preventing the PBD from identifying its substrates. Another possibility which must be considered is a direct
role for Nek2A in activating Plk1. Plx1 has been shown to be activated by an upstream kinase(s) (Kelm et al., 2002; Qian et al., 1998b), and in S. pombe Fin1 is implicated in recruiting Plo1 to the SPB. However, overexpressing Fin1 has no effect upon Plo1 activity (Grallert and Hagan, 2002). No direct phosphorylation of mammalian Plk1 by Nek2 has been shown despite a number of attempts. Furthermore, the experiments performed have used a hyperactive Plk1 mutant suggesting that kinase dead Nek2A cannot be preventing activation of Plk1. Importantly, overexpressing Nek2A-K37R also interfered with displacement of endogenous Nlp from the mitotic spindle pole. There was a partial decrease in Nlp at metaphase spindle poles but the abundance of Nlp remaining was significantly higher than for control cells. Ultimately, it will be necessary to deplete Nek2, for instance by RNAi, and determine whether this leads to maintenance of Nlp at spindle poles.

Co-expression of Nek2A with recombinant GFP-Nlp assemblies provides further evidence that Nlp can interact with Nek2 in vivo. Active Nek2A also causes fragmentation of these assemblies supportive of Nlp being an in vivo substrate. These fragments fail to associate with the centrosome implying that Nlp phosphorylated by Nek2A loses the ability to interact with other centrosomal components. Nek2A could also phosphorylate, fragment and displace GFP-NlpΔ8 from the centrosome, indicating that the Nek2 phosphorylation sites are different to those recognized by Plk1. Plk1 phosphorylation of Nlp also inhibits the association of Nlp with the γ-TuRC, whereas Nek2A phosphorylation of Nlp did not prevent this association. Therefore, the alternative phosphorylation sites targeted by Nek2A and Plk1 have different effects upon Nlp. This also indicates that Nlp is not bound to the centrosome as a result of interaction with γ-TuRCs. An attractive model is that Nek2A has a direct role in displacing Nlp from the centrosome, but also acts as a priming kinase for Plk1 which inhibits the association of Nlp with the γ-TuRC and may further stimulate the dissociation of Nlp from the centrosome. The γ-TuRC then becomes relocalized to other spindle pole components (e.g. NuMA or dynactin) to participate in spindle formation.

Like active Nek2A, overexpressing GFP-Nlp induced centriole splitting. The GFP-Nlp aggregates can recruit endogenous Nek2A, but this is unlikely to be the cause of premature centriole splitting as co-expressing GFP-Nlp with Nek2A-K37R is as efficient at splitting centrioles as GFP-Nlp alone. It is possible that the expression of GFP-Nlp perturbed C-
Nap1 localization at the centrioles although the current evidence is not convincing for this hypothesis. Centriole splitting may simply result from the large Nlp assemblies that surround the mother centriole, forcing the centrioles apart.

Here, we have shown that Nek2A and Plk1 can dissociate Nlp from the interphase centrosome and that Nek2A-K37R interferes with Nlp displacement from spindle poles. We have also established that Nek2A and Plk1 appear to phosphorylate alternative sites. Nek2A may perform a priming kinase event for Plk1. Alternatively, the phosphorylation events may be independent and/or redundant pathways to displace Nlp from the mitotic centrosome. The severe mitotic phenotypes that result from aberrant localization of Nlp during mitosis highlights the importance of its displacement at the G2/M transition.
CHAPTER 7

FINAL DISCUSSION
7. Final Discussion

The Nek2 kinase is implicated in the processes of centrosome disjunction, centrosome organization and cell cycle progression. However, only one bona fide centrosomal substrate has been identified, C-Napl (Fry et al., 1998a; Mayor et al., 2002). The aims of this work were to identify a full-length *Xenopus laevis* cDNA of C-Napl then raise a variety of antibodies which could be used to assess the importance of centrosome cohesion during early development as well as the importance of C-Napl in spindle assembly/maintenance. We also wished to identify further substrates or regulators of the Nek2 kinase by using a yeast two hybrid screen with Nek2 as the bait. Characterization of novel Nek2 substrates would then open the door to understanding how deregulation of Nek2 expression might contribute to malignant progression of cancer cells.

7.1 Rootletin, a centrosomal substrate of Nek2?
Attempts to isolate an orthologue of C-Napl in *Xenopus laevis* have so far proved unsuccessful. However, database screening identified a *Xenopus* protein with high similarity to C-Napl, although full-length sequencing of the EST revealed similarity was greatest to another novel centrosomal protein called rootletin (Yang et al., 2002). Rootletin homologues are present in vertebrates and *Drosophila*, while C-Napl homologues are only apparent in mammals. Hence, it is possible that mammalian systems might have evolved to encode C-Napl as well as rootletin, while other eukaryotes contain only rootletin. This raises the possibility that rootletin and C-Napl might have overlapping or even redundant functions in centrosome cohesion. Due to the high protein sequence similarity between rootletin and C-Napl, it will be of interest to establish whether rootletin has a role in centrosome cohesion in mammalian systems and whether Nek2 can phosphorylate and regulate rootletin *in vivo*.

7.2 Nlp is a mother centriole specific protein involved in microtubule anchorage during interphase
Polo-like kinases are known to influence many aspects of mitosis, but the molecular details are only beginning to be understood (Dai et al., 2002; Glover et al., 1998; Nigg, 1998). In searching for further substrates of Polo-like kinases, the Nigg group identified Nlp as a novel Plk1 interacting centrosomal protein. Their data revealed a role for Nlp in interphase microtubule nucleation as microinjection of antibodies against Nlp caused a delay in
microtubule polymerization in interphase, but not mitotic, cells. Indeed, Nlp is normally absent from the mitotic centrosome and overexpressing Nlp in mitotic cells causes the formation of abnormal spindles.

With the aid of database searching, we identified ESTs corresponding to a full-length *Xenopus laevis* Nlp homologue based on alignment to human Nlp. Expressed cDNA fragments allowed us to generate antibodies that could be used to characterize X-Nlp in a different vertebrate system. We obtained no evidence that X-Nlp is required during early development as X-Nlp is absent in eggs and sperm and was also undetectable during embryogenesis. However, X-Nlp was detected in adult mitotic tissues such as skin and transformed cell lines. X-Nlp is associated with the mother centriole during interphase and shares high similarity to another maternal centriole protein called ninein. Ninein is believed to anchor microtubules to the mother centriole as well as apical sites in epithelial cells. By providing an anchoring site, ninein may also cap and stabilize microtubules at the minus end (Mogensen et al., 2000). As X-Nlp is associated with the mother centriole only, this would support a role in anchorage. But Casenghi et al. (2003) showed that Nlp interacts with GCP4 and γ-tubulin rather implying a role in microtubule nucleation. Khodjakov and Rieder (1999) demonstrated using GFP-γ-tubulin that two discrete subpopulations of γ-tubulin exist at the centrosome, one is static and the other is in dynamic exchange with the cytoplasmic pool.

Therefore, we propose that Nlp may associate with the static population and be a component of a γ-TuRC anchoring complex during interphase. As a component of a γ-TuRC anchoring complex Nlp could contribute to microtubule nucleation by providing a docking site to which the γ-TuRC will adhere. If the nucleated microtubule is not severed it will remain anchored to the centrosome. Nlp may also directly or indirectly anchor microtubules by non-γ-TuRC associated complexes. As Nlp is associated with the mother centriole and forms a barrel-like structure identical to ninein, it may also localize exclusively to the appendages (Figure 7.1). This would suggest that Nlp has a more important role in anchoring microtubules to the centrosome, rather than simply anchoring γ-TuRCs in the PCM.
Figure 7.1 Proposed model for ninein and Nlp function at the interphase centrosome

Ninein and Nlp localize at the appendages upon the mother centriole. **Complexes:** γ-TuRC consists of the known human components GCP1-GCP6; γ-TuRC anchor consists of pericentrin, kendrin or CG-NAP; Nlp or ninein may exist complexed to other unidentified proteins or self aggregate. The γ-TuRC nucleates microtubules which become either severed or released. These microtubules may then be translocated to the appendages and anchored by a ninein containing complex (1); or the entire γ-TuRC is released from the γ-TuRC anchor which may then translocate to the appendages and become anchored by an Nlp containing complex (2).
7.3 Regulation of microtubule anchorage at G2/M

Protein phosphorylation is no doubt used to regulate changes in microtubule organization at the MTOC between interphase and mitosis. The yeast γ-TuSC anchoring protein Spc110p is phosphorylated during mitosis by Mps1. A non-phosphorylateable Spc110p combined with Spc97p mutants is lethal suggesting that Spc110p phosphorylation may enhance binding to Spc97p/Spc98p/Tub4p during mitosis (Friedman et al., 2001). Spc98p can also be phosphorylated by Mps1 but only at the inner plaque, this event may aid microtubule attachment on the nuclear side of the envelope (Pereira et al., 1998). No kinases have been identified which can phosphorylate kendrin, the closest mammalian homologue of Spc110p, but kendrin can associate with calmodulin which is centrosomal during mitosis implying that calcium dependant kinases may in part control γ-TuRC anchoring (Flory et al., 2000; Li et al., 1999).

Another mammalian γ-TuRC anchoring protein, CG-NAP, may help to regulate microtubule organization through recruitment of multiple protein kinases and phosphatases. CG-NAP can associate with and thus localize to the centrosome PKN, PKA, PKC-ε, PP1 and PP2A, it can also bind to the GTPase Ran (Gruss et al., 2001; Keryer et al., 2003; Takahashi et al., 2000; Takahashi et al., 1999). However, how these kinases and phosphatases are involved in microtubule organization or bipolar spindle formation awaits elucidation. The phosphorylation of Nlp is required to displace it from the centrosome to facilitate bipolar spindle formation, a similar situation may also exist for ninein where Aurora A and PKA may facilitate its displacement from the spindle pole (Casenghi et al., 2003; Chen et al., 2003). Phosphorylation of large centrosomal proteins to displace them from the centrosome during mitosis may be a common mechanism in cells. Degradation and resynthesis is energetically costly, especially when the protein is large and displaced only for a short period as in the case of C-Nap1, ninein and Nlp.

Nlp is displaced from the spindle pole in both human (Casenghi et al., 2003) and *Xenopus* cells. Overexpressing Nlp during mitosis causes aberrant spindles to form which may be monopolar or multipolar (Casenghi et al., 2003). Interestingly, in human cells ninein is also displaced from the mitotic centrosome (Chen et al., 2003; Ou et al., 2002). These findings would suggest that removal of Nlp, and perhaps also ninein, from the spindle pole is required to establish bipolarity.
7.4 Nlp is a novel Nek2 substrate
Experiments performed both in *Xenopus* and human cells *in vitro* and *in vivo* showed that Nlp is a novel substrate of Nek2. Furthermore, elevated kinase activity of Nek2 is sufficient to displace Nlp from the interphase centrosome. In addition, overexpressing catalytically inactive Nek2A delays Nlp displacement from the centrosome in mitotic cells. Overexpressing Nek2A-K37R from an inducible promoter has previously been reported to cause monopolar spindles (Faragher and Fry, 2003). Considering the phenotype caused by Nlp overexpression, Nek2 phosphorylation of Nlp may be one mechanism by which Nek2A affects spindle formation. Nlp can be displaced from the centrosome independently by overexpressing active forms of Nek2 and Plk1. This is despite Nek2 phosphorylating Nlp at distinct residues to Plk1. However, only Plk1 can interrupt the association between Nlp and the γ-TuRC (Casenghi et al., 2003). Therefore, the actions of each kinase on Nlp are not identical. To establish exactly what Nek2 phosphorylation of Nlp facilitates will require the identification of Nek2 phosphorylation sites (see section 7.5) as well as further proteins which may form part of an Nlp containing complex.

7.5 Nek2 as a potential Plk1 priming kinase
Both Nek2 and Plk1 can phosphorylate and displace Nlp from the interphase centrosome when overexpressed. The critical question is whether one or other or both kinases are required in the physiological setting. Depletion experiments using an RNAi approach would help to answer this. Intriguingly, overexpressing Nek2A-K37R significantly reduced the ability of hyperactive Plk1 to displace Nlp from the centrosome. This raises the possibility that Nek2 kinase activity may be required for Plk1 to regulate Nlp. The mechanism by which Polo-like kinases recognize their substrates suggests that a priming phosphorylation event must occur, and that the S-pS/pT must be exposed in order to be detected (Elia et al., 2003a; Elia et al., 2003b; Neef et al., 2003). Therefore, our data highlight Nek2 as a potential priming kinase for Plk1 upon Nlp (Figure 7.2A). In this case, overexpressing kinase-dead Nek2A would cause the PBD binding site to remain unphosphorylated by outcompeting endogenous Nek2, thus preventing Plk1 from identifying its substrate (Figure 7.2B). Alternatively, Nek2A-K37R could bind directly to Nlp at sites that the PBD binds, or to sites which Plk1 phosphorylates, thereby masking phosphorylation (Figure 7.2C).
Figure 7.2 A model for co-ordinate regulation of Nlp by Nek2A and Plk1

(A) Under physiological conditions Nek2A binds to and phosphorylates the PBD target site (S-S). Upon Nek2A dissociation Plk1 can recognize the phosphoepitope via the PBD allowing the catalytic domain (CAT) to phosphorylate Nlp at a D-x-S site. (B) When Nek2A-K/R is overexpressed, it binds to Nlp and competitively inhibits the action of Nek2A. The PBD target site remains unphosphorylated thus Plk1 cannot recognize Nlp. (C) Alternatively Nek2A-K/R binds to Nlp but cannot be displaced due to lack of ATP hydrolysis. Nek2A-K/R binding to Nlp causes masking of Plk1 binding sites (▲).
Sequence analysis of Nlp identifies fifteen potential S-pS/pT motifs, ten of which are S-S and five S-T (Figure 7.3). The consensus phosphorylation motif for Nek2 is unknown but the sequence I-R-R-L-S-T-R-R-R is a good substrate (Fry et al., 1995). Likewise, NIMA has been shown to target F-x-x-S/T and the potential autophosphorylation site in the Nek2 T-loop is F-A-K-T (de Souza et al., 2000; Lu et al., 1994; Songyang et al., 1996). Alignment of the different S-S and S-T motifs in Nlp shows that a hydrophobic residue -3 to the potential phosphorylated serine/threonine is common. Therefore \( \phi \)-x-S-S/T (\( \phi \) represents a hydrophobic residue) may be a feature of the consensus sequence for the priming kinase. This is consistent with Nek2 recognizing this sequence. Nek2 can phosphorylate C-Napl \textit{in vitro} and \textit{in vivo} (Fry et al., 1998a; Mayor et al., 2002); work in our laboratory has specifically shown that the N-terminal (amino acids 1-400) and C-terminal domains (amino acids 2000-2442) of C-Napl can be phosphorylated by Nek2 \textit{in vitro}, but the C-terminal domain is a better substrate. Interestingly, C-Napl contains twenty \( \phi \)-x-x-S motifs and sixteen are present within the C-terminal domain. Therefore, \( \phi \)-x-x-S may be a component of the Nek2 consensus phosphorylation motif. \( \phi \)-x-S-S assumes that the first S is not necessary for Nek2 phosphorylation, but when present creates a potential PBD recognition site.

Plk1 may also phosphorylate the PBD recognition site itself and promote its own binding. Two potential sites exist for this model within Nlp (Figure 7.3). In support of this, Plk1 can phosphorylate Mklp2 at S528, and this site appears to be required for Plk1 to bind Mklp2 so that Plk1 can be transported to the midbody (Neef et al., 2003). However, the phosphorylation site is E-H-S-L which matches the consensus phosphorylation motif for Plk1 (D/E-x-S/T)(Kelm et al., 2002; Nakajima et al., 2003), but does not match a PBD binding site (H-pS, not S-pS)(Neef et al., 2003). Further identification of PBD binding sites will determine whether this exception is frequent or an isolated case.

During mitosis other kinases are active (such as the Aurora kinases and CDK1) making them additional candidates for priming Plk1 substrates. The phosphorylation consensus sequence for the Aurora kinases is unknown, but CDK1 is postulated to phosphorylate K/R-S-P-R/P-R/K/H (Songyang et al., 1994). Proline at +1 from the phosphorylated site can enhance Plk1 binding (two fold) but is not essential, whereas serine -1 from the phosphorylation site enhances Plk1 binding by over ten fold (Elia et al., 2003a). Whether
Figure 7.3 Sequence alignment of potential PBD binding sites in Hs-Nlp
(A) Putative PBD binding sites that contain S-pS. (B) Putative PBD binding sites that contain S-pT. Yellow boxes show the PBD binding sites, P, phosphorylation site, hydrophobic amino acids are red, polar amino acids are blue, charged amino acids are green. Circles show two Plk1 phosphorylation sites identified in Nlp which could create PBD binding sites resulting in positive feedback. Numbers denote the amino acid residue which would be phosphorylated to create the PBD binding site.
the binding coefficients are indicative of in vivo interactions has yet to be shown, but CDK1 is therefore a potential priming kinase for Plk1.

7.6 Concluding remarks

The work presented here has identified two further potential centrosomal substrates of the Nek2 kinase, rootletin and Nlp. The function of rootletin is unknown but it bears striking similarity to another Nek2 substrate C-Napl. C-Napl is involved in centriolar cohesion and phosphorylation by Nek2 is postulated to lead to centrosome disjunction at the G2/M transition (Fry et al., 1998b; Mayor et al., 2002). Nlp may function in the process of microtubule nucleation and anchorage to the interphase centrosome. Overexpression of Nek2A-K37R from a tetracycline inducible cell line leads to abnormal spindle formation. The abnormal spindles may arise due to a failure in centrosome disjunction (Faragher and Fry, 2003). However, bearing in mind the results in this thesis, we suggest that, in addition to centrosome disjunction, Nek2 may also function in bipolar spindle assembly by causing the displacement of Nlp from the centrosome. Nek2 phosphorylation of Nlp causes a protein involved in interphase microtubule organization to be removed from the centrosome during mitosis, thereby aiding the redistribution of microtubules and γ-TuRCs to form the bipolar spindle. The action of Nek2 upon Nlp may be a discrete event, but may also be a priming event for phosphorylation by Plk1. This would explain why Nek2A-K37R overexpression, microinjection of anti-Plk1 antibodies and overexpressing Nlp all cause the same mitotic phenotype.

We are still at the beginning of unraveling the regulations and multiple function of the Nek2 kinase at the centrosome. Much more work will be needed to elucidate the molecular mechanisms which govern Nek2 actions. However, the fact that upregulated Nek2 mRNA (de Vos et al., 2003; Wai et al., 2002) and protein (D. Hayward and A. Fry, personal communication) have been detected in various tumors means that these processes may well contribute to abnormal cell division in cancer.
CHAPTER 8

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Cloning Strategy:
Isolate $X$-Nlp$^{174-282}$ from the original yeast two hybrid clone as an $Ncol/Ncol$ fragment
Linearise pET32a using $Ncol$
Ligate and sequence

Sequencing primer
S.tag: CGG TTC TGG TTC TGG CCA ATA
Cloning Strategy:
Isolate X-Nlp\textsubscript{262-552} from the original yeast two hybrid clone as an EcoRI/XhoI fragment
Linearise pET32a using EcoRI and XhoI
Ligate and mini-prep

Sequencing primer
S.tag: CGG TTC TGG TTC TGG CCA ATA
Cloning Strategy:
PCR amplify X-Nlp$_{800-1397}$ from XL076 using XL076F and XL076R
Digest PCR product and pET32a using EcoRI and HindIII
Ligate and sequence

Cloning primers:
**XL076F:** CGC GGA ATT CGA GGA TAA CGG CTT ACT C
**XL076R:** CGC GAA GCT TTC AGA CTG CAA GAG AGG C

Sequencing primer
**S.tag:** CGG TTC TGG TTC TGG CCA ATA
Cloning Strategy:
Isolate X-Nek2A-K37R from pGAD-X-Nek2A-K37R as a *BamHI/SalI* fragment
Linearise pGBDU using *BamHI* and *SalI*
Ligate and mini-prep
Digest mini-prep DNA with *EcoRV* to produce DNA fragments of 5638 and 1741 bp
Cloning Strategy:
Isolate X-Nek2A-K37R from pGAD-X-Nek2A-K37R as a BamHI/SalI fragment
Linearise pGBDU using BamHI and SalI
Ligate and mini-prep
Digest mini-prep DNA with EcoRV to produce DNA fragments of 5638 and 1741 bp
Cloning Strategy:
PCR amplify X-Nlp{}_{262-552} from original yeast two hybrid clone using X-Nlp1F and X-Nlp1R
Digest PCR product and pMAL-c2 using XbaI and HindIII
Ligate and sequence

Cloning primers:
X-Nlp1F: GCG CTC TAG AAT GGA GCA ACT CAA TGA AAG C
X-Nlp1R: GCG CAA GCT TCT GCT GTT TAG TGA GCT CTG C

Sequencing primer
malE: TCG AGC TCG AAC AAC AAC AAC AAT
Cloning Strategy:
Site directed mutagenesis to introduce a *SalI* site at nucleotides 2488-2493 in XL076 and XL082 using Nlp-joinF and Nlp-joinR
Mini-prep both plasmids
Digest XL082 mutant mini-prep DNA with *SalI* and *EcoRI*
Digest XL076 mutant mini-prep DNA with *SalI* and *XhoI*
Isolate X-Nlp<sub>262-861</sub> from the mutant XL088 as an *EcoRI/SalI* fragment
Linearise mutant XL076 using *EcoRI* and *SalI*
Ligate and sequence
Site directed mutagenesis to remove the *SalI* site from pBS SK- X-Nlp<sub>262-1397 ΔSalI</sub> using Nlp-joinR and Nlp remutate
Mini-prep plasmid and digest with *EcoRI* and *SalI*
Sequence

Cloning primers:
**Nlp-join F**: AAA CAC AGG AAG GAG GTC GAC CAT TTA AAG AAA
**Nlp-join R**: CTC CTT CCT GTG TTT GTT AGC TTG ATC TTC
**Nlp remutate**: AAA CAC AGG AAG GAG GTG GAA CAT TTA AAG AAA

Sequencing primers:
**T7**: AAT ACG ACT CAC TAT AGG G
**T3**: ATT AAC CCT CAC TAA AGG G