CHARACTERISATION OF THE NEK6 AND NEK7 MITOTIC PROTEIN KINASES

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by

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Declaration

The accompanying thesis submitted for the degree of Doctor of Philosophy, entitled *"Characterisation of the Nek6 and Nek7 mitotic protein kinases"* is based on work conducted by the author in the Department of Biochemistry at the University of Leicester mainly during the period October 2004 and September 2007. All of 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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Characterisation of the Nek6 and Nek7 Mitotic Protein Kinases

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Summary

Entry into mitosis results in a dramatic reorganization of the cellular architecture to allow for segregation of duplicated DNA to nascent daughter cells. This complex biomechanical feat is orchestrated by members of the Cyclin-dependent, Aurora, Polo-like and NIMA-related kinase families. Four NIMA-related kinase proteins are implicated in regulation of mitotic progression, Nek2, Nek9, Nek6 and Nek7. Nek6 and Nek7 are closely related in sequence, encode little more than a catalytic domain and have been implicated in a mitotic NIMA-related kinase cascade downstream of Nek9. Functional data on Nek9 has implicated it in regulation of mitotic spindle architecture and thus Nek6 and Nek7 are also thought likely to function in spindle organization. However, functional data validating such a role is sparse and the roles of Nek6 and Nek7 remain poorly defined. In this thesis I set out to carry out a detailed functional analysis of Nek6 and Nek7, focusing on their proposed roles in mitotic progression. We show that expression of Nek6 and Nek7 mutants whose kinase activity is compromised results in mitotic arrest leading to apoptosis. However, whilst mutants with no activity cause an arrest at metaphase with fragile mitotic spindles, hypomorphic mutants, which retain intermediate levels of activity, result in an arrest in late mitosis. Nek6 and Nek7 interact with γ-tubulin, and interference with Nek6 or Nek7 disturbs the centrosomal localization of γ-tubulin. Nek6 localizes to the microtubules of the mitotic spindle and RNAi depletion of Nek6 leads to destabilization of the spindle microtubules. Thus, Nek6 and Nek7 may function during metaphase to regulate microtubule nucleation both from the spindle poles and from within the spindle itself. Furthermore, we identified the spindle components, Hsp70 and β-tubulin as Nek6 substrates, providing a possible mechanism by which Nek6 may achieve such a role. Finally, we also identified Cortactin A, a regulator of actin dynamics, as a Nek6 substrate. Cortactin A and Nek6 localize to the cleavage furrow of late mitotic cells raising the possibility that Nek6 may be involved in the regulation of membrane dynamics during cytokinesis. Together, the functional data suggests that Nek6 and Nek7 may regulate multiple events during mitosis and the identification of Nek6 substrates provides possible mechanisms by which they might achieve these functions.

Acknowledgements

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Contents

Abbreviations used in this Manuscript

γ-TURC	γ-tubulin ring complex
aa	amino acid
APS	ammonium persulphate
APC/C	Anaphase promoting complex/cyclosome
ATP	adenosine triphosphate
bp	base pairs
BCA	biciochoic acid
BSA	Bovine Serum Albumin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
C-	carboxy
Cdk	cyclin-dependent kinase
CPC	Chromosome passenger complex
DMSO	dimethylsulfoxide
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EST	expressed sequence tag
FACS	fluorescence activated chromosome sorter
FBS	foetal bovine serum
GFP	green fluorescent protein
HRP	horseradish peroxidase
INCEP	Inner centromere protein
IVT	*in vitro* translation
kDa	kilo Daltons
KESTREL	kinase substrate tracking and elucidation
K-fiber	kinetochore fiber
LB	Luria Bertani
MAP	microtubule-associated protein
MBP	myelin basic protein
mRNA	messenger ribonucleic acid
mg	milligrams
mM	millimolar
µM	micromolar
µg	microgram
MT	microtubule

MTOC	microtubule organizing centre
NBT	nitroblue tetrazolium
N-	amino
Nek	NIMA-related kinase
NIMA	Never in mitosis A
NP40	nonidet P-40
ng	nanogram
nM	nanomolar
OD	optical density
O/N	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCM	pericentriolar material
Plk	polo-like kinase
PMSF	phenylmethylsulphonly fluoride
$p70^{S6K}$	p70 ribosomal S6 kinase
rpm	revolutions per minute
RNA	ribonucleic acid
RCC1	regulator of chromatin condensation-1
SAC	Spindle assembly checkpoint
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIN	septation initiation network
SPB	spindle pole body
TACC	transforming acidic coiled coil
TEMED	N, N, N, N,-tetramethylethylenediamine
TBS	tris-buffered saline
WT	Wild-type
µl	microlitre
v/v	volume/volume
w/v	weight/volume

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Chapter 1

Introduction

1.1 The Eukaryotic Cell Cycle

1.1.1 Interphase

Somatic cells progress through four stages during the process of dividing to give rise to new daughter cells (Figure 1.1). Three of these four stages, G1, S and G2 are collectively known as interphase. The fourth is M-phase, which comprises the interlinked mitosis and cytokinesis. G (gap) 1 is a stage of growth following the completion of the previous cell division cycle. S (synthesis) phase, is characterized by DNA synthesis in which the genome is duplicated in preparation for cell division. There is then a second gap phase, G2, which allows further cell growth and synthesis of all the components necessary for cell division before the cell enters mitosis and divides.

1.1.2 Mitosis

Mitosis sees the segregation of duplicated DNA into two nascent daughter cells, such that each receives a complete chromosome complement. The process can be divided into 5 distinct stages (Figure 1.2) (Pines and Rieder, 2001). The first is prophase, when chromatin condenses into distinct chromosomes and centrosomes migrate apart and begin the process of nucleating the highly dynamic microtubules that ultimately will become the mitotic spindle. This is followed by prometaphase, in which the nuclear envelope breaks down, allowing the microtubules of the growing mitotic spindle to be captured by kinetochores. By metaphase the unstable monopolar microtubule-kinetochore attachments have become stable bipolar attachments as a result of the interaction of paired sister chromatids with microtubules emanating from opposite poles of the cell. These chromosomes then congress to the metaphase plate. Anaphase is triggered by the sudden loss of sister chromatid cohesion with the sister chromatids pulled towards opposite poles of the cells in anaphase A, before the poles themselves start to separate towards the cell cortex in anaphase B. Telophase sees the completion of chromosome segregation, the reassembly of the nuclear envelope and the decondensation of the daughter chromosomes.

The metaphase to anaphase transition and thus initiation of mitotic exit is brought about as a result of the Cdc20-dependent activation of the anaphase-promoting complex/cyclosome (APC/C). This multisubunit E3 ubiquitin ligase, when activated, ubiquitinates and thus targets a number of key cell cycle regulators for 26S proteasomal destruction and ultimately brings about the activation of the separase that removes the link between sister chromatids as well as destruction of cyclins (Fang *et al.*, 1998; Peters, 2002; Malmanche *et al.*, 2006). It is essential that equal and accurate chromosome segregation is accomplished during mitosis in order to avoid the aneuploidy and genetic instability characteristic of cancer cells. Thus, at metaphase,

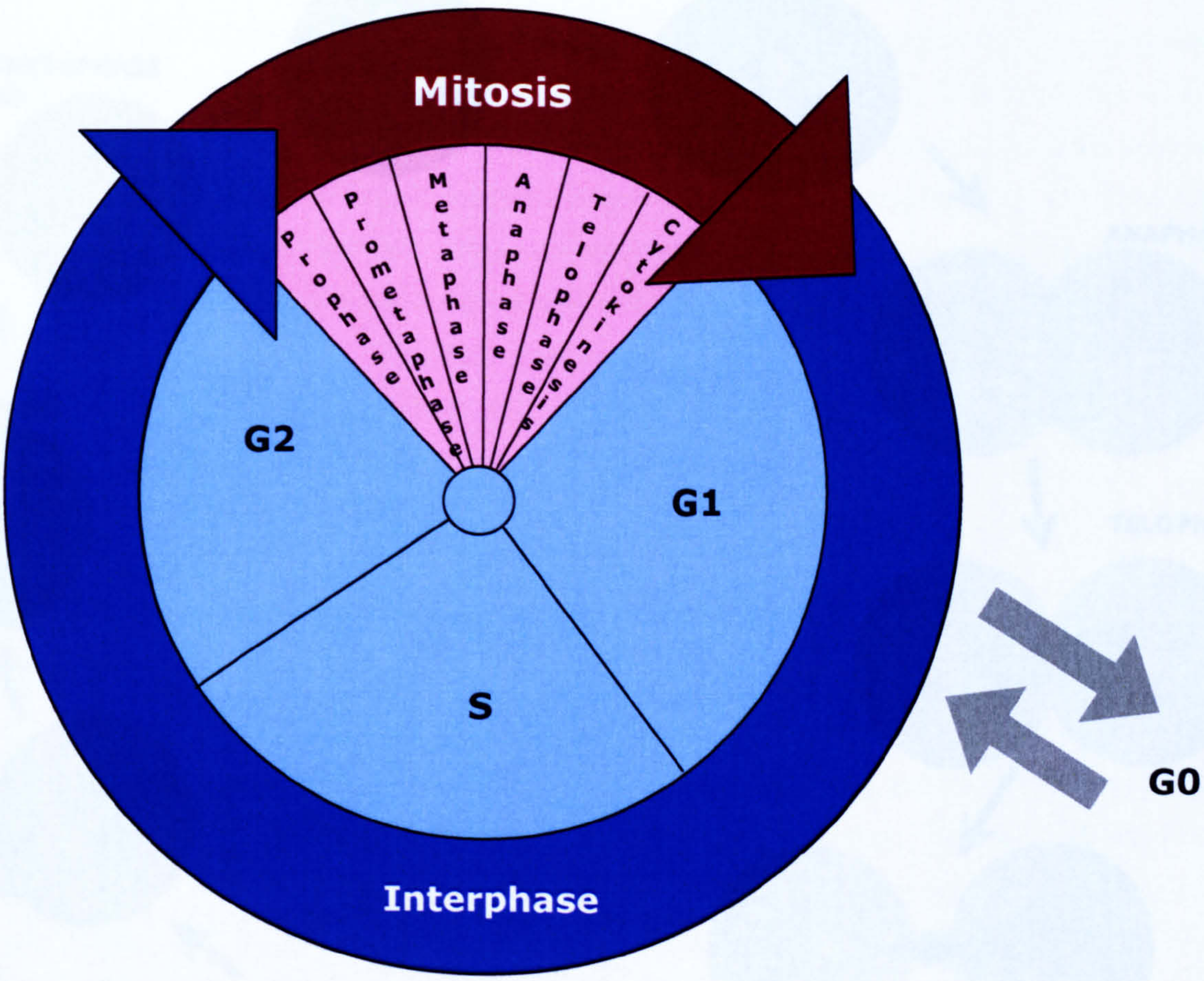

Figure 1.1 The eukaryotic cell cycle
The eukaryotic cell cycle comprises interphase which, itself can be divided into two gap phases (G1 and G2) and a period of DNA synthesis (S) and mitosis, which can be divided into the discrete phases of prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. Cells which receive no stimulus to grow enter quiescence (G0) and return to G1 phase following a restored growth signal.

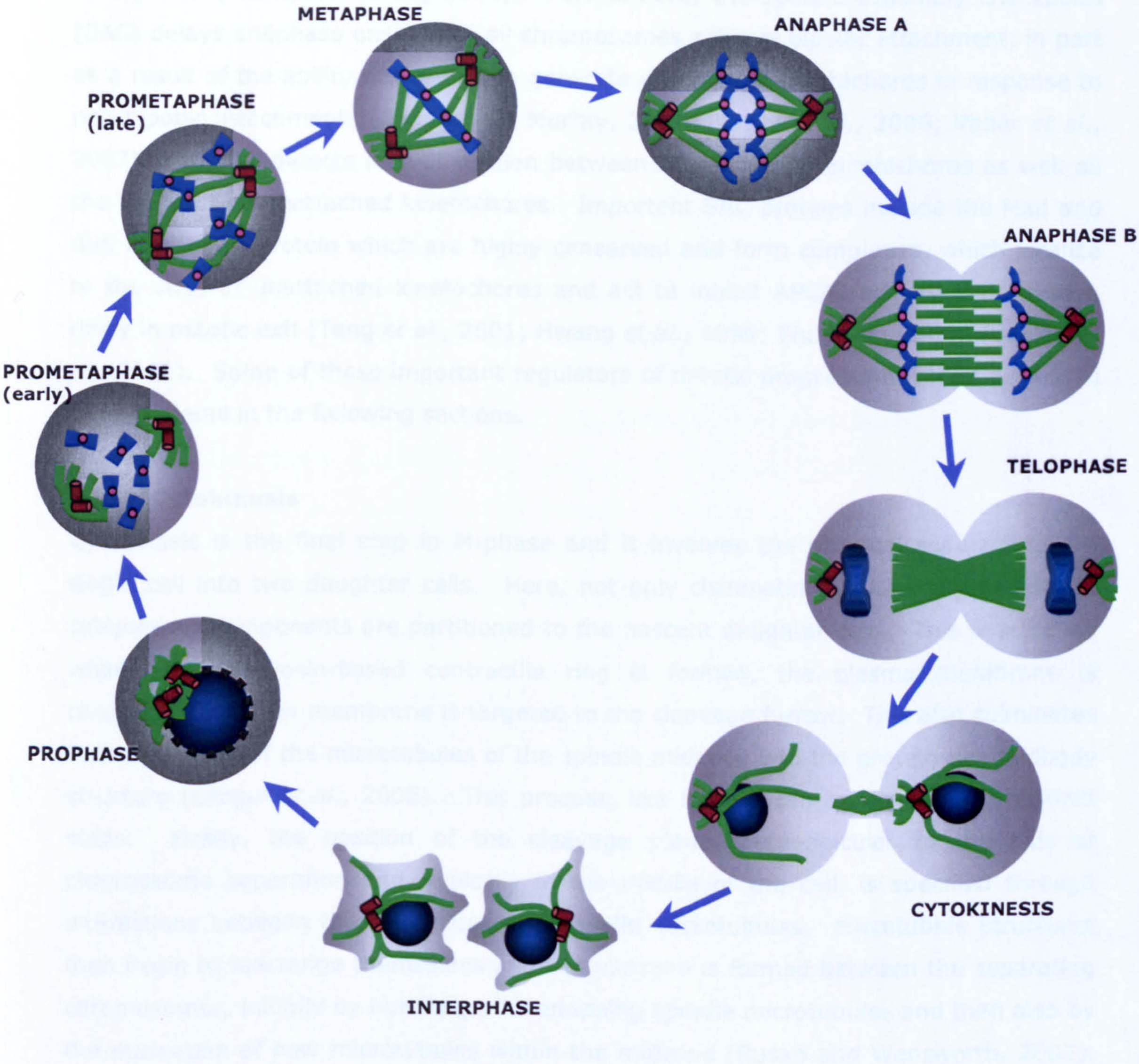

Figure 1.2 Mitosis
Schematic representation of the various stages of mitosis highlighting the dramatic reorganization that occurs within the cell cytoskeleton and chromatin that lead to the creation of the mitotic spindle and sister chromatid separation. Microtubules are shown in green, DNA in blue and centrioles in red. Kinetochores appear in pink.

mitotic exit can only be initiated when all chromosomes have properly aligned at the metaphase plate and achieved bipolar spindle attachment and a series of surveillance mechanisms exist to ensure that this is the case. The chromosomal passenger complex (CPC), for example, localizes to centromeres and destabilizes any non-bipolar spindle attachments to allow for new rounds of attachment (Biggins and Murray, 2001; Tanaka *et al.*, 2002; Lampson *et al.*, 2004). Furthermore, the spindle assembly checkpoint (SAC) delays anaphase onset until all chromosomes achieve bipolar attachment, in part as a result of the ability of the CPC to generate unattached kinetochores in response to non-bipolar attachments (Biggins and Murray, 2001; Pinsky *et al.*, 2006; Vader *et al.*, 2007). The SAC detects lack of tension between two opposing kinetochores as well as the presence of unattached kinetochores. Important SAC proteins include the Mad and Bub families of protein which are highly conserved and form complexes, which localize to the sites of unattached kinetochores and act to inhibit APC/C activity and cause a delay in mitotic exit (Tang *et al.*, 2001; Hwang *et al.*, 1998; Wu *et al.*, 2000; Sudakin *et al.*, 2001). Some of these important regulators of mitotic progression will be discussed in more detail in the following sections.

1.1.3 Cytokinesis

Cytokinesis is the final step in M-phase and it involves the physical separation of a single cell into two daughter cells. Here, not only chromatin but also organelles and cytoplasmic components are partitioned to the nascent daughter cells. This is achieved when an actomyosin-based contractile ring is formed, the plasma membrane is constricted and new membrane is targeted to the cleavage furrow. This also culminates in the bundling of the microtubules of the spindle midzone into the protein rich midbody structure (Otegui *et al.*, 2005). This process, like mitosis can be divided into distinct steps. Firstly, the position of the cleavage plane, perpendicular to the axis of chromosome separation and, typically in the middle of the cell, is specified through interactions between the cell cortex and spindle microtubules. Microtubule structures then begin to rearrange themselves and the midzone is formed between the separating chromosomes, initially by bundling of overlapping spindle microtubules and then also by the nucleation of new microtubules within the midzone (Rusan and Wadsworth, 2005). The contractile ring then assembles and begins to contract. This leads to cleavage furrow ingression which also requires an increase of plasma membrane surface area. Once the contractile ring has fully constricted and the cleavage furrow ingressed to create an intercellular bridge, the final step in cytokinesis, completion or abscission, occurs, in which the two daughter cells separate. Overall, cytokinesis, although considered distinct from mitosis, is intimately linked with mitotic structures; the mitotic spindle specifies the position of the cleavage furrow (Bringmann and Hyman, 2005;

Glotzer, 2004), while evidence is emerging that centrosomes are important in cleavage plane specification and possibly also abscission (Piel *et al.*, 2001; Gromley *et al.*, 2003).

The final step of cytokinesis, abscission, also requires careful coordination of actomyosin ring contraction with membrane delivery (Albertson *et al.*, 2005). Whilst it is not implicit in the traditional 'purse-string' model of actomyosin ring constriction that membrane addition and cleavage furrow ingression are temporally and spatially linked, it is now becoming clear that targeted membrane addition during cleavage furrow formation is a fundamental mechanism of cytokinesis (Glotzer, 2005; Albertson *et al.*, 2005). Both secretory and endocytic pathways have been implicated in the later stages of cytokinesis, with a number of proteins within endocytic, exocytic and recycling pathways having been identified as essential for cytokinesis (e.g. Skop *et al.*, 2001; Sisson *et al.*, 1999; Feng *et al.*, 2002; for comprehensive reviews see Albertson *et al.*, 2005; Glotze *et al.*, 2005; Konopka *et al.*, 2006); these include clathrin, syntaxin, endobrevin, Arf1 and dynamin II (Gerald *et al.*, 2001; Xu *et al.*, 2002; Burgess *et al.*, 1997; Conner and Wessel, 1999; Low *et al.*, 2003; Altan-Bonnet *et al.*, 2003; Feng *et al.*, 2002; Thompson *et al.*, 2002).

Cytokinesis was first described more than 100 years ago and it has been more than 20 years since the key role that actin and myosin have in cleavage, as well as the importance of spindle microtubules in specifying the cleavage plane, were first described. However, the mechanisms underlying cytokinesis remain among the major unsolved questions in cell biology (Eggert *et al.*, 2006). Recently, significant progress has been made in the identification of proteins that regulate cytokinesis due to genome-wide, systematic RNAi screens carried out in model organisms, specifically *C. elegans* and *Drosophila*. Such screens have identified a range of proteins including components of the actin cytoskeleton, microtubule associated proteins, vesicle transport proteins and regulatory proteins (for comprehensive review see Eggert *et al.*, 2006). Moreover, protein kinases that function in earlier stages of mitosis have been shown to also have roles in cytokinesis. For example, the Aurora B/survivin/INCEP/Borealin CPC has been shown to be involved in cytokinesis (Carmena and Earnshaw, 2003), as has Polo kinase (Carmen *et al.*, 1998; Descombes and Nigg, 1998; Seong *et al.*, 2002; Lindon and Pines, 2004; Lee *et al.*, 2005; Santamaria *et al.*, 2007). However, identifying proteins which are involved in both mitosis and cytokinesis is a challenge, as the cytokinesis defect that may arise through interfering with protein function may be masked by the earlier defect in mitosis (Eggert *et al.*, 2006).

1.2 Microtubules and the Mitotic Spindle

Equal and accurate distribution of chromosomes to daughter cells at mitosis is critical. The consequences of errors in this process are disastrous, resulting in non-viable, aneuploid cells, a key stage in tumorigenesis. Chromosome segregation at mitosis is brought about primarily by the action of the mitotic spindle, which is composed of two radial arrays of microtubules as well as hundreds of other microtubule-associated proteins (MAPs) that function together to orchestrate chromosome segregation (Gadde and Heald, 2004; O'Connell and Khodjakov, 2007)

1.2.1 Microtubules

Microtubules are highly dynamic, macromolecular structures with important roles in diverse cellular functions including chromosome segregation, structural support, cell migration, cell shape determination, intracellular transport and positioning of membrane bound organelles such as the Golgi Apparatus and mitochondria. To perform these functions, they can be arranged into different structures including the radial cytoplasmic network, the ciliary axoneme, centrioles and the mitotic spindle (Verhey and Gaertig, 2007). Structurally, microtubules are hollow cylindrical elements, 25 nm in diameter, composed of heterodimers of α- and β-tubulin arranged into 13 parallel protofilaments surrounding a central lumen. As the tubulin subunits polymerise end-to-end, with one α-tubulin subunit connected to the adjacent β-tubulin subunit, and protofilaments all bundled parallel to one another, the microtubule structure has an overall polarity with one end ringed by α-tubulin (the minus end) and the other by β-tubulin (the plus end). These singlet microtubules are the most ubiquitous form of the polymer although they can be fused laterally into doublets or triplets as is the case in cilia and centrioles, respectively.

Singlet microtubules are highly dynamic structures, which undergo rapid turnover known as dynamic instability in which the ends of microtubules undergo rapid switching between growth and shrinkage (Mitchison and Kirschner, 1984). Whilst both α- and β-tubulin subunits are GTP-bound, only the β-tubulin subunit is able to hydrolyse GTP to GDP. The hydrolysis and availability of GTP has a major impact upon microtubule dynamics: β-tubulin containing GTP supports microtubule growth whilst β-tubulin containing GDP supports microtubule shrinkage (Job *et al.*, 2003).

Within the cytoplasmic network, there are also subpopulations of microtubules which appear to be stabilized as a result of the acquisition of a variety of post-translational modifications. This is also true of the microtubules of the ciliary axoneme and centrioles

(Schultz and Kirschner, 1987). Such modifications are facilitated by microtubule-associated proteins, some of which either bind to the microtubule plus or minus ends, and which also function to facilitate assembly, speed up polymerization and otherwise stabilize microtubules (Dammermann *et al.*, 2003; Mimori-Kiyosue and Tsukita, 2003).

1.2.2 Microtubule nucleation at the centrosome

The majority of microtubules of the mitotic spindle (and indeed the interphase cytoskeleton) in an animal cell are nucleated by the centrosome. Centrosomes are small, perinuclear organelles, 1-2 μm in diameter. They are composed of a pair of short, linked barrels of nine triplet microtubules called centrioles, surrounded by a fibrous network of proteins called the pericentriolar material (PCM) (Figure 1.3) (Doxsey, 2001). The composition of the centrosome is still largely unknown although proteomic analysis is allowing rapid progress in identification of centrosomal proteins (Andersen *et al.*, 2003; Mack *et al.*, 2000; Andersen, 1999). However, typically, the proteins of the PCM are both structural and regulatory ranging in size from 13-461 kDa and a great many of them contain coiled-coils (Andersen *et al.*, 2003).

Centrioles form the central scaffold of the centrosome to which other components adhere. They are typically 200-400 nm in length and are composed of 9 blades of triplet microtubules comprising post-translationally modified α- and β-tubulin subunits (Lange and Gull, 1996; Bobinnec *et al.*, 1998). They replicate in a semi-conservative manner and thus each centriole pair contains one older, mother, centriole and a nascent, daughter centriole. These two are distinct from each other in that the mother possesses distal and sub-distal appendages, that are thought to facilitate microtubule anchoring and membrane attachment, as well as the antigens ε-tubulin and cenexin (Doxsey, 2001; Lange and Gull, 1996; Marshall, 2001; Bornens, 2002).

Throughout interphase, mother and daughter centrioles are tethered to one another via a putative intercentriolar linkage. Evidence from both electron micrographs and the purification of paired centrosomes from cultured cells suggests the presence of a physical link (Fry, 2002). The nature and identity of this link is still under investigation, however, a structural protein, C-Nap1, localizes to proximal ends of centrioles and has been shown to be involved in centriolar cohesion (Fry *et al.*, 1998). This protein is though to provide a platform for linkage via additional proteins including rootletin and Cep68 (Bahe et al., 2005; Mayor et al., 2002; Mayor et al., 2000).

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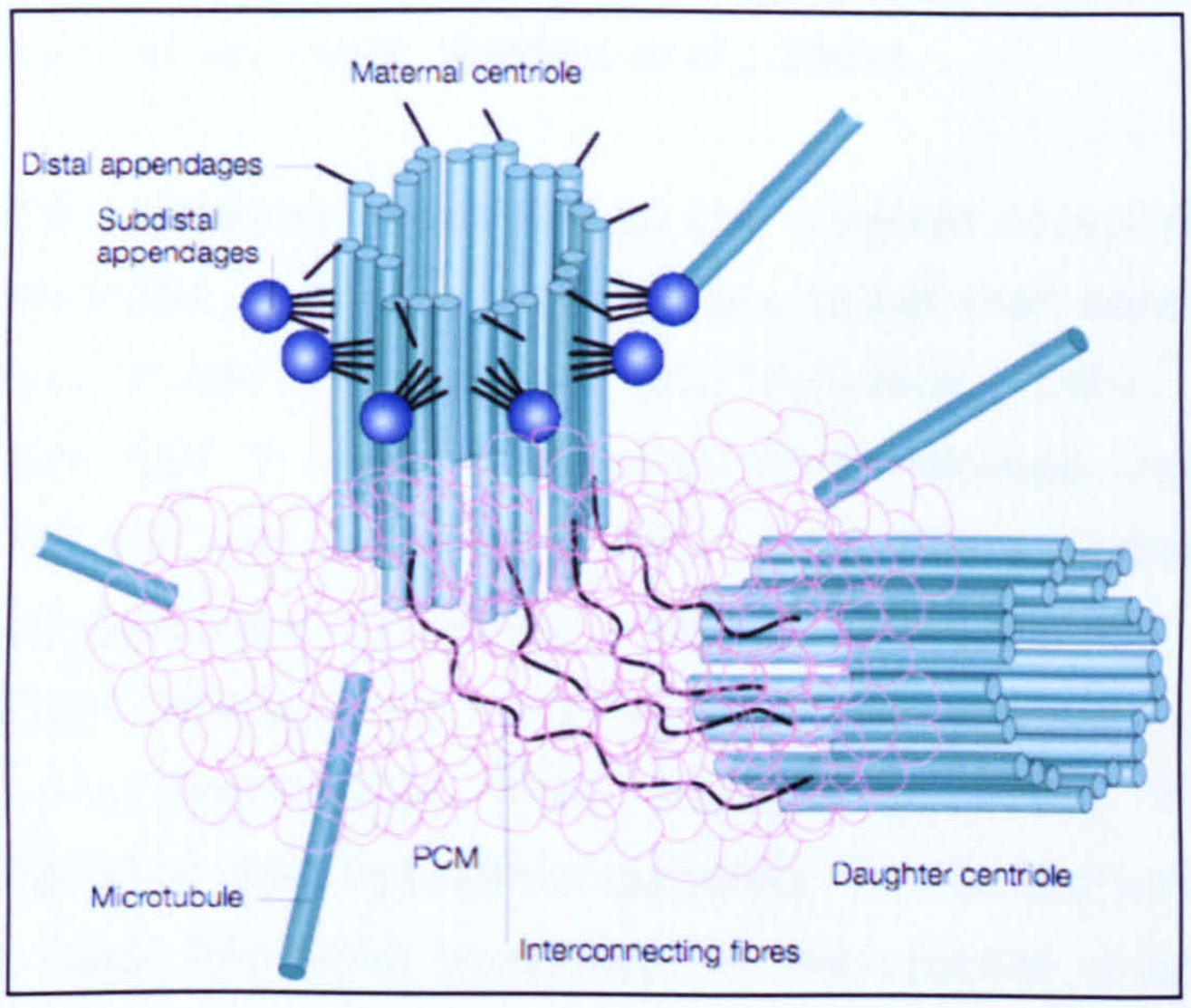

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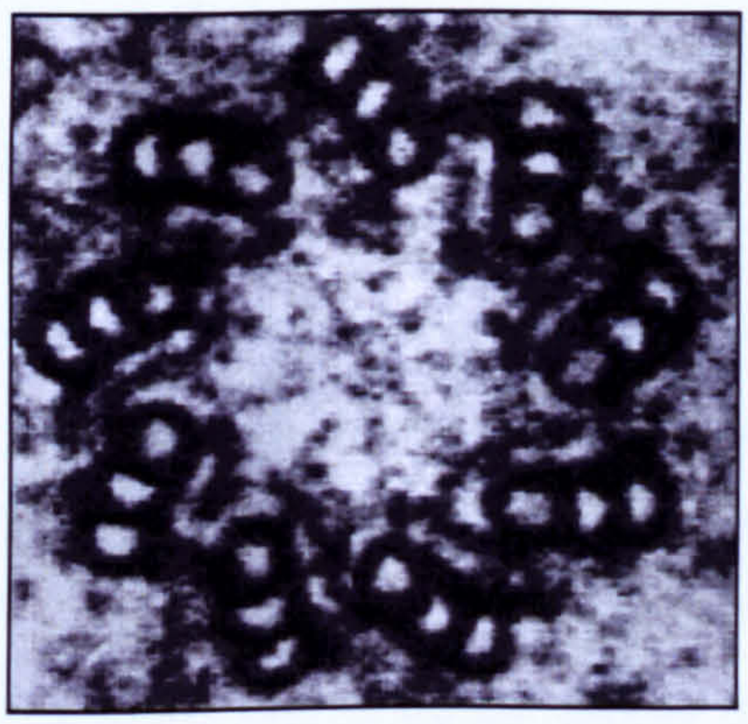

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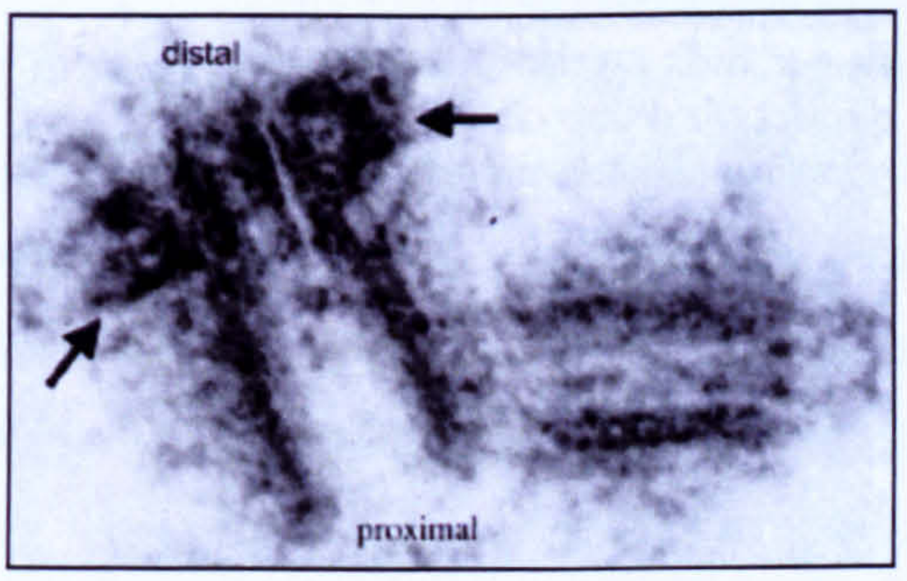

Figure 1.3 The mammalian centrosome

(**A**) The mammalian centrosome is composed of a mother and a daughter centriole each of which comprises a barrel-like structure of nine triplet microtubules connected by a linker comprising C-Nap1 and rootletin and possibly other, as yet undetermined, proteins. These are surrounded by the proteinaceous PCM which contains structural and signaling elements required for microtubule nucleation and anchoring. Taken from Doxsey *et al.* (2001). (**B**) Transmission electron micrograph of a cross section of the centriole showing the nine triplet microtubule barrel structure. Taken from http://www.users.rcn.com. (**C**) Electron micrograph of a duplicated centriole showing the distal appendages that mark the mother centriole (arrowheads). Taken from Delattre and Gonczy (2004).

Centriolar satellites, that contain components such as the protein PCM-1, also exist in close proximity to the PCM and are believed to be involved in recruitment of proteins, such as ninein, to the centrosomes via microtubules (Vorobjev and Chentsov, 1982; Dammermann and Merdes, 2002; Balczon *et al.*, 1994).

The PCM provides nucleation sites at which the initiation of microtubule assembly can occur at concentrations of tubulin heterodimers below that normally required for *de novo* microtubule assembly (Mitchison and Kirschner, 1984). This microtubule nucleation is mediated through a centrosomally-associated multi-protein complex, known as the γ-tubulin ring complex (γ-TuRC). Here, approximately 13 subunits of the conserved tubulin-superfamily member, γ-tubulin, associate with numerous γ-tubulin interacting proteins (GRIPS) to form a 25 nm open ring structure capable of nucleating microtubules *in vitro* (Moritz *et al.*, 1998; Zheng *et al.*, 1995). The open ring shape, centrosomal localization and nucleation capability of γ-TuRCs has led to them being considered the major functional component of microtubule assembly at microtubule organizing centers (MTOCs) (Cuschieri *et al.*, 2007). The γTuRC, though present in the cytoplasm, predominantly nucleates microtubules at the centrosome (Weise and Zheng, 2000).

There are two possible mechanisms by which γ-TuRCs nucleate microtubules, the template and profilament models, although the bulk of evidence favours the template model (Mortiz *et al.*, 2000; Keating and Borisy, 2000; Weise and Zheng, 2000; Job *et al.*, 2003). In the 'template' model, the 25 nm diameter γ-TuRC ring structure acts as a template to mimic a microtubule end and thus provide a seed onto which α/β-tubulin heterodimers can assemble (Zheng *et al.*, 1995). The alternative 'profilament' model suggests that tubulin dimers bind to the γ-TuRC, creating a sheet which then grows and curls to form a microtubule whilst rotating around the γ-TuRC (Erikson and Stoffler, 1996).

Centrioles also serve as microtubule nucleating centres in non-dividing, ciliated cells. Here, centrioles migrate to the apical cell surface and acquire accessory structures and in this context, they are known as basal bodies. Once at the cell surface the basal body can begin nucleating the ciliary axoneme. In multiciliated cells, multiple rounds of centriole duplication occur to provide to numerous basal bodies, although the primary cilium is nucleated from the basal body formed from the mother centriole (Dawe *et al.*, 2006). As centrioles/basal bodies form the mitotic spindle poles, in ciliated cells, cilia

disassembly is an important first step in commitment to mitosis (Quarmby and Parker, 2005).

The centrosome clearly has a well documented role as a microtubule organizing centre, however, beyond this canonical role, the centrosome is emerging as a key regulator of an increasing number of mitotic processes including the G2/M transition, mitotic exit and cytokinesis as well as spindle assembly and positioning (Nigg, 2004; Doxsey, *et al.*, 2005). As discussed, the centrosome nucleates and anchors not only the K-fibers which radiate towards the centre of the cell and capture kinetochores of sister chromatids but also interpolar microtubules, which overlap at the central spindle and are important in cytokinesis, and astral microtubules which extend away from the spindle towards the cell cortex and are emerging as important regulators of spindle and cleavage plane positioning. Moreover, the centrosome itself may be important for cytokinesis: the mother centriole appears to move into the midbody following anaphase and this is believed to be in order to regulate the release of microtubules from this structure to allow abscission (Piel *et al.*, 2000; Gromley *et al.*, 2003).

1.2.3 The mitotic spindle

The segregation of duplicated DNA to daughter cells at mitosis is a complex biomechanical process achieved in large part through the action of the bipolar microtubule-based scaffold known as the mitotic spindle (McIntosh *et al.*, 2002; Walczak and Heald, 2008).

The mitotic spindle is essentially composed of centrosomes, chromosomes and microtubules (O'Connell and Khodjakov, 2007). By prophase, a series of events have occurred which allow spindle formation and metaphase chromosome alignment: centrosomes and chromosomes have duplicated, centrosomes have separated, chromosomes have condensed and the nuclear envelope has broken down (Gadde and Heald, 2004). There is also an increased frequency of microtubule catastrophes and a decrease in microtubule growth, which contributes to the dismantling of the interphase microtubule array and the formation of the bipolar spindle (Belmont *et al.*, 1990; Rusan *et al.*, 2001).

The bipolar mitotic spindle is fully formed at metaphase when the centrosomes and kinetochores are linked via the specialized microtubules called kinetochore or K-fibers. Two interrelated mechanisms are primarily responsible for the formation of these connections (O'Connell and Khodjakov, 2007). Firstly, the capture of kinetochores by

dynamic astral microtubules nucleated by MTOCs, the so-called search-and-capture mechanism (Kirschner and Mitchison, 1986), and secondly, the chromatin mediated nucleation of microtubules driven by an RCC1-mediated, Ran-GTP gradient present around mitotic chromosomes (Heald *et al.*, 1996; Karsenti *et al.*, 1984; Khodjakov *et al.*, 2000; Basto *et al.*, 2006; Carazo-Salas *et al.*, 2001; Carazo-Salas *et al.*, 1999). This second process is thought to act in two ways to promote spindle formation (O'Connell and Khodjakov, 2007); firstly, by directly stimulating microtubule nucleation around chromatin (Heald *et al.*, 1996; Karsenti and Vernos, 2001) and, secondly, by creating a local concentration of microtubule stabilizing factors around the chromosomes to promote the capture of astral microtubules (Bastiaens *et al.*, 2006). Ran-GTP serves to release spindle assembly factors such as TPX2 and NuMA from the importin-α inhibitor thus promoting spindle assembly (Gruss *et al.*, 2001; Di Fiore *et al.*, 2003).

Two other populations of microtubules also contribute to the bipolar mitotic spindle structure in addition to K-fibers. Firstly, interpolar microtubules overlap in the spindle midzone and, secondly, astral microtubules interact with the cell cortex (Gadde and Heald, 2004). Thus, in the mitotic spindle, microtubule minus ends are tethered at the spindle poles (centrosomes) and microtubules extend to the centre of the cell to either attach to kinetochores (K-fibers), or overlap and form antiparallel arrays (interpolar microtubules) or they extend away from the spindle towards the cell cortex (astral microtubules) (Figure 1.4A) (Gadde and Heald, 2004).

Upon satisfaction of the SAC, the dynamic nature of microtubules is exploited to effect segregation of sister chromatids to opposite poles of the cell. Moreover, as chromosomes move away from each other, interpolar microtubules in the spindle midzone bundle into the central spindle (Glotzer, 2005) and this results in the initiation of the cleavage furrow (Wheatley and Whang, 1996; Durcan *et al.*, 2008). As furrow ingression occurs, these central spindle microtubules become highly compacted into the transient midbody which connects the two daughter cells until abscission. Hence, the midbody is comprised of the overlapping, compacted and remarkably stable microtubules, surrounded by an electron dense structure, known as the midbody matrix (Mullins and Biesele, 1977) which is thought to contain microtubule stabilizing elements (Figure 1.4B) (Mullins and McIntosh, 1982; Durcan *et al.*, 2008).

Although the centrosome, when present, is the major site of microtubule nucleation and anchorage, alone it is insufficient for establishment of a stable bipolar spindle. Indeed, the creation and maintenance of this highly organized, bipolar array during mitosis requires numerous microtubule-associated motor and non-motor proteins (Manning and

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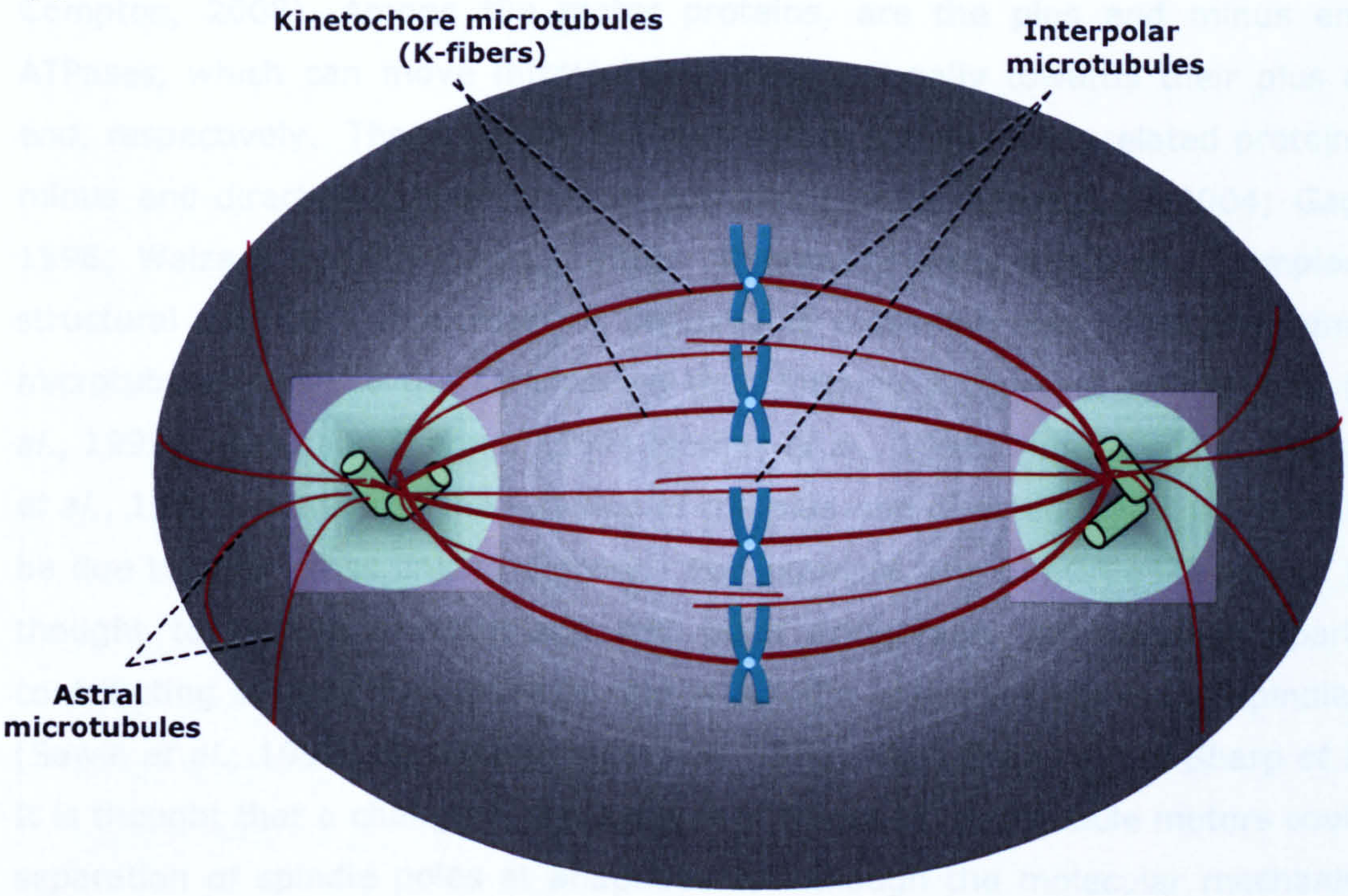

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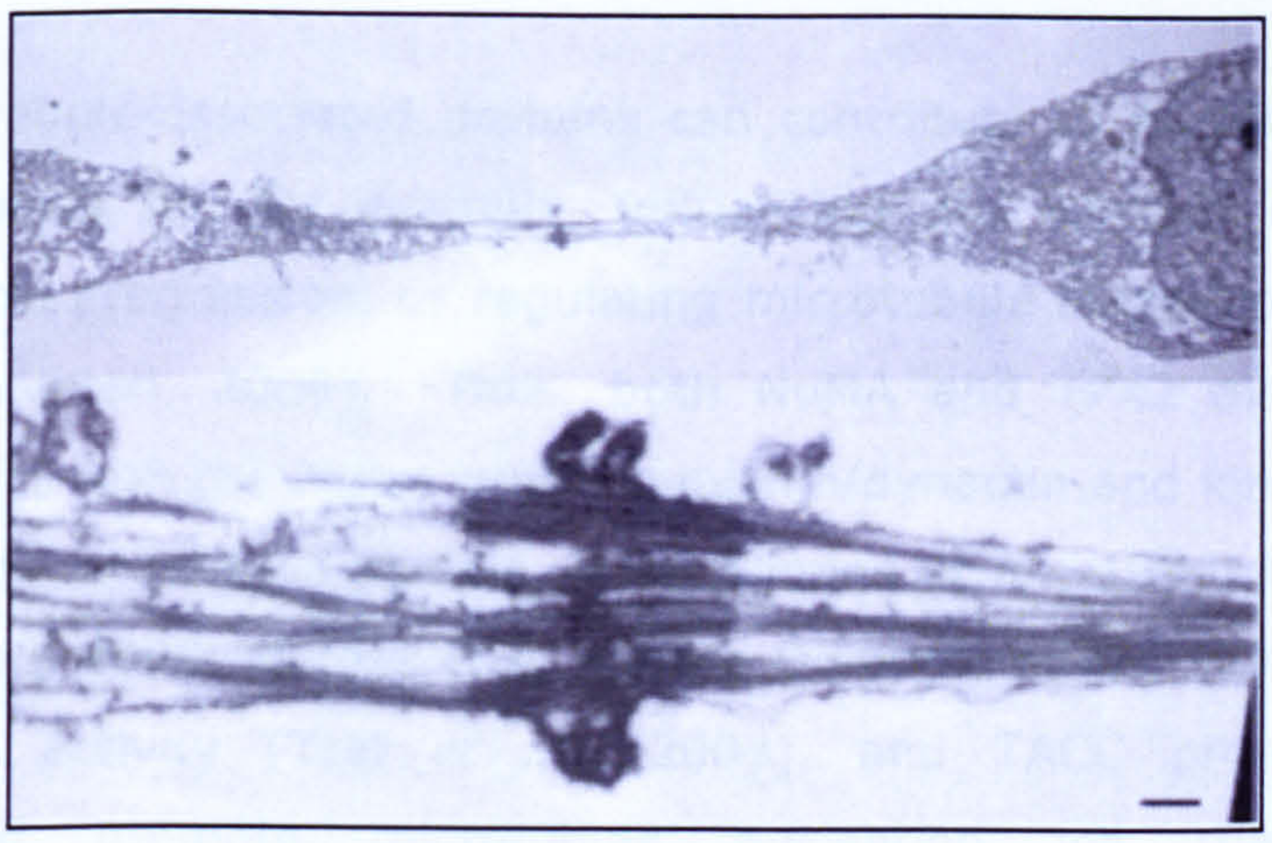


Figure 1.4 Microtubules and the mitotic spindle at metaphase and cytokinesis
(**A**) Schematic representation of the various microtubule populations present in the mitotic spindle. Microtubules are tethered at their minus ends at the centrosomes. Kinetochore microtubules, or K-fibers, extend to the centre of the cell to capture the kinetochores of sister chromatids. Interpolar microtubules overlap at the central spindle to form antiparallel microtubule arrays that eventually will become compacted into the midbody. Astral microtubules extend away from the spindle to make contact with the cell cortex and thus contribute to spindle positioning and cleavage plane specification. Adapted from Gadde and Heald (2004). (**B**) Electron micrograph of the bundled microtubules in the spindle midbody of a dividing HeLa cell. Lower panel, higher resolution image of the same bridge. Taken from Eggert *et al.* (2006). During mitotic exit the midzone is initially formed by bundling of interpolar microtubules, although it eventually becomes self-organizing and new microtubules are thought to be nucleated within the midzone. The midzone microtubules then become highly stabilized and compacted into the midbody structure and these microtubules are critical to keeping separated genomes apart and in the final step of cytokinesis, abscission.

Compton, 2008). Among the motor proteins, are the plus and minus end-directed ATPases, which can move microtubules unidirectionally towards their plus and minus end, respectively. These include the plus end directed kinesin-related proteins, and the minus end-directed dyenin/dynactin complex (Gadde and Heald, 2004; Gaglio *et al.*, 1996; Walzack and Mitchison, 1996). Dyenin/dynactin exists in a complex with the structural protein, NuMA, which is believed to cross-link and tether the minus ends of microtubules close to the centrosome thus forming a compact spindle pole (Dionne *et al.*, 1999; Gaglio and Dionne, 1997; Merdes *et al.*, 1996; Merdes *et al.*, 2000; Quintyne *et al.*, 1999). Similarly, the function of the plus-end directed kinesins is also thought to be due to their cross linking activity. For example, the BimC/Eg5-family of kinesins is thought to bundle microtubules and push antiparallel microtubules apart, thereby contributing to their fundamental role in spindle pole separation and spindle bipolarity (Sawin *et al.*, 1992; Hagan and Yanagida, 1990; Hoyt *et al.*, 1993; Sharp *et al.*, 1999). It is thought that a change in the balance of forces of microtubule motors could result in separation of spindle poles at anaphase B, although the molecular mechanism behind this is not understood (Gadde and Heald, 2004).

Non-motor microtubule-associated proteins can contribute to microtubule organization in a variety of ways by, for example, influencing the function of motor proteins, regulating cell cycle progression, or regulating microtubule nucleation and organization (Manning and Compton, 2008). Thus, both NuMA and TPX2 bind to microtubules directly and interact with the motor proteins dyenin/dynactin and kinesins, respectively, to ensure formation of focused spindle poles (Dionne *et al.*, 1999; Gaglio and Dionne, 1997; Merdes *et al.*, 1996; Merdes *et al.*, 2000; Quintyne *et al.*, 1999). TPX2 activates Aurora A kinase activity (Tsai *et al.*, 2003), and TACC proteins directly bind microtubules and promote microtubule nucleation via the recruitment of Msps/XMAP215/TOG protein to the centrosome (Gergely *et al.*, 2000; Peset *et al.*, 2005).

Importantly, the nucleation, organization and stabilization of mitotic spindles is regulated largely by post-translational modifications of microtubule-, as well as centrosome-associated, proteins. Foremost among these modifications is protein phosphorylation and dephosphorylation and hence, I will now go on to discuss the enzymes responsible for these events.

1.3 Mitotic Protein Kinases

Temporal and spatial regulation of the mitotic machinery are key to ensuring the accuracy and fidelity of mitosis. Indeed, the importance of the regulation of this process is highlighted by the fact that both the mechanisms of regulation and the regulators involved are highly conserved throughout evolution (Nigg, 2001).

Protein phosphorylation is perhaps the key mechanism of mitotic regulation, as it is for many critical cellular processes. The addition or removal of a phosphate group on a protein can induce several changes in its properties including charge, activity and conformation, which in turn, regulate enzymatic activity, polymerisation and complex formation. In particular serine/threonine protein kinases have been shown to be involved in regulation of mitotic entry and progression, operating in complex regulatory cascades involving many proteins to activate and inactivate mitotic regulatory molecules at critical times (Nigg, 2001).

A number of different protein kinase families are involved in modulation of mitotic progression, arguably the best known of which are the cyclin-dependent protein kinases. Other important protein kinase families include the Aurora kinases, the Polo-like kinases and the NIMA-related kinases (summarized in Figure 1.5). Each of these will be discussed in detail below.

1.3.1 Cyclin-dependent kinases

The canonical cell cycle regulators are the cyclin-dependent kinases (Cdks) and their attendant cyclins (Norbury and Nurse, 1992). The cyclin-dependent protein kinases are a family of heterodimeric protein kinases which require, in addition to activatory phosphorylation, to be associated with their appropriate cyclin partner in order to become activated and coordinate key events/processes in the cell cycle. A number of different cyclin partners are accumulated and degraded at specific stages in the cell cycle. In this way the same molecule can act as the key trigger for a number of distinct events occurring at separate phases of the cell cycle. For example, the major mammalian cell cycle regulator Cdk1 functions in association with both A- and B-type cyclins (Nigg, 2001). The other protein kinase families important in mitotic regulation often, although not always, act downstream of the cyclin-dependent kinases in protein phosphorylation cascades responsible for mitotic control (O'Connell *et al.*, 2002).

To date 12 loci encoding Cdks have been identified in the mammalian genome, although only Cdk1, 2, 4 and 6 have been directly implicated in regulation of cell cycle progression and, of these, only Cdk1 is generally considered to be a mitotic kinase

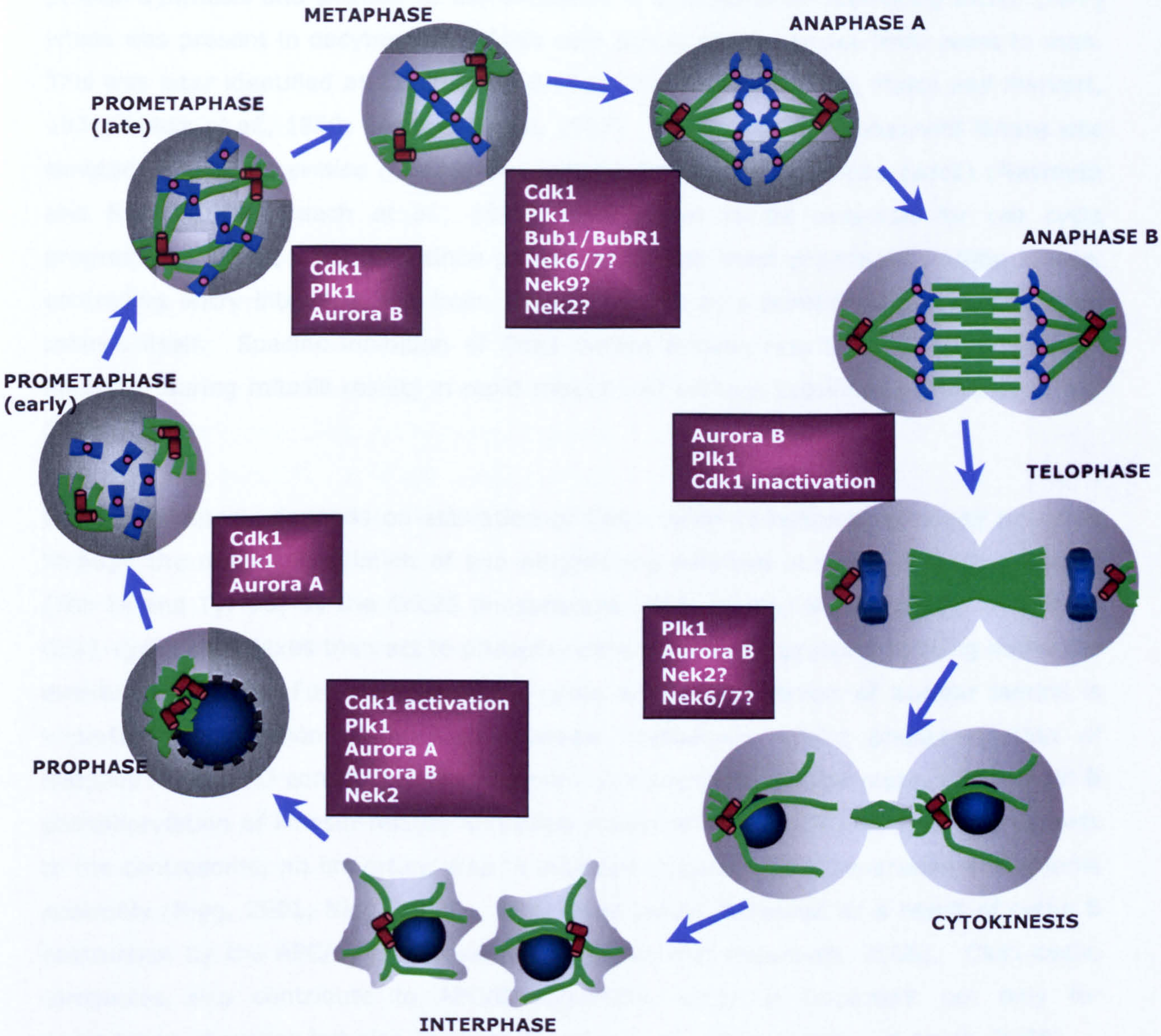


Figure 1.5 Protein kinases in control of mitosis
Scehmatic diagram of the different stages of mitosis indicating the contributions that the Cyclin-dependent, Aurora, Polo-like and NIMA-related kinases make to each stage. Activation of Cdk1-cyclin B is absolutely required for entry into mitosis and its inactivation is absolutely required for mitotic exit. Adapted from Nigg, 2001.

(Malumbres and Barbacid, 2001; Malumbres and Barbacid, 2007). Moreover it is the only Cdk absolutely required for cell cycle progression (Tetsu *et al.*, 2003; Ortega *et al.*, 2003). Early experiments showed that injection of cytoplasm from maturing oocytes or eggs into immature oocytes resulted in their maturation even in the absence of new protein synthesis and pointed to the existence of a maturation-promoting factor (MPF) which was present in oocytes and mitotic cells across many species from yeast to man. This was later identified as Cdk1-cyclin B (Rao and Johnson, 1970; Masui and Markert, 1971; Lohka *et al.*, 1988; Lee and Nurse, 1987). In parallel, this conserved kinase was isolated from *S. cerevisiae* (Cdc28) and subsequently from *S. pombe* (cdc2) (Nasmyth and Reed, 1980; Beech *et al.*, 1982), and shown to be essential for cell cycle progression. Thus, Cdk1 has since proven to be the most prominent mitotic kinase, controlling entry into, and exit from, mitosis as well as a number of processes during mitosis itself. Specific inhibition of Cdk1 before mitosis results in G2 arrest, whilst inhibition during mitosis results in rapid mitotic exit without cytokinesis (Vassilev *et al.*, 2006).

Entry into mitosis depends on activation of Cdk1-cyclin complexes, achieved primarily through the dephosphorylation of two neighboring residues in the ATP-binding pocket (Thr-14 and Tyr-15) by the Cdc25 phosphatase (Norbury and Nurse, 1992). Activated Cdk1-cyclin complexes then act to phosphorylate key mitotic proteins to bring about the events of mitosis. For example, Cdk1-cyclin B phosphorylation of nuclear lamins is important in initiation of nuclear envelope breakdown, whilst phosphorylation of histones H1 and H3 contributes to chromatin condensation. Furthermore, Cdk1-cyclin B phosphorylation of kinesin-related and other motor proteins facilitates their recruitment to the centrosome, an important step in initiation of centrosome separation and spindle assembly (Nigg, 2001, Nigg, 1995). Cdk1 inactivation, achieved as a result of cyclin B destruction by the APC/C, is necessary for mitotic exit (Nasmyth, 2005). Ckd1-cyclin complexes also contribute to APC/C activation, which is important not only for degradation of cyclins but also securins (Kramer *et al.*, 2000; Rahal and Amon, 2008).

Also important in terms of mitotic progression, Cdk2 has been proposed to function as a master regulator of centrosome duplication (Duensing *et al.*, 2006). To ensure successful mitosis and preserve the fidelity of chromosome segregation, each cell must possess only two centrosomes at the onset of mitosis. Each of which forms one pole of the bipolar spindle. Thus each daughter cell inherits a single centrosome which must then duplicate precisely once prior to mitosis in synchrony with the cell division cycle (Doxsey, 2002; Hinchcliffe and Sluder, 2001). Centriole duplication, and indeed DNA replication, is triggered by the Cdk2-cyclin E/A complex (Mussman et al., 2000).

Inhibition of Cdk2 in various model systems, including *Xenopus* egg extracts and cultured mammalian cells, blocks the initiation of centrosome duplication (Lacey *et al.*, 1999; Matsumoto *et al.*, 1999, Meraldi *et al.*, 1999). Active Cdk2-cyclin A/E complexes are thought to phosphorylate centrosomal proteins including nucleophosmin/B23, Mps1 and CP110, thus initiating centrosomal duplication (Okuda *et al.*, 2000; Fisk and Winey, 2001; Chen *et al.*, 2002).

1.3.2 Polo-like kinases

Polo-like kinases (Plks) represent a highly conserved family of serine/threonine kinases implicated in cell division and centrosome regulation (Barr et al., 2004). Polo, the founding member of the Plk family, was identified in *Drosophila melanogaster* as the product of the *polo* gene. Mutation of the *polo* gene resulted in abnormal mitosis, specifically, abnormal spindle formation and abnormal chromosome congression on the metaphase plate resulting in aneuploidy (Sunkel and Glover, 1998; Llamazares *et al.*, 1991). Subsequently, Plks have been identified in many eukaryotes (Barr *et al.*, 2004), including Plo1 and Cdc5 in fission and budding yeast, respectively, Plc1-3 in *C. elegans*, Plx1-3 in *Xenopus laevis* and Plk1-4 in mammals (Kitada *et al.*, 1993; Ohkura et al., 1995; Chase *et al.*, 2000a; Chase *et al.*, 2000b; Descombes and Nigg, 1998; Kumagai and Dunphy, 1996; Duncan *et al.*, 2001; Donohue et al., 1995; Golsteyn et al., 1994; Hamanaka et al., 1994; Simmons et al., 1992; Ma *et al.*, 2003; Kauselmann *et al.*, 1999; Fode *et al.*, 1994). Thus, the number of Plks present in the genome has increased with increasing evolutionary complexity.

Designation as a Plk is based on structural similarity and all Plks possess a canonical amino-terminal serine/threonine kinase domain and a carboxy-terminal regulatory domain containing highly conserved polo box motifs (Figure 1.6A). The polo box is structurally important for targeting polo-like kinases to their substrates by facilitating binding to phosphoepitopes (Lee et al., 1998; Cheng *et al.*, 2003).

The abnormal spindle phenotype seen in *Drosophila polo* mutants pointed to a role for Polo in centrosome maturation and separation (Llamazares *et al.*, 1991; Snukel and Glover, 1988). However, Polo-like kinases have since been demonstrated to regulate many stages of mitotic progression including activation of Cdc25 at the G2/M transition, bipolar spindle formation, activation of the APC/C and thus the metaphase/anaphase transition and cytokinesis (Qian *et al.*, 1998; Qian *et al.*, 1999; Lee *et al.*, 1998; Glover *et al.*, 1996; Nigg, 1998; Erikson *et al.*, 2004). Whilst all polo-like kinases influence aspects of mitosis, they do not perform identical functions in all species, although mammalian Plk1 is believed to carry out the majority of the functions attributed to Polo,

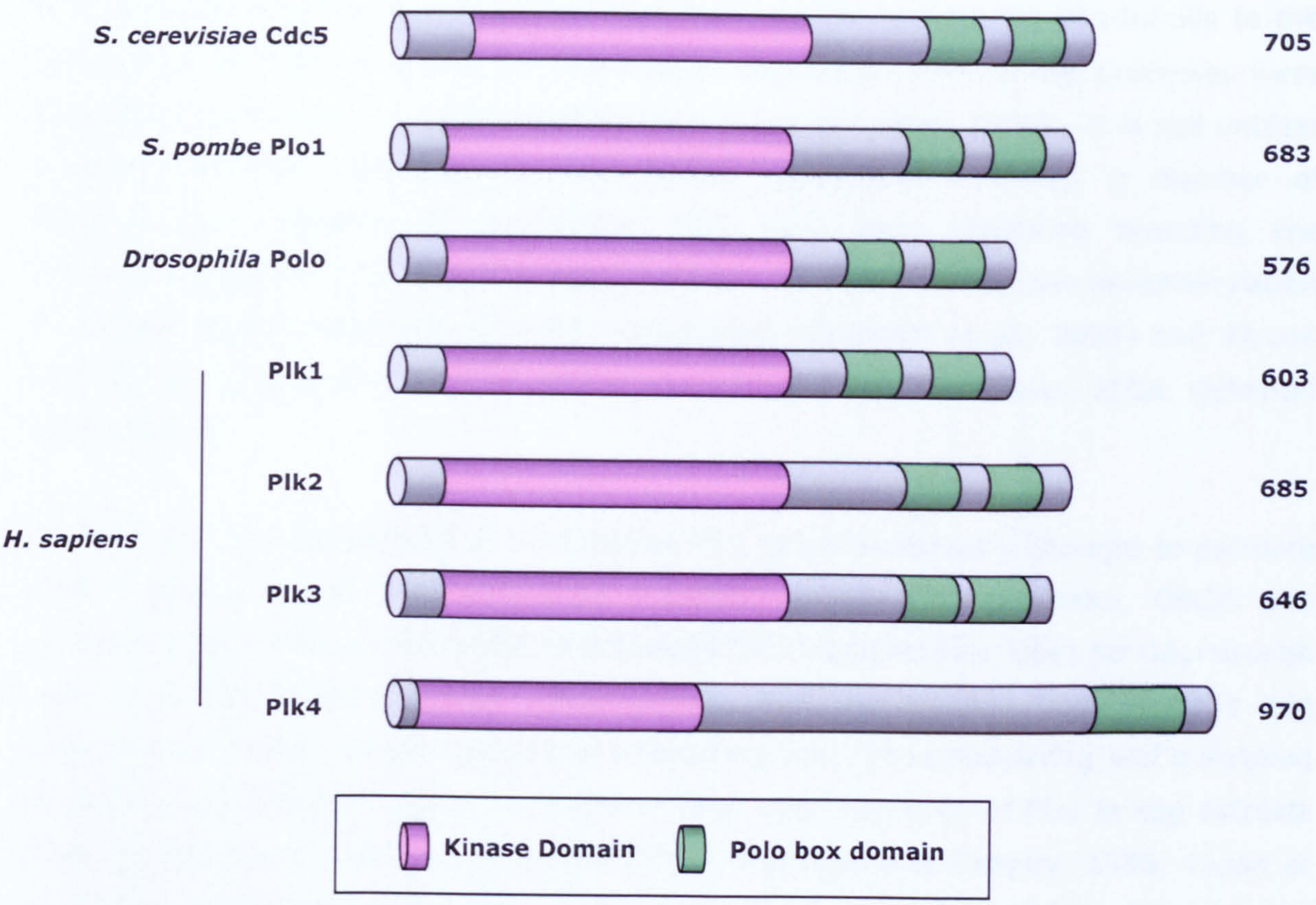
Cdc5 and Plo1 (Barr *et al.*, 2004). Indeed, overexpression of mammalian Plk1 was able to rescue the lethal phenotype in *cdc5* loss of function mutants in budding yeast (Ouyang *et al.*, 1997).

Plk cell cycle abundance and localization are not identical across species. The abundance of Polo, Plo1 and Plx1 is constant throughout the cell cycle, although their catalytic activity is restricted to mitosis (Descombes and Nigg, 1998; Llamazares *et al.*, 1991; Mulvihill and Hyams, 2002; Tanaka *et al.*, 2001). On the other hand, Cdc5 and Plk1 abundance, as well as activity, are maximal in mitosis (Cheng *et al.*, 1998; Golsteyn *et al.*, 1995; Golsteyn *et al.*, 1994; Shirayama *et al.*, 1998; Lee *et al.*, 1995). A striking feature of Plk1, Plx1 and Polo is their dynamic localization. Early in mitosis they associate with the centrosome and kinetochores before being redistributed to the spindle midzone and central spindle at anaphase and telophase and the midbody in cytokinesis (Figure 1.6B) (Goldsteyn *et al.*, 1995; Logarinho and Sunkel, 1998; Lee *et al.*, 1995; Moutinho-Santos *et al.*, 1999; Arnaud *et al.*, 1998). Plo1 and Cdc5 similarly localize to the spindle pole body although there is some variation in the timing of this localization; Cdc5 localizes to SPBs from G1 until late in mitosis whilst the SPB localization of Plo1 is restricted to the window in which Cdc2 is active (Shirayama *et al.*, 1998; Song *et al.*, 2000; Mulvihill *et al.*, 1999). Moreover, yeast Plks localize to the bud neck in late mitosis but there is no apparent localization to the spindle midzone or central spindle (Bahler *et al.*, 1998; Mulvihill *et al.*, 1999; Sakahaisri *et al.*, 2004). The mitotic subcellular localization of Plks is dependent upon the polo-box domain and essential for Plk activity (Song *et al.*, 2000; Seong *et al.*, 2002; Jang *et al.*, 2000; Lee *et al.*, 1998). Thus, disruption of the normal cell cycle localization of Cdc5 or Plk1 by mutation of residues with the polo-box domain, whilst having no effect on kinase activity, was sufficient to disrupt the mitotic functions of each Plk (Lee *et al.*, 1998; Song *et al.*, 2000; Seong *et al.*, 2002).

1.3.2.1 Polo-like kinases at the centrosome

The cell cycle-dependent regulation of Polo kinases is consistent with their roles in regulating multiple mitotic events. The abnormal mitotic spindle phenotypes observed in *Drosophila* mutants provided the first evidence that Plks may play an important role at the centrosome and thus in bipolar spindle formation (Sunkel and Glover, 1988; Llamazares *et al.*, 1991). This was supported by the cell cycle-dependent centrosomal localization of the various Plks. In common with *Drosophila polo* mutants, fission yeast *plo1* mutants fail to separate SPBs at mitosis onset and form monopolar spindles in a phenotype resembling that seen upon mutation of genes encoding the kinesin proteins required for SPB separation (Ohkura *et al.*, 1995). It was later shown that *polo* mutants

A



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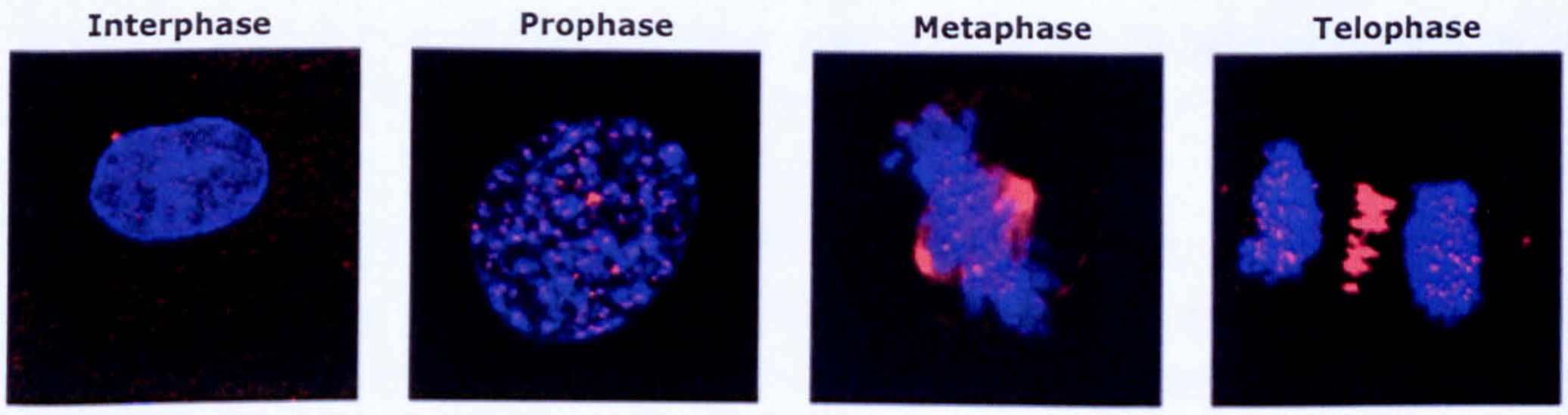


Figure 1.6 The Polo-like kinases

(**A**) Schematic representation of different members of the Polo-like kinase family with the kinase domain highlighted in pink and the polo-box domain shown in green. The length of each protein in amino acids is indicated on the right. Adapted from Dai (2005). (**B**) Immunofluorescence microscopy showing the dynamic cell cycle-dependent localization of Plk1 (red). DNA is stained in blue. Taken from van Vught and Medema (2005).

in *Drosophila* failed to recruit γ-tubulin to the mitotic centrosome (Donaldson *et al.*, 2001; do Carmo Avides *et al.*, 2001). Similarly, mammalian Plk1 was shown to be important for centrosome maturation, that is to say the recruitment of γ-tubulin to the centrosome at mitosis as well as centrosome disjunction when these processes were disrupted by anti-Plk1 antibody microinjection (Lane and Nigg, 1996). It is still unclear exactly how Plks contribute to centrosome maturation; however, a number of centrosomal substrates of mammalian Plk1 have been identified including the centrosomal protein, Nlp, which is displaced from the centrosome upon phosphorylation in mitosis possibly facilitating γ-TuRC recruitment (Casenghi *et al.*, 2003) and Kizuna (Kiz) which is required to ensure spindle pole integrity (Fry and Baxter, 2006; Oshimori et al., 2006).

Furthermore, the recruitment of mammalian Plk1 to centrosomes is thought to promote mitotic entry through the coordinated regulation of the Cdc2 activator, Cdc25 and inhibitor, Wee1: Plk1 phosphorylation activates Cdc25 and targets Wee1 for degradation (van Vugt and Medema, 2005). Furthermore, Plx1 was purified from *Xenopus* egg extracts as a protein kinase capable of interacting with, phosphorylating and activating Cdc25 and the delay in mitotic commitment seen upon depletion of Plx1 in egg extracts could be rescued by addition of excess Cdc25 (Kumagai and Dunphy, 1996; Quian *et al.*, 1998). A similar role has been proposed for Cdc5 (Lee *et al.*, 2005). Although the potential function of Plo1 at the G2/M transition was initially unclear, as this kinase only appears to become active following Cdk1 activation, it seems that Plo1 likewise acts in a positive feedback loop to further activate Cdc25 following its own activation by MPF (Mulvihill *et ai,* 1999; Tanaka *et al.*, 2001; MacIver *et al.*, 2003).

1.3.2.2 Polo-like kinases and sister chromatid separation

Plk1 localizes not only to centrosomes in prometaphase but also to kinetochores, raising the possibility of a function here, perhaps in sister chromatid attachment and separation (Arnaud *et al.*, 1998). Cohesins are a class of conserved proteins which mediate the attachment of sister chromatids in early mitosis and the association between chromosomes and cohesins must be disrupted to allow sister chromatid separation at anaphase onset (Nasmyth *et al.*, 2000). Both Cdc5 and Plk1 have been shown to phosphorylate cohesins prior to anaphase onset thus promoting separation of sister chromatids (Alexandru *et al.*, 2001; Sumara *et al.*, 2002). Cdc5 phosphorylates residues close to the cleavage site in the budding yeast cohesin, Scc1, thus enhancing its cleavage (Alexandru *et al.*, 2001). Plx1 and Plk1 have likewise been shown to phosphorylate cohesins although this appears to act to decrease the affinity of cohesin for chromosomes early in mitosis, reflecting the differences in cohesin dissociation

pathways between vertebrates and yeast (Sumara *et al.*, 2002; Hauf *et al.*, 2005). Plk1 localization to kinetochores may also reflect a role for Plk1 in the formation of K-fibers and thus regulation of chromosome congression at metaphase (Sumara *et al.*, 2004; Hanisch *et al.*, 2006).

1.3.2.3 Polo-like kinases and exit from mitosis

As cells progress through mitosis, Plk1 and related kinases are redistributed away from the MTOC to the spindle midzone and central spindle and finally to the midbody of cytokinetic cells, raising the possibility of a role in regulation of mitotic exit. Indeed evidence has accumulated that Plks may regulate several aspects of late mitosis including sister chromatid separation, APC/C activity and contractile ring assembly and cytokinesis.

Evidence for roles for Plks in later stages of mitosis first came from the localization of vertebrate Plk1 to the central spindle and midbody, as well as from the phenotypes of *cdc5* mutants, which arrest with separated chromosomes and elongated spindles and *plo1* mutants, which showed septation defects with neither F-actin ring formation nor septal material deposition (Kitada *et al.*, 1993; Golsteyn *et al.*, 1995; Ohkura *et al.*, 1995). Subsequently, Cdc5 was shown to play a critical role in activation of the mitotic exit network in budding yeast by phosphorylating and negatively regulating the spindle checkpoint component, Bfa1 (Geymonat *et al.*, 2003). Cdc5 was also shown to be a component of the FEAR (cdc fourteen early anaphase release) network that acts early in mitotic exit in budding yeast to initiate the exit machinery (Kitada *et al.*, 1993). Similarly, Plk1 and Plx1 are important for the activation of the APC/C. Addition of catalytically-inactive Plx1 to mitotic *Xenopus* egg extracts prevented their Ca^{2+}-induced release into interphase as well as the proteolytic destruction of several targets of the APC/C, including cyclin B, and thus Cdk1 inactivation. Moreover depletion of Plx1 from mitotic *Xenopus* egg extracts blocked the metaphase-anaphase transition whilst add-back of Plx1 rescued this block (Descombes and Nigg, 1998). APC/C activation by Plks may be mediated via either direct or indirect mechanisms. There is evidence that Plks may directly phosphorylate APC/C components (Kotani *et al.*, 1998; Rudner and Murray, 2000; Shirayama *et al.*, 1998; Golan *et al.*, 2002). Furthermore, Plo1 has been shown to interact with the fission yeast APC/C component Cut23 (May *et al.*, 2002). Moreover, Plks may indirectly regulate APC/C activity during mitotic exit by regulating inhibitors of the APC/C. Both Plk1 and Plx1 have been shown to phosphorylate the APC/C inhibitor, Emi1, thus facilitating its ubiquitination and degradation (Moshe *et al.*, 2004; Schmidt *et al.*, 2005).

1.3.2.4 Polo-like kinases and cytokinesis

There is a significant body of evidence that Plks play important roles in cytokinesis in a number of organisms. Early analyses to identify Polo homologues in fission yeast identified the Plo1 protein which, in *plo1* mutants, not only failed to form a bipolar mitotic spindle, but also showed septation defects with neither F-actin ring formation nor septal material deposition (Ohkura *et al.*, 1995). Conversely, overexpression of Plo1 resulted in the formation of multiple septa in interphase cells (Ohkura *et al.*, 1995). Plo1 mutants have also been identified which are defective in placement and organization of the contractile medial ring (Bahler *et al.*, 1998) and it has been suggested that Plo1 acts upstream of the septation initiation network (SIN) to induce septation (Tanaka *et al.*, 2001). Generation of localization-defective *cdc5* mutants in budding yeast showed that the proper localization of this protein to the SPB, but interestingly not the bud neck, was essential for cytokinesis (Park *et al.*, 2004). A role for *Drosophila* Polo in cytokinesis was demonstrated by observation of cytokinesis defects in hypomorphic *polo* mutants during spermatogenesis in which midzone and midbody formation, as well as localization of kinesin-like proteins required for cytokinesis and contractile ring formation, were compromised (Carmena *et al.*, 1998). Recently, experiments in which Plk1 was specifically inhibited at the metaphase/anaphase transition by addition of rapidly acting small molecule inhibitors have highlighted potential roles for mammalian Plk1 in cytokinesis. Inhibition of Plk1 at this point blocked cleavage furrow ingression and thus cytokinesis as a result of a failure to assemble the contractile ring or accumulate the upstream regulator of contractile ring function, RhoA (Brennan *et al.*, 2007; Petroczki *et al.*, 2007; Santamaria *et al.*, 2007). Paradoxically, there is also evidence that Plk1 is a target for the APC/C and that its proteolytic degradation is required for mitotic exit. Expression of a Plk1 mutant lacking the D-box motif responsible for targeting it for degradation results in a delay in mitotic exit (Lindon and Pines, 2004). It is thought that, whilst Plk1 is necessary for recruitment of contractile ring components and regulators to the cleavage furrow, excessive Plk1 impairs microtubule bundling as cytokinesis progresses (Pines and Lindon, 2005).

Thus, Plks are cell cycle-regulated protein kinases with multiple functions in mitosis. Furthermore, the localization of Plks throughout the cell cycle, mediated by the polo-box domains, is clearly an important mode of regulation. Studies in budding yeast and human cultured cells have shown that the MTOC localization of Cdc5 and Plk1, respectively, is dependent upon the polo-box and moreover, that this localization is essential for Plk function in mitosis (Park *et al.*, 2004; Seong *et al.*, 2002). In addition, like many key cell cycle regulators, protein phosphorylation and degradation are

essential to Plk function although exactly how is unclear (Barr *et al.*, 2004). The increase in Plk kinase activity seen upon entry into mitosis is the result of phosphorylation events, however which kinases are responsible for these remain unclear (Tanaka *et al.*, 2001; Lee *et al.*, 1995). An important question in Plk regulation concerns the relationship between Plk1 and Cdk1-cyclin B. As discussed above, there is significant evidence that Plk regulates Cdk1 activity at the G2/M transition; however, what remains unclear is whether Cdk1 regulates Plk activity. Although Plx1 was identified as an *in vitro* substrate of Cdk1, Plk1 lacks any Cdk1 phosphorylation sites suggesting it is not an *in vivo* substrate of this kinase (Barr *et al.*, 2004).

1.3.2.5 Plk2

Mammalian Plk2, identified as a result of its significant homology to Plk1, is expressed maximally in G1 and early S phase with kinase activity peaking at the G1/S transition (Simmons *et al.*, 1992; Ma *et al.*, 2003; Warnke *et al.*, 2004). Plk2 is thought to be involved in centriole duplication as it localizes to the centrosome and interference with Plk2 function, either by depletion or expression of catalytically-inactive Plk2 blocks centriole duplication (Warnke et al., 2004). Moreover, cultured $Plk^{-/-}$ MEFs exhibit a delay in S-phase progression (Ma *et al.*, 2003). However, $Plk2^{-/-}$ embryos are viable and whilst cultured $Plk2^{-/-}$ MEFs grow more slowly, they do divide (Ma *et al.*, 2003). Furthermore, depletion of Plk2 in either U2OS or HeLa cells does not prevent cell cycle progression, thus Plk2 function is not essential, although this may be due to the ability of other Plks to functionally compensate for loss of Plk2 (Burns *et al.*, 2003). Nonetheless, Plk2 is postulated to have important functions in the response to spindle or DNA damage; Plk2 expression is upregulated in response to DNA damage and there is significant induction in apoptosis if Plk2-depleted cells are treated with microtubule destabilizing agents (Burns *et al.*, 2003; Shimizu-Yoshida *et al.*, 2001).

1.3.2.6 Plk3

Plk3 expression is constant throughout the cell cycle, although its kinase activity appears to peak at S and G2 phases of the cell cycle (Ouyang et al., 1997). In common with Plk1, Plk3 can complement *cdc5* temperature sensitive mutants in *Saccharomyces cerevisiae* suggesting some conservation of functionality between these two kinases (Ouyang et al., 1997, Lee and Erikson, 1997). Plk3 localizes to the midbody in late mitosis and ectopic expression of Plk3 induces apoptosis following incomplete cytokinesis (Conn *et al.*, 2000). Moreover, Plk3 can phosphorylate Cdc25 and thus is thought to function similarly to Plk1 with respect to this protein at entry into mitosis (Myer et al., 2005).

1.3.2.6 Plk4

Plk4 expression is increased late in G1 and remains high through S phase and mitosis before falling in early G1 (Fode *et al.*, 1996). Northern blot analysis in mouse embryos and adult tissues demonstrated an association of Plk4 mRNA expression with mitotic and meiotic cell division (Fode *et al.*, 1994). Moreover, Plk4 function appears to be essential for cell viability: RNAi depletion of Plk4 induces apoptosis in HeLa cells (Li *et al.*, 2005). The most well characterized function of Plk4 appears, in common with Plk2, to be regulation of centrosome duplication in cooperation with Cdk2: RNAi depletion of Plk4 reduces centrosome number with successive cell cycles and overexpression of Plk4 results in centriole overduplication (Habedanck et al., 2005). Similar results with the *Drosophila* Plk4 homologue Sak support this hypothesis (Bettencourt-Diaz *et al.*, 2005). It is also postulated that Plk4/Sak are orthologues to the Zyg-1 kinase that is required for centriole duplication in *C. elegans* (O'Connell *et al.*, 2001).

1.3.3 Aurora kinases

Aurora kinases were originally identified as part of a group of temperature-sensitive mutants of *Saccharomyces cerevisiae* which showed increases in chromosome number and designated increase-in-ploidy (Ipl) (Chan and Botstein, 1993). Subsequently, Ipl1 was identified as a homologue of the *Drosophila* serine/threonine kinase, Aurora which, when mutated, results in monopolar spindle formation as a result of a failure in centrosome separation (Glover *et al.*, 1995). At around the same time, a serine/threonine kinase encoded within the amplicon 20q13 frequently detected in human breast tumours was identified and named variously as Breast Tumour Activated Kinase (BTAK), Aurora2 and serine/threonine kinase 15 (STK15) (Sen *et al.*, 1997; Bischoff et al., 1998; Zhou et al., 1998). Since this time two other Aurora homologues have been identified within the human genome and whilst these similarly have been given a number of different names, the unifying nomenclature of Aurora-A (BTAK/Aurora2/STK15), -B and –C has widely been adopted (Nigg, 2001).

As for Plks, the number of Aurora kinases present in the genome increases with increasing evolutionary complexity: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* each have a single Aurora kinase, Ipl1 and Ark1, respectively; *Caenorhabditis elegans, Drosophila melanogaster* and *Xenopus laevis* each have two Aurora kinases (AIR1 & 2, Aurora & IAL, Eg2 & AIRK2, respectively); mammals have at least 3 Aurora kinases, as described (Chan and Botstein, 1993; Petersen *et al.*, 2001; Schumacher *et al.*, 1998a; Schumacher *et al.*, 1998b; Glover *et al.*, 1995; Mesilaty-Gross *et al.*, 1999; Roghi *et al.*, 1998; Adams *et al.*, 2000; Sen *et*

al., 1997; Bischoff et al., 1998; Zhou et al., 1998; Kimura *et al.*, 1997; Bernard *et al.*, 1998; Tseng *et al.*, 1998; Hu *et al.*, 2000; Nigg, 2001).

Aurora kinases have been implicated in chromosome condensation, spindle assembly and chromosome segregation with the different family members being localized variously to centrosomes, spindle poles and microtubules, and to centromeres/kinetochores (Meraldi *et al.*, 2004). Evidence also suggests that with their various roles in spindle assembly, SAC function and accurate chromosome segregation, deregulation of Aurora kinase function may be an important factor in malignant transformation (Nigg, 2001; Katayama *et al.*, 2003).

Aurora-B and –C appear to have overlapping functions as CPC proteins important for ensuring correct syntelic chromosome attachment to the mitotic spindle at metaphase (Adams *et al.*, 2000; Adams *et al.*, 2001; Giet and Glover, 2001; Kimura *et al.*, 1999; Li *et al.*, 2004; Sasai *et al.*, 2004). Aurora-A on the other hand appears to have distinct functions in centrosome duplication, maturation and separation, mitotic entry and spindle assembly. Consistent with this, the localization and cell cycle regulation of Aurora-A and –B are likewise distinct. It seems that the individual Aurora kinases in lower eukaryotes achieve the functions attributed to both Aurora-A and Aurora-B in these organisms. Indeed, a recent systematic analysis of the functions of the single *Dictyostelium* Aurora kinase has clearly demonstrated that this kinase has properties of both Aurora-A and Aurora-B (Li *et al.*, 2008). Whilst there has been significant interest in Aurora-A and –B function due to their expression mainly in proliferating tissues and their potential role in tumorigenesis, Aurora-C, although identified as a CPC protein, is less well characterized than either Aurora-A or –B due to its restricted expression in the male germline (Hu *et al.*, 2000; Tseng *et al.*, 1998).

Structurally, Aurora kinases comprise a C-terminal catalytic domain and an N-terminal regulatory domain which contain the destruction-box (D-box) and D-box activating domain (A-box), respectively, responsible for Aurora degradation. Designation as an Aurora kinase is based mainly on sequence homology within the catalytic domain, and regulatory domains vary significantly in length and sequence between family members (Figure 1.7A) (Nigg, 2001).

Aurora-A and-B show distinct cell cycle-dependent patterns of localization and regulation which reflect the distinct functions of these kinases. Aurora-A localizes to centrosomes from S-phase and becomes heavily concentrated on centrosomes and spindle poles as well as being detectable on spindle microtubules during early mitosis. Consistent with

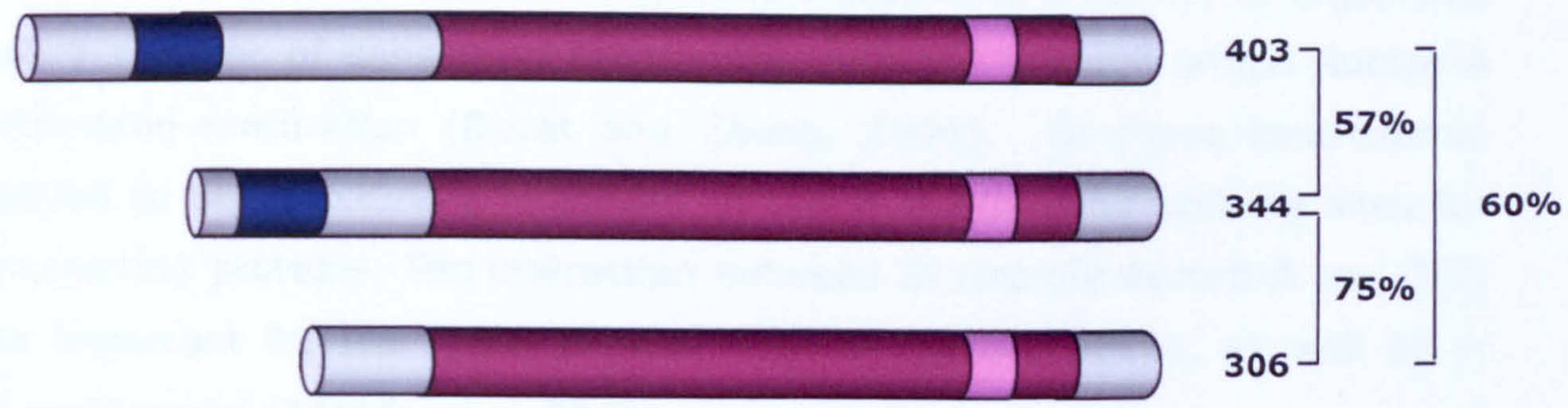
this, Aurora-A binds to taxol-stabilized microtubules in *vitro* (Roghi *et al.*, 1998; Giet and Prigent, 2001). The centrosomal portion of Aurora-A appears to be in dynamic equilibrium with the cytoplasmic Aurora-A and there is a rapid turnover, suggesting that its centrosomal localization reflects a signaling, rather than a structural, role (Stenoien *et al.*, 2003). Aurora-B, on the other hand localizes to chromosomes in prophase before becoming concentrated on centromeres in prometaphase and metaphase and at the central spindle in late mitosis (Figure 1.7B) (Stenoien *et al.*, 2003; Adams *et al.*, 2001). Myc-Ipl1 showed a punctate mitotic spindle stain in mitosis in budding yeast (Biggins *et al.*, 1999). Fission yeast Ark1 associated with chromatin and is particularly concentrated at centromeres/kinetochores following commitment to mitosis in this organism. Ark1 then becomes redistributed along the spindle during anaphase, finally concentrating at the midzone in late mitosis (Petersen *et al.*, 2001). Levels of Ipl1p are low in G1 and peak during S phase and mitosis (Biggins *et al.*, 1999). Aurora-A expression and kinase activity levels are low during G1 and S phase but both rapidly rise through G2 to a peak in early mitosis, before its degradation by the APC/C^{Cdh1} begins in anaphase B (Bischoff, *et al.*, 1998; Kimura *et al.*, 1997; Zhou *et al.*, 1998; Honda *et al.*, 2000; Taguchi *et al.*, 2001; Lindon and Pines, 2004; Stenoien *et al.*, 2003). Aurora-B peak expression is at the G2/M transition and kinase activity is maximal during mitosis but occurs slightly later than that of Aurora-A (Bischoff *et al.*, 1998; Terada *et al.*, 1998).

1.3.3.1 Aurora A function at the centrosome

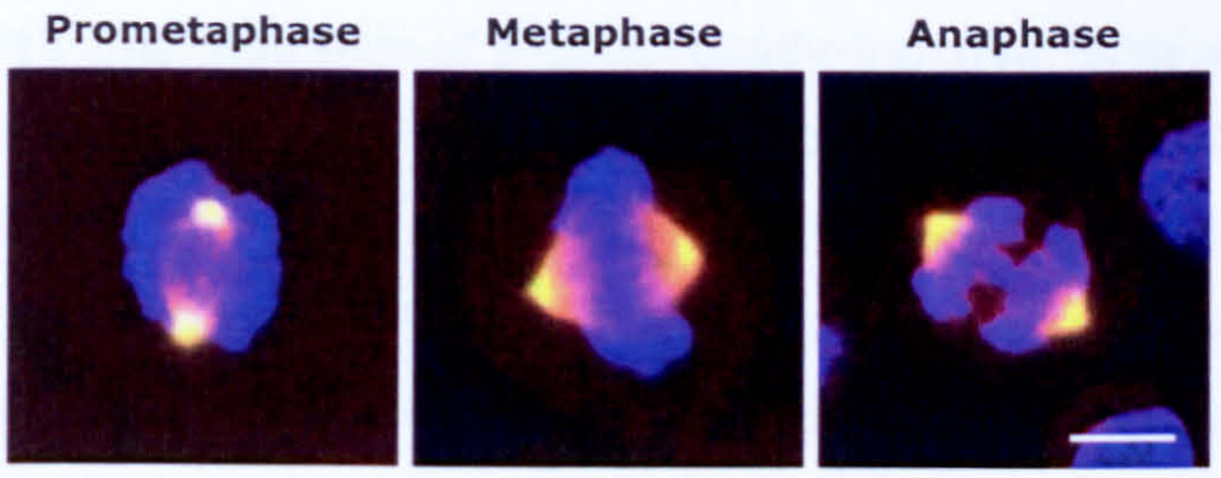
The phenotypes of *aurora* mutants of *Drosophila* pointed to a function for Aurora kinases in centrosome separation and bipolar spindle formation. *aurora* mutants display closely paired centrosomes at inappropriate mitotic stages resulting in chromosomal congression in a circle around monopolar spindles (Glover *et al.*, 1995). Loss of Ark1 from *S. pombe* likewise resulted in monopolar spindle formation (Petersen *et al.*, 2001). Moreover, a similar phenotype is displayed upon immunodepletion of the *Xenopus* Aurora-A protein, Eg2, from egg extracts (Roghi *et al.*, 1998). Similarly, interference with Aurora-A in cultured human cells via RNAi depletion or small molecule inhibitors resulted in monopolar spindles (Marumoto *et al.*, 2003; Girdler *et al.*, 2006). Furthermore, antibody microinjection of anti-Aurora-A antibodies into cells in late G2 resulted in a failure of centriole pairs to separate (Marumoto *et al.*, 2003).

These phenotypes appear to reflect a role for Aurora-A in centrosome maturation. Studies of the effect of disruption of Aurora function in *Drosophila* showed that Aurora mutation or RNAi depletion disrupts normal centrosome organization and recruitment of PCM components including γ-tubulin (Giet *et al.*, 2002; Berdnick and Knoblich, 2002;

A



B



C

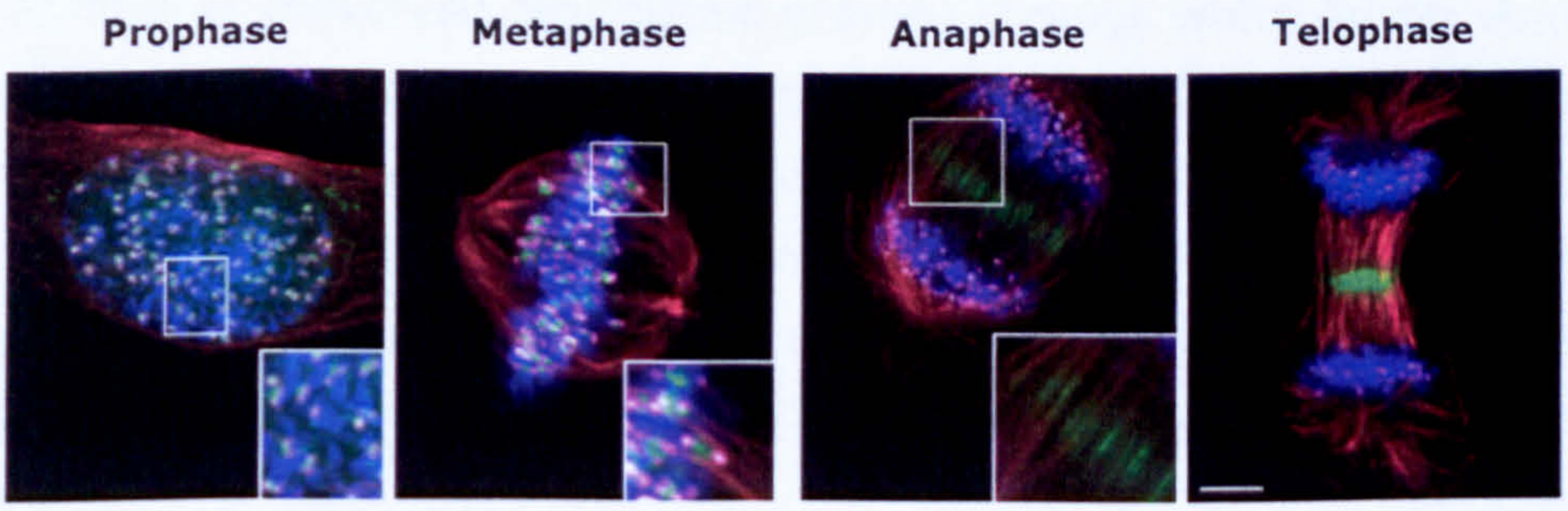


Figure 1.7 The Aurora Kinases

(**A**) Diagramatic representation of the domain structure of the human Aurora kinases. The amino acid sequence length and percentage of sequence homology between the human Auroras is indicated on the right. The D-box and the D-box activating domain promote Aurora degradation at the end of mitosis. Adapted from Quintas-Cardama *et al.* (2007). (**B**) Immunofluorescence microscopy showing the subcellular localization of Aurora-A (green), DNA is blue and microtubules are red. Taken from Barr and Gergely, 2007. (**C**) Immunofluorescence microscopy showing the subcellular localization of Aurora-B (green). Kinetochores are shown in pink, α-tubulin in red and DNA in blue. Taken from Ruchaud *et al.* (2007).

Terada *et al.*, 2003). RNAi depletion of Aurora-A from *C. elegans* embryos results in a decreased density of centrosome-associated microtubules and reduced γ-tubulin recruitment to the centrosome (Hannak *et al.*, 2001). Studies in a variety of organisms have identified a number of conserved centrosome proteins through which Aurora-A mediates centrosome maturation (Ducat and Zheng, 2004). Of these centrosomin (CNN), is believed to be structurally important in the PCM providing docking sites for microtubule-nucleating proteins. The interaction between *Drosophila* Aurora-A and CNN appears to be important for the localization of both of these proteins, as well as γ-TuRCs, to the centrosome (Terada *et al.* 2003).

The monopolar spindle phenotype seen upon interference with Aurora-A function also appears to reflect a more direct role in centrosome separation and maintenance of spindle bipolarity. For example, in *C .elegans* live imaging of cells depleted of Aurora-A via RNAi showed that whilst centrosomes initially separate, cells fail to maintain this separation and they rapidly coalesce resulting in a single spindle pole (Hannak *et al.*, 2001). Consistent with this, addition of a catalytically-inactive form of *Xenopus* Aurora-A to egg extracts which have already assembled bipolar spindles results in their collapse (Giet and Prigent, 2000). *Xenopus* Aurora-A appears to phosphorylate Eg5, a kinesin required for spindle pole separation during mitosis, raising the possibility that Aurora-A mediates centrosome separation through this interaction (Giet *et al.*, 1999).

Aside from its role in centrosome maturation, studies in a variety of organisms have identified a number of conserved centrosome proteins through which Aurora-A mediates the formation of a stable bipolar spindle (Ducat and Zheng, 2004). These include the transforming acidic coiled-coil proteins (TACCs), which stabilize microtubules during mitosis by recruiting minispindles (Msps/XMAP215/TOG) proteins to the centrosome. Both *Drosophila* and *Xenopus* Aurora-A proteins have been shown to phosphorylate and thus recruit TACC proteins to the centrosome (Barros et al., 2005; Peset et al., 2005).

1.3.3.2 Aurora-A and mitotic entry

Another phenotype seen upon interference with Aurora-A function in human cells and in *C. elegans* embryos is a delay in mitotic entry, indicating a possible role for Aurora-A in regulating entry into mitosis (Hirota *et al.*, 2003; Marumoto *et al.*, 2002; Marumoto *et al.*, 2003; Hannak *et al.*, 2001). The first evidence for this came from experiments in G2 arrested *Xenopus* oocytes in which overexpression of Aurora-A resulted in accelerated progesterone-stimulated entry into M-phase of meiosis (Andresson and Rudermann, 1998). This is believed to be a result of activation of Cdk1-cyclin B, as interference with Aurora-A function in *Xenopus* egg extracts also delays Cdk1 activation

and mitotic entry (Liu and Rudermann, 2006). Similarly, RNAi depletion of Aurora-A in human cells has been reported to result in a delay in Cdk1-cyclin B activation (Hirota *et al.*, 2003). Aurora-A has been shown to phosphorylate Cdc25B and this phosphorylation is believed to activate this protein and thus promote Cdk1-cyclin B activation and mitotic entry (Duerte *et al.*, 2004). Furthermore, the centrosomal localization of Cdk1-cyclin B, which appears to be important for correct mitotic entry, is mediated by Aurora-A (Hirota *et al.*, 2003).

1.3.3.3 Aurora-A and chromatin-mediated spindle assembly

Studies in *Xenopus* egg extracts have highlighted a further role for Aurora-A in the Ran-GTP and chromatin-mediated spindle assembly pathway. Ran-GTP serves to release spindle assembly factors, such as TPX2 and NuMA, from the importin-α inhibitor thus promoting spindle assembly (Gruss *et al.*, 2001; Di Fiore *et al.*, 2003). Experiments in *Xenopus* uncovered an interaction between Aurora-A and TPX2, which is important in this pathway, and demonstrated that Aurora-A coated beads were able to act as MTOCs and promote microtubule assembly in egg extracts lacking both centrosomes and chromatin (Tsai *et al.*, 2003; Tsai and Zheng, 2005). Subsequently, a protein complex involved in stimulating this pathway was isolated from *Xenopus* extracts and shown to contain Aurora-A (Koffa *et al.*, 2006).

1.3.3.4 Aurora-A and cytokinesis

Ectopic expression of Aurora-A provokes centrosome amplification (Meraldi *et al.*, 2002; Zhou *et al.*, 1998). However, Aurora-A is only detected on duplicated centrosomes from the end of S-phase (Goplan *et al*, 1997; Kimura *et al.*, 1997; Roghi *et al.*, 1998). In view of the fact that centriole duplication occurs around the time of the G1/S transition (Doxsey, 2001), it seems unlikely that this phenotype reflects a direct involvement in centriole duplication, but rather a failure in cell division resulting in tetraploidization. This suggests that Aurora-A may also be involved in regulation of cytokinesis (Meraldi *et al.*, 2002) and, consistent with this, interference with Aurora-A function by antibody microinjection into metaphase cells resulted in cytokinesis defects (Marumoto *et al.*, 2003).

1.3.3.5 Aurora-B

Neither murine nor human Aurora-A is capable of complementing an *ipl1* mutation in budding yeast (Gopolan *et al.*, 1997; Kimura *et al.*, 1997). Similarly, *Xenopus* Aurora-A is unable to functionally complement an *ark1* mutation in *S. pombe*, suggesting functional divergence (Petersen *et al.*, 2001). Furthermore, in these organisms, which

have only one Aurora-related kinase, this protein appears to mediate more functions than those performed by Aurora-A. Indeed the main functions of the yeast kinases appear to be chromosome segregation and cytokinesis (For example, Biggins and Murray, 2001; Petersen and Hagan, 2003; Leverson *et al.*, 2002). Thus, Ipl1 and Ark1 kinases are required for the SAC response (Biggins and Murray, 2001; Petersen and Hagan, 2003) and correct sister chromatid separation (Kim *et al.*, 1999; Leverson *e al.*, 2002), and interfering with Ark1 function inhibited cytokinesis resulting in elongated, multiseptate cells (Leverson *et al.*, 2003). These are functions mediated by Aurora-B kinases in metazoans. Aurora-B is the catalytic component of the CPC, along with inner centromere protein (INCENP), survivin and borealin. It has multiple mitotic functions, including mediating chromosome-microtubule interactions, sister chromatid cohesion and cytokinesis (Kim *et al.*, 1999; Adams *et al.*, 2000; Kaitna *et al.*, 2000; Adams *et al.*, 2001). Both fission and budding yeast Aurora kinases likewise associate with yeast survivin and INCENP orthologues (Kim *et al.*, 1999; Leverson *et al.*, 2002).

1.3.3.6 Aurora-B and chromatin condensation

Very early in mitosis, Aurora-B is seen all over the chromatin and *Drosophila aurora-B* mutants exhibit defects in chromosome structure and compaction (Adams *et al.*, 2001; Giet and Prigent, 2000). Furthermore, a conserved Aurora function carried out by Aurora-B in metazoans, is phosphorylation of S10 on Histone H3, which may reflect a role in chromosome condensation at mitosis (Biggins *et al.*, 1999; Sassoon *et al.*, 1999; Hsu *et al.*, 2000; Adams *et al.*, 2001; Giet and Glover, 2001; Petersen and Hagan, 2001). Alternatively, the defects in chromosome structure seen could reflect a role for Aurora-B in loading condensin, a protein complex essential for maintenance of the structural integrity of mitotic chromosomes, onto the chromosomes. Interference with Aurora-B function in *Drosophila* or Ark1 function in fission yeast, results in condensin mislocalization (Giet and Prigent, 2000; Petersen and Hagan, 2001).

1.3.3.7 Aurora-B at the inner centromere

In early mitosis, the CPC is localized to the inner centromeres and this in part reflects the highly conserved function of these proteins in regulation of kinetochore attachment as well as in the SAC (Gassman *et al.*, 2004; Adams *et al.*, 2001; Kaitna *et al.*, 2000; Giet and Glover, 2001; Tanaka *et al.*, 2002; Hauf *et al.*, 2003; Murata-Hori and Wang, 2002). Inhibition of Aurora-B leads to an increased number of incorrectly attached kinetochores during prometaphase, moreover, there is an increased number of cells which enter anaphase with misaligned chromosomes suggesting a defective SAC (Hauf *et al.*, 2003; Ditchfield *et al.*, 2003). Studies in a range of model systems have demonstrated that the CPC acts to detect and destabilize incorrect microtubule-

kinetochore attachments, thus preventing errors in chromosome segregation. Aurora-B specifically appears to function by phosphorylating key kinetochore-centromere components (Ducat and Zheng, 2004; Cimni, 2007). In budding yeast, Ipl1 detects non-bipolar attachments and prevents stable microtubule-kinetochore attachments occurring under such circumstances (Biggins and Murray, 2001; Tanaka *et al.*, 2002). Similarly, partial inhibition of Aurora-B resulted in a higher frequency of merotelically oriented kinetochores at metaphase and an increase in lagging chromosomes at anaphase (Cimini *et al.*, 2006; Cimini, 2007). Moreover, in *Xenopus* cultured cells, Aurora-B becomes enriched at centromeres which possess merotelic attachments (Knowlton *et al.*, 2006). Yeast Aurora substrates include the Ndc10p kinetochore protein, whose phosphorylation state regulates kinetochore/spindle attachment (Biggins *et al.*, 1999). Subsequently, vertebrate Aurora-B was shown to phosphorylate Hec1, the vertebrate homologue of Ndc10, and this phosphorylation is believed to mediate the release of microtubules by kinetochores in the case of the formation of inappropriate attachment (DeLuca *et al.*, 2003). Furthermore, Aurora B kinase activity has been shown to control the centromeric localization and catalytic activity of the microtubule depolymerase, MCAK, and thus regulate microtubule stability (Andrews *et al.*, 2004; Lan *et al.*, 2004; Knowlton *et al.*, 2006). MCAK itself is part of an inner centromere protein complex also implicated in correction of merotelic attachments before anaphase onset (Desai *et al.*, 1999; Kline-Smith *et al.*, 2004). This may thus represent another pathway through which Aurora B may mediate microtubule release from kinetochores.

Studies in yeast have demonstrated that the CPC is required for the SAC response when spindle tension is aberrant (Biggins and Murray, 2001). Specific inhibition of Aurora B ablates the SAC and results in the mislocalisation of kinetochore-associated SAC components including Bub1 and Mad2, highlighting a pathway through which the CPC may be directly involved in the checkpoint response (Ditchfield *et al.*, 2003).

Another important emerging function of Aurora-B and the CPC is in the assembly and stability of the mitotic spindle. Disruption of CPC function in *Drosophila* cells or *Xenopus* egg extracts appears to interfere with bipolar spindle formation, whilst depletion of components of the CPC in human cells results in the formation of unstable mitotic spindles (Gassmann *et al.*, 2004; Adams *et al.*, 2001; Sampath *et al.*, 2004). This may also involve interaction of Aurora-B with the microtubule depolymerase, MCAK (Andrews *et al.*, 2004; Lan *et al.*, 2004).

1.3.3.8 Aurora-B and cytokinesis

Finally, the redistribution of Aurora-B to the spindle midzone and midbody during late mitosis reflects a role in regulation of mitotic exit. Aurora-B depletion in *C. elegans* embryos and *Drosophila* cells results in cytokinesis failure (Schumacher *et al.*, 1998; Giet and Glover, 2001). Studies in a variety of organisms have shown that CPC components, whilst not essential for furrow initiation, are absolutely required for the completion of cytokinesis (Terada *et al.*, 1998; Gassman *et al.*, 2004; Adams *et al.*, 2001; Carvalho *et al.*, 2003; Schumacher *et al.*, 1998). Although the exact mechanism by which Aurora-B may regulate cytokinesis completion remains to be elucidated, a number of conserved substrates of Aurora-B which may mediate this function have been identified and include kinesins such as ZEN-4/MKLP1 and the intermediate filament protein, vimentin (Severson *et al.*, 2000; Guse *et al.*, 2005; Goto *et al.*, 2003).

1.3.4 NIMA-related kinases

1.3.4.1 NIMA

NIMA is a serine/threonine protein kinase from the filamentous fungus *Aspergillus nidulans*, encoded by the *nimA* (never-in-mitosis A) gene, whose activation and degradation are essential for mitotic entry and exit, respectively (Osmani *et al.*, 1991; Pu & Osmani, 1995). NIMA was first identified in a genetic screen for cell cycle mutants in *Aspergillus*. The screen resulted in the identification of two major classes of mutation: never-in-mitosis, or *nim*, genes, whose mutants were blocked in interphase at the restrictive temperature and unable to enter mitosis; and blocked-in-mitosis, or *bim*, genes, whose mutants arrested in mitosis with condensed chromosomes and a metaphase array of microtubules (Morris, 1975).

nimA was one of the *nim* mutants isolated and, at the restrictive temperature, these mutants arrested in G2 with uncondensed chromosomes, intact cytoplasmic microtubule structures and duplicated, but not separated, SPBs. Upon shifting to the permissive temperature cells entered mitosis within 5 minutes, rapidly undergoing chromosome condensation and spindle formation, suggesting that the *nimA* mutation blocks cells at a point immediately before initiation of chromosome condensation and spindle assembly (Oakley and Morris, 1983). This gene was subsequently shown to encode the NIMA serine/threonine protein kinase (Lu *et al.*, 1993; Osmani *et al.*, 1987).

NIMA is a 79 kDa protein with an N-terminal catalytic domain and a C-terminal non-catalytic domain which contains a predicted coiled-coil region immediately after the catalytic domain followed by two PEST sequences. The coiled-coil domain is believed to be important for the formation of NIMA oligomers and the PEST sequences direct NIMA

for ubiquitin-dependent proteolysis (Lu *et al.*, 1994; Pu & Osmani, 1995; O'Connell *et al.*, 2003).

Analysis of the cell cycle-dependent levels of mRNA and protein expression demonstrated that, consistent with a role in regulation of mitotic entry, both mRNA and protein levels increase as cells progress through G2, peaking during late G2 and early mitosis, after which NIMA levels drop as cells progress through mitosis. The kinase activity of NIMA reflects the abundance of the protein with peak levels in late G2 and early M-phase and progression through mitosis resulting in NIMA inactivation (Osmani *et al.*, 1987; Osmani *et al.*, 1991; Lu *et al.*, 1993; Ye *et al.*, 1995). Kinase activity of NIMA is regulated not only at the level of protein abundance but also by phosphorylation and, the increase in NIMA kinase activity is concomitant with an increase in autophosphorylation (Ye *et al.*, 1995). Furthermore, NIMA is degraded during progression through mitosis and this degradation is essential for mitotic exit (Figure 1.8 A) (O'Connell *et al.*, 1994; Pu and Osmani, 1995).

The phenotype of *nimA* temperature sensitive mutants provided clear evidence that NIMA activity is required for the initiation of all cytological aspects of mitosis (Osmani *et al.*, 1978). Moreover, overexpression of NIMA caused the induction of a pseudo-mitotic state in which chromosome condensation occurred from any stage of the cell cycle (Osmani et al., 1988; O'Connell et al., 1994). Conversely, degradation of NIMA is absolutely required for mitotic exit and expression of a truncated form of NIMA which lacks the PEST sequences that mediate NIMA proteolysis is highly toxic and results in an arrest in mitosis (Pu and Osmani, 1995). Mutation of *nimA*, whilst preventing mitotic entry, does not prevent activation of cdc2, but active cdc2 alone cannot induce any of the cytological rearrangements necessary for mitotic progression (Osmani *et al.*, 1991). Thus, although NIMA clearly plays no role in cdc2 activation, the activity of neither cdc2 nor NIMA alone is sufficient for mitotic entry (Osmani *et al.*, 1991). Indeed, there is evidence that NIMA is activated by cdc2/cyclin B during mitosis initiation (Ye *et al.*, 1995). Alternatively, NIMA activity may be required to localize cdc2/cyclin B to the nucleus at the time of mitotic entry, a process essential for mitotic progression in the closed mitosis of *Aspergillus*, and to promote localization of cdc2/cyclin B to the SPB (Wu *et al.*, 1998). Indeed, there is some evidence for interactions between NIMA and two nuclear pore complex components, SONA and SONB (De Souza *et al.*, 2003). The functions of NIMA are summarized in Figure 1.8B.

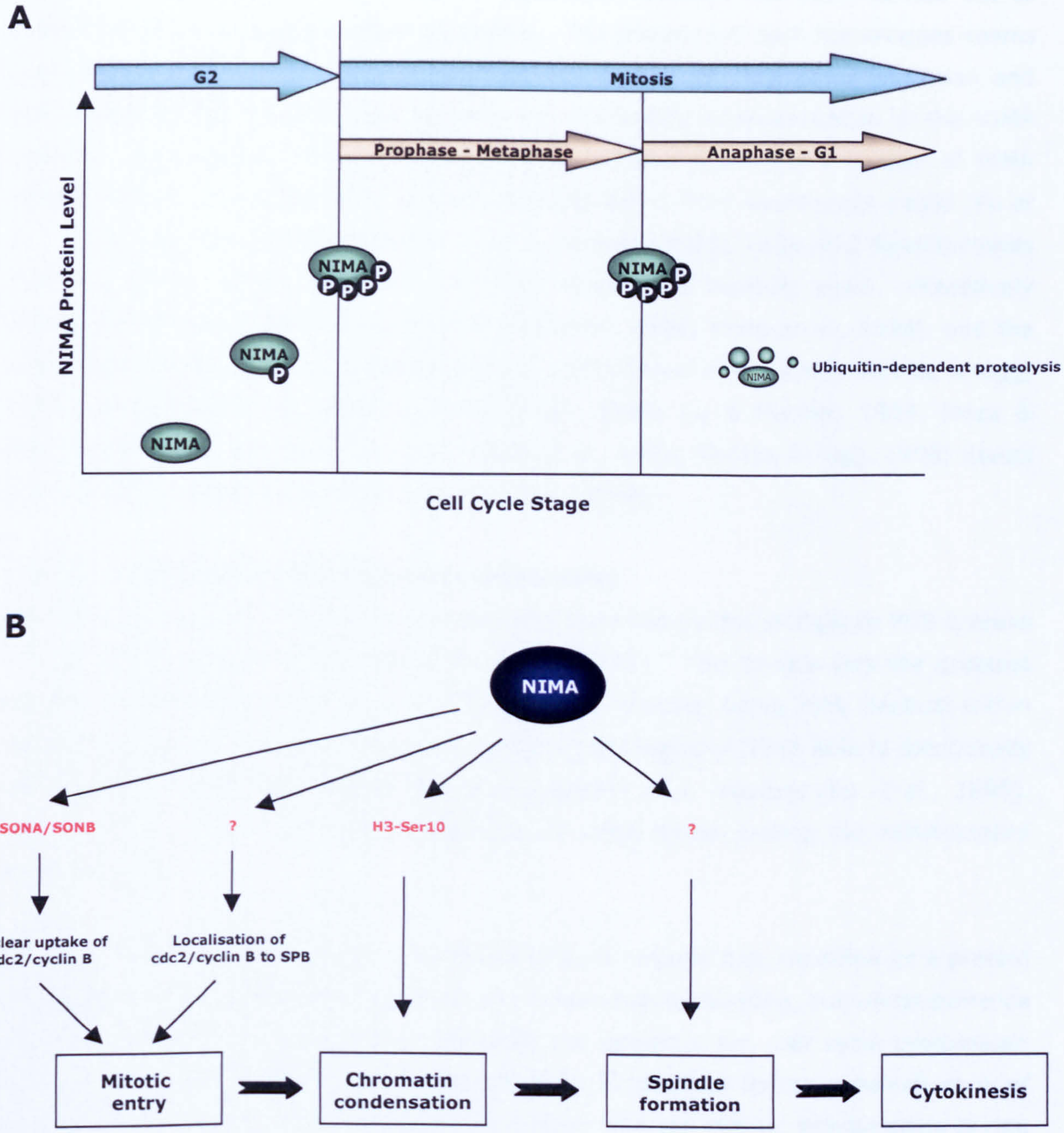


Figure 1.8 NIMA cell cycle-dependent regulation and functions
(**A**) Cell-cycle dependent regulation of NIMA. NIMA protein levels increase during G2, peaking at the G2/M transition concomitant with NIMA hyperphosphorylation. NIMA protein and phosphorylation levels remain high until the metaphase/anaphase transition when NIMA is subject to ubiquitin-dependent proteolyosis. Adapted from O'Connell *et al.* (2003). (**B**) Schematic representation of what is known about the multiple mitotic events regulated by the NIMA kinase. NIMA regulates entry into mitosis by regulating the localization and/or activation of MPF. Only in *Aspergillus* is this conserved fungal NIMA-related kinase function critical. NIMA promotes chromatin condensation by phosphorylating Histone H3. NIMA is also believed to be involved in regulation of mitotic spindle formation although by what mechanism remains unclear. NIMA is not known to function at cytokinesis. Adapted from O'Regan *et al.*, 2007.

Since its isolation and characterization, significant research has been carried out to identify NIMA homologues in other organisms. The presence of such homologues seems likely as the mitotic machinery is generally well-conserved throughout evolution and early experimental evidence also supports the probability of conservation of the NIMA pathway (Lu & Hunter, 1995). This work led to the identification of a number of NIMA related kinases from a variety of species including NIM-1 from *Neurospora crassa* (Pu *et al.*, 1995), the Nrks in trypanosomes (Gale & Parsons, 1993), Fa2p in *Chlamydomonas* (Mahjoub *et al.*, 2002), Fin1 and Kin3 from fission and budding yeast, respectively (Jones & Rosamond, 1990; Schweitzer & Philippsen, 1992; Krien *et al.*, 1998), and the mammalian Neks (Figure 1.9) (Letwein *et al.*, 1992; Cance *et al.*, 1993; Schultz & Nigg, 1993; Levedakou *et al.*, 1994; Schultz *et al.*, 1994; Lu & Hunter, 1995; Rhee & Wolgemuth, 1997, Arama *et al.*, 1998; Chen *et al.*, 1999; Tanaka & Nigg, 1999; Kandli *et al.*, 2000; Holland *et al.*, 2002; Noguchi *et al.*, 2002).

1.3.4.2 NIMA-homologues in lower eukaryotes

The NIM-1 protein from *Neurospora crassa* was identified by low stringency PCR screens of a *N. crassa* genomic DNA library (Pu *et al.*, 1995). This protein has the greatest sequence similarity to NIMA of all the NIMA-related kinases, being 75% identical within the kinase domain. It is furthermore a functional homologue of NIMA able to functionally complement a *nimA* temperature sensitive mutation in *A. nidulans* (Pu *et al.*, 1995). This is the only true functional homologue of NIMA found among the NIMA-related kinases to date.

Fa2p is a NIMA-related kinase in *Chlamydomonas reinhardtii* first identified as a protein which acts during deflagellation to bring about microtubule severing, but which evidence suggests may also be involved in, although not essential for, cell cycle progression (Finst *et al.*, 1998; Mahjoub *et al.*, 2002). Fa2p is localized to the proximal ends of flagella during interphase but becomes associated with the mitotic spindle poles during mitosis (Mahjoub *et* al., 2004). *Fa2* mutants are not only defective in deflagellation but also have subtle cell cycle progression defects with such cells being delayed in passage through the cell cycle at a number of points including at the G2/M transition (Mahjoub *et al.*, 2002). Two possible explanations for these observations have been put forward: firstly, Fa2p may be involved in the centrosome cycle or, secondly, the delay in cell cycle progression may be due to a delay in microtubule severing, a process which has been implicated in cell cycle progression, as many ciliated cells disassemble their cilia in order to utilize basal bodies/centrioles as spindle poles (Mahjoub *et al.*, 2002). More recently, a second of the 10 NIMA-related kinases present in the *Chlamydomonas* genome, Cnk2p, was characterized and shown to localize to the ciliary axoneme and be

Figure 1.9 The NIMA-related kinases
Schematic representation of the NIMA-related kinases. Designation as a NIMA-related kinase is based on sequence homology to *A. nidulans* NIMA within the kinase domain. Regulatory domains, although typically C-terminal, can be highly divergent between family members. Regulatory motifs act to promote protein-protein interactions (coiled-coil, RCC1-like domain), or to promote proteolytic degradation (PEST-sequence, KEN-box, D-box). There are 11 mammalian Neks, although Nek2 exists in three isoforms (Nek2A, Nek2B and Nek2C) and Nek11 in two isoforms (Nek11L and Nek11S). Adapted from O'Connell *et al*. (2003).

involved in regulating flagellar length and cell size in *Chlamydomonas* (Bradley and Quarmby, 2005). Ectopic expression of Cnk2p resulted in small cells with short flagella whilst RNAi depletion of Cnk2p resulted in large cells with long flagella. This is thought to reflect a role for Cnk2p in cell cycle control by promoting flagellar disassembly and monitoring cell size prior to commitment to mitosis (Bradley and Quarmby, 2005)

Early low stringency PCR screens identified two NIMA-related kinases in the protozoan parasite, *Trypanosoma brucei*, which were named NRKA and NRKB, However functional analysis of these proteins has been limited although they are thought to be involved in parasite differentiation (Gale and Parsons, 1993; Gale *et al.*, 1994; Pradel *et al.*, 2006). Recently, a third *T. brucei* NIMA-related kinase has been identified through database searching and named NRKC (Pradel *et al.*, 2006). This kinase is localized to the basal body of *T. brucei* cells and is believed to be important for regulation of cell cycle progression. Interference with *Tb*NRKC function by RNAi depletion or expression of kinase-dead versions of the protein results in the accumulation of four basal bodies and, occasionally, a failure in basal body separation. NRKC overexpression results in accumulation of multinucleate cells and supernumary basal bodies indicative of failed cytokinesis (Pradel *et al.*, 2006). This data suggests that *Tb*NRKC is involved in the cytokinesis pathway in which basal body separation is necessary for cytokinesis to occur in *Trypanosomes*.

The fission and budding yeasts each have one NIMA-related kinase in their genome, Fin1 and Kin3, respectively (Jones & Rosamond, 1990; Schweitzer & Philippsen, 1992; Krien *et al.*, 1998). Kin3 is one of the most closely related Neks to NIMA, sharing 48% sequence identity, although *kin3* mutations are not lethal and it cannot functionally complement a *nimA* mutation. Indeed, mutation of *kin3* or overexpression of the protein does not result in an observable phenotype and thus, elucidation of the function of Kin3 has proved difficult (Barton et al., 1992; Kambouris et al., 1993). However, there is some evidence for a role in catalyzing the phosphorylation of another cell cycle regulatory protein, Hec1, a step essential for chromosome separation (Chen *et al.*, 2002).

Fin1, like Kin3 in budding yeast, is a non-essential gene in fission yeast, although its mutation does delay mitotic entry (Krien *et al.*, 1998; Grallert and Hagan, 2002). Fin1 activity is cell cycle-regulated by gene expression and proteolysis, with maximal levels of Fin1 abundance and kinase activity at mitosis. Notably, however, Fin1 kinase activity does not appear to peak until after the metaphase/anaphase transition, suggesting a role in late mitosis (Krien *et al.*, 2002). The protein localizes to the SPB in mitosis,

accumulating there from late in G2 and, as cells progress through mitosis, Fin1 becomes localized to the region between separating chromosomes (Krein *et al.*, 1998; Krien *et al.*, 2002; Grallert *et al.*, 2004). Consistent with its cell cycle-dependent activity and localization, Fin1 is believed to have roles in regulation of commitment to mitosis and mitotic spindle formation in fission yeast and in mitotic exit (Grallert & Hagan, 2002; Krein *et al.*, 1998; Grallert *et al.*, 2004). Like NIMA, overexpression of Fin1 promotes premature chromatin condensation from any point in the cell cycle independently of Cdc2 function (Krein *et al.*, 1998), although given the timing of Fin1 activation in mitosis and the fact that there is no recruitment of condensin and topoisomerase II, whether this reflects a true physiological function of Fin1 is unclear (Krien *et al.*, 2002). Temperature-sensitive *fin1* mutants cannot form a mitotic spindle at the restrictive temperature. Moreover, the identification of epistatic interactions between Fin1 and spindle checkpoint mutants in the same study, also implicates Fin1 in spindle formation (Grallert and Hagan, 2002). Fin1 appears to promote the localization of the *S. pombe* polo-like kinase, Plo1, to the SPB suggesting a potential role for Fin1 in mitotic commitment is as much as Plo1 acts in the cdc2/cyclin B activation feedback loop (Grallert and Hagan, 2002). This would explain the delay in mitotic entry seen upon mutation of Fin1 (Krien *et al.*, 1998; Grallert and Hagan, 2002; O'Regan *et al.*, 2007). Finally, Fin1 appears to have a key role in control of mitotic exit by modulating the activity of the septum initiation network (SIN) in *S. pombe* (Grallert *et al.*, 2004). Specifically, Fin1 activity appears to be necessary for restricting the distribution of active SIN components on only one, the younger, SPB. Interference with Fin1 function results in active SIN components on both SPBs and an inappropriate SIN signal, suggesting that Fin1 inhibits SIN activity on the older SPB. This activity appears to be as a result of interaction with the SIN inhibitors, Byr4 and Cdc16 (Grallert *et al.*, 2004).

1.3.4.3 Mammalian NIMA homologues not directly implicated in mitosis: Nek1, Nek3, Nek8, and Nek11

Nek1 was the first mammalian Nek to be isolated and characterized, although its precise function remains unknown. Its kinase domain shares 42% identity with that of NIMA and, like NIMA, it also possesses a coiled-coil domain, albeit larger than that of NIMA, and PEST sequences (O'Connell *et al.*, 2003). Nek1 was originally proposed to be a dual specificity serine/threonine and tyrosine kinase, as it was isolated using an anti-phosphotyrosine screening approach (Letwin *et al.*, 1992). However, it is more likely to be a standard serine/threonine kinase. It is highly expressed in meiotic germ cells as well as in peripheral and motor neurons and thus was proposed to have a role in meiosis and/or differentiation (Letwin *et al.*, 1992; Arama *et al.*, 1998). More recently, it has been implicated in polycystic kidney disease (PKD); positional cloning studies have

demonstrated that it is the gene which is altered in a laboratory mouse strain with this condition (Upadhya *et al.*, 2000; Vogler *et al.*, 1999). Furthermore, Nek1 has been reported to interact in a yeast-two-hybrid assay with proteins previously described to be involved in the development of PKD, such as the motor transport protein, KIF3A, and the signalling protein, tuberine (Surpili *et al.*, 2003). Primary cilium proteins have been implicated in the etiologies of various PKDs suggesting that Nek1 may be involved in cilium control. In support of such a function, Nek1 has been shown to localize to primary cilia, basal bodies and centrosomes and its overexpression in canine kidney epithelial cells inhibited ciliogenesis, whilst MEF cell lines mutated for Nek1 exhibit abnormal cilia (Shalom *et al.*, 2008; White and Quarmby, 2008; Mahjoub *et al.*, 2005). This is postulated to be a result of defective centrosome/basal body function: expression of a dominant-negative form of Nek1 resulted in disruption of centrosome organization (White and Quarmby, 2008).

Additionally, interactions between Nek1 and the DNA damage repair/checkpoint proteins, MRE11 and 53BP1, have been identified and there is evidence for a putative role in the response to ionizing radiation (IR)-induced DNA damage (Surpili *et al.*, 2003; Polci et al., 2004). IR appears to rapidly induce Nek1 kinase activity and the redistribution of Nek1 to sites of DNA double strand breaks, whilst interference with Nek1 function appears to make cells more sensitive to IR (Polci *et al.*, 2004). To date, little evidence has suggested a direct role for Nek1 in mitosis, although recently it has been reported that, like NIMA, ectopic expression of Nek1 results in chromatin condensation, however, the physiological relevance of this is, as yet, unclear (Feige *et al.*, 2006).

Structurally, Nek8 is quite divergent from NIMA. Aside from the prerequisite N-terminal kinase domain similarity, it has no coiled-coil or PEST motifs but instead it possesses an RCC1-like domain (O'Connell *et al.*, 2003). Like Nek1, Nek8 has been implicated in the etiology of PKD and much of the data surrounding Nek8 has focused on this. Positional cloning identified the *nek8* gene to be the one mutated in *jck* mouse models of PKD and injection of zebrafish embryos with a morpholino anti-sense oligonucleotide corresponding to the orthologue of Nek8 resulted in the PKD phenotype (Liu *et al.*, 2002). Nek8 is thought to be involved in regulation of the local cytoskeletal structure in collecting duct epithelial cells as overexpression of inactive mutants of Nek8 in such cells results in enlarged, multinucleated cells with abnormal actin cytoskeletons and a reduced number of actin stress fibres (Liu *et al.*, 2002). Moreover, overexpression of kinase-deficient Nek8 in U2OS cells interfered with actin expression (Bowers and Boylan, 2004). Nek8 has since been reported to localize to the proximal region of the

primary cilium in IMCD-3 mouse kidney epithelial cell lines and mutated versions of the protein are mislocalized. This indicates that Nek8 may have a role in ciliary signaling, which is emerging as an important factor in the etiology of PKD (Mahjoub *et al.*, 2005; Trapp *et al.*, 2008; Otto *et al.*, 2008). It is not yet clear whether Nek8 has any role in regulation of mitotic progression, although Nek8 was only observed on primary cilia during interphase and was notably absent from dividing cells (Mahjoub *et al.*, 2005). Interestingly, *Hs*Nek8 mRNA is reported to be upregulated in primary human breast tumors (Bowers and Boylan, 2004).

Mammalian Nek3 similarly does not appear to have an obvious mitotic role. Indeed, functional studies of mammalian Nek3 provide little evidence to support a role for Nek3 in cell cycle control. Murine Nek3 shows little or no cell cycle- or tissue-specific variation in expression or activity levels and antibody microinjection or overexpression of either wild type or catalytically inactive forms of Nek3 produced no obvious phenoytpes. Furthermore, murine Nek3 shows no distinct pattern of subcellular localization and is predominantly cytoplasmic (Tanaka & Nigg, 1999). However there is one report of increased murine Nek3 expression in mitotically active cells (Chen *et al.*, 1999). Human Nek3 did appear to be more highly expressed in testis, ovary and brain tissue, although there was no cell cycle-dependent variation in expression (Kimura and Okanu, 2001). More recent evidence suggests that Nek3 may act to activate the Vav2 guanine nucleotide exchange factor in a prolactin-mediated signaling pathway important in cytoskeletal reorganization in breast cancer cells, thus implicating it in the etiology of breast cancer (Miller *et al.*, 2005; Miller *et al.*, 2007).

Nek11 also contains an N-terminal NIMA-related catalytic domain and, like NIMA, its C-terminus contains predicted coiled-coil and PEST sequences (Noguchi *et al.*, 2002). The protein exists in two isoforms, Nek11Long (Nek11L) and Nek11Short (Nek11S), probably as a result of alternative splicing. Of these, Nek11L is the most abundant protein in those somatic cells tested. Nek11L shows a cell cycle-dependent pattern of expression and localization, suggesting a potential role in cell cycle regulation although there is no direct evidence as yet of such a role. Nek11L mRNA increases in abundance as cells progress through S, G2 and M-phase. As cells enter mitosis Nek11L appears to be redistributed from the nucleus, where it resides in interphase, to become associated with the microtubules of the mitotic spindle. The Nek11L signal then becomes undectable as cells exit mitosis (Noguchi *et al.*, 2002). Nek11L appears to be regulated by the DNA replication/damage stress response pathways and may be involved in checkpoint responses: the kinase activity of the protein appears to be activated in response to DNA-damaging agents and DNA replication inhibitors. Moreover, expression

of kinase-deficient Nek11L resulted in abrogation of an aphidicolin-induced S-phase arrest and subsequent cell lethality (Noguchi *et al.*, 2002). There is also evidence that this activation of Nek11 in response to DNA damage/replication blocks may be as a result of interaction with Nek2A at the nucleolus of G1/S arrested cells. Nek11 was reported to colocalise with Nek2A in the nucleoli of U2OS cells and both proteins coimmunoprecipitated from the nuclear fractions of U2OS and HeLa cell lysates, with complex formation increasing in G1/S arrested cells. Furthermore, Nek2A can phosphorylate the C-terminal region of Nek11 *in vitro*, increasing its activity (Noguchi *et al.*, 2004).

1.3.4.4 Mammalian NIMA homologues implicated in mitosis: Nek2

Mammalian Nek2 is among the best-characterized of the NIMA-related kinases. It is also the most closely-related to NIMA structurally; the two proteins share 47% sequence identity in their catalytic domains and, like NIMA, Nek2 has a C-terminal extension containing a coiled-coil motif through which it forms heterodimers (Fry *et al.*, 1999; Fry *et al.*, 1995). Moreover, Nek2 and NIMA share many biochemical properties and both proteins display similar cell cycle-dependent patterns of expression, activity, and localization (Lu et al, 1993; Fry *et al.*, 1995; Schultz *et al.*, 1994; Fry *et al.*, 1998). Nonetheless, there is no evidence that Nek2 can complement a NIMA temperature-sensitive mutation and as such it cannot be considered a true functional homologue of NIMA (O'Connell *et al.*, 2003).

Nek2 exists as three different splice variants, Nek2A, Nek2B and the recently identified Nek2C (Hames and Fry, 2002; Fardilha et al., 2004; Wu *et al.*, 2007). Nek2A and Nek2B result from the presence of an alternative polyadenylation signal within the last intron of the mRNA, which prevents splicing to the final exon in Nek2B. As a result Nek2A and Nek2B differ in their extreme C-termini, with Nek2B specifically lacking sequences important for Nek2 regulation and, as a result, cell cycle expression (Hames and Fry, 2002). Nek2C contains a short deletion at the position of this splice site and thus differs from Nek2A by only 8 amino acids. This deletion results in the presence in Nek2C of a strong nuclear localization sequence, which is absent from Nek2A and Nek2B and this provides a mechanism for regulating Nek2 localization and thus function (Fardilha *et al.*, 2004; Wu *et al.*, 2006).

Nek2 expression is maximal in S and G2 phases of the cell cycle as a result of cell cycle dependent transcription (Twomey *et al.*, 2004). However, Nek2A is rapidly degraded upon entry into mitosis whilst Nek2B persists throughout mitosis. This is due to the alternative splicing, which results in the absence in Nek2B of the degradation motifs

which direct Nek2A for proteolysis (Schultz *et al.*, 1994; Fry *et al.*, 1995; Hames and Fry, 2001). Nek2B is nonetheless a relatively short-lived protein, although the mechanism of Nek2B degradation remains unclear. Nek2A is hyperactivated by autophosphorylation at the onset of mitosis and this is thought to be dependent upon the inactivation of protein phosphatase 1 (PP1) (Helps *et al.*, 2000; Rellos *et al.*, 2005). Nek2A possesses a KVHF motif in its C-terminal tail to which PP1 can bind, maintaining Nek2A in a dephosphorylated, inactive state (Hames and Fry, 2002). On entry into mitosis PP1 is inactivated, both by inactivatory phosphorylation and by the competitive binding of inhibitor 2 (Ihn-2) (Helps *et al.*, 2000; Eto *et al.*, 2002). The focal adhesion scaffold protein, HEF1, may also negatively regulate Nek2 (Pugacheva and Golemis, 2005). The difference in the regulation of Nek2A and Nek2B points to potential differing substrates and/or roles. The major portion of both endogenous and ectopically expressed recombinant Nek2 localizes to the centrosome throughout the cell cycle with a small portion of the protein localizing to microtubules of the cell cytoskeleton. This may reflect a trafficking role for the cytoplasmic microtubules in delivering Nek2 to the centrosome (Fry *et al.*, 1998; Faragher and Fry, 2003; Hames *et al.*, 2005).

The major role of Nek2 at the centrosome appears to be in initiating the separation of duplicated centrosomes upon entry into mitosis, thus enabling bipolar spindle formation (Fry, 2002; Fargher & Fry, 2003). Thus, overexpression of wild-type Nek2A causes premature centrosome separation in interphase cells, whilst expression of catalytically-inactive Nek2A mutants results in monopolar spindle formation (Faragher and Fry, 2003). Similarly, RNAi depletion of Nek2 inhibits centrosome separation without significantly interfering with entry into mitosis (Fletcher *et al.*, 2005). Mechanistically, Nek2 can interact with and phosphorylate C-Nap1 and rootletin, two core components of the intercentriolar linkage (Bahe *et al.*, 2005; Fry *et al.*, 1998; Mayor *et al.*, 2000). Both of these proteins can interact with one another, are present at the centrosome during interphase but are displaced late in G2, and expression of exogenous Nek2 has been shown to stimulate this dissociation in a kinase-dependent manner (Bahe *et al.*, 2005; Mayor *et al.*, 2002). In summary, the current data supports a model in which C-Nap1, tethered to the proximal ends of centrioles, interacts with the globular N-terminal portion of rootletin leaving the C-termini of rootletin loose to form a dynamic intercentrosomal linker. Activation of Nek2 in late G2 results in phosphorylation of C-Nap1 and rootletin and their subsequent dissociation from the centrosome and loss of centrosomal cohesion, allowing centrosomes to be driven apart by kinesin motor proteins (O'Regan *et al.*, 2007). Another component of the intercentriolar linkage, Cep68, has recently been described but it is not known whether it also acts as a substrate for Nek2 (Graser *et al.*, 2007). A further substrate of Nek2, β-catenin has

also been described which may be an additional component of the intercentriolar linkage. β-catenin localizes to the centrosome in mitosis, specifically co-localizing with rootletin, with which it appears to exist in a complex (Bahmanyar *et al.*, 2008).

A second potential role for Nek2 in centrosomal regulation was revealed by the identification of two other centrosomal substrates of Nek2, Ninein-like protein (Nlp) and centrobin. Both of these are large coiled-coil proteins believed to be structural components of the PCM involved in anchoring microtubules at the centrosome during interphase (Jeong *et al.*, 2007; Rapley *et al.*, 2005). Endogenous Nlp is phosphorylated and displaced from the centrosome at the G2/M transition and overexpression of Nlp results in the formation of aberrant mitotic spindles, presumably as a result of its persistence at the centrosome following entry into mitosis (Casenghi *et al.*, 2003; Rapley *et al.*, 2005). Not only can Nek2 phosphorylate Nlp, but coexpression of Nlp and Nek2 results in Nlp mislocalization (Rapley *et al.*, 2005). Nlp is also a substrate for Plk1 and one hypothesis proposes that phosphorylation of Nlp by Nek2 might prime it for phosphorylation by Plk1.

The centrosomal localization of Nek2 is consistent with its roles in maintenance and modulation of centrosomal architecture (Fry *et al.*, 1998). However, Nek2 has also been reported to localize to the nucleus during S-phase and to associate with chromosomes and kinetochores in early mitosis and the midbody during telophase, hinting at non-centrosomal functions for Nek2 (Kim *et al.*, 2002). First of all, Nek2 has been implicated in chromatin condensation as a result of an interaction with the chromatin-bound high mobility group protein A2 (HMGA2). It appears that Nek2 interacts with HMGA2 via its C-terminus and its phosphorylation resulted in a decreased affinity for chromatin, leading to its release and eventual chromatin condensation (Di Agostino *et al.*, 2004). The Nek2C splice variant of Nek2 localizes to the nucleus, but, as overexpression of Nek2C does not promote chromatin condensation or phosphorylation of histone H3 in interphase cells, the significance of this localization is unclear (Wu *et al.*, 2007).

Nek2 has also been reported to localize to kinetochores and be involved in the SAC. Nek2 has been shown to specifically bind Hec1 and phosphorylate it on Ser165 in human cells and this phosphorylation appears to be essential for accurate chromosome segregation in both human and budding yeast cells (Chen *et al.*, 2002). An interaction between Nek2 and Mad1 was identified in a yeast two-hybrid screen and Nek2 was subsequently shown to co-localize with Mad1 at kinetochores, although the association between Nek2 and Mad1 was not necessary for Mad1 localization and RNAi depletion of

Nek2 had no effect on the localization of Mad1 to kinetochores (Lou *et al.*, 2004). Thus the functional significance of these interactions remains unclear.

A final putative role in the regulation of cytokinesis is suggested by the localization of Nek2 to the midbody of cells in late mitosis. Nek2B persists in late mitotic cells and specific depletion of Nek2B has been reported to delay mitotic exit (Fletcher *et al.*, 2005). Moreover, *Drosophila* Nek2 localizes to the midbody in late mitosis and its overexpression was reported to result in ectopic cleavage furrow formation and cytokinesis failure, possibly as a result of mislocalization of actin and anillin (Prigent *et al.*, 2005). However, as for many of the non-centrosomal roles, more work is required to substantiate these proposed functions.

1.3.4.5 Mammalian NIMA homologues implicated in mitosis: Nek9

Nek9 was originally identified in two separate studies: firstly, as a β-casein kinase activity from rabbit lung (Nek9 was originally called Nek8 in this study and then renamed Nek9) and secondly, as a binding partner of Nek6 (Nek9 was originally called Nercc1 in this study) (Roig *et al.*, 2002; Holland *et al.*, 2002). Like the majority of the mitotic protein kinases described here, Nek9 has a cell cycle-regulated pattern of localization and expression, implicating it in regulation of mitotic progression (O'Regan *et al.*, 2007). Nek9 possesses an N-terminal catalytic domain with 33% sequence identity to the catalytic domain of NIMA and 39-44% sequence identity to the catalytic domain of other mammalian Neks. This is followed by an RCC1-like domain and a predicted coiled-coil motif (Roig *et al.*, 2002). Evidence suggests that Nek9 can interact with Nek6 and Nek7 and that together these proteins regulate mitotic progression, with specific roles in spindle dynamics and chromosome separation (Roig *et al.*, 2002; Roig *et al.*, 2005).

Whilst Nek9 expression levels are relatively unchanging throughout the cell cycle, Nek9 is avidly activated during mitosis as a result of a phosphorylation event on a conserved threonine residue within the activation loop of the catalytic domain (Roig *et al.*, 2002; Holland *et al.*, 2002). Whether this is the result of phosphorylation by an exogenous kinase or an autophosphorylation event remains unclear, but Nek9 mutants which lack the coiled-coil dimerization domain are impaired in their catalytic activity. Notably, however, the Nek9 sequence contains a number of predicted Cdk1 phosphorylation motifs and Cdk1 can phosphorylate and activate Nek9 *in vitro*, although the *in vivo* significance of this remains unclear (Roig *et al.*, 2002). Nek9 localization is cell cycle-regulated in as much as the mitotic, active, form of Nek9 appears to localize to the centrosome and spindle poles during early mitosis, before becoming associated with

chromosomes following the metaphase/anaphase transition and, finally, with the midbody during late mitosis (Roig *et al.*, 2005). Consistent with this, *Xenopus* Nek9 was detectable on the poles of spindles assembled *in vitro* in *Xenopus* egg extracts (Roig *et al.*, 2005). Moreover, *Hs*Nek9 coimmunoprecipitates with γ-tubulin and XNek9 coimmunoprecipitates in a multiprotein complex that contains γ-tubulin as well as other components of the γ-TuRC, suggesting that active Nek9 may, like Nek2, play a direct role in regulating microtubule nucleation and mitotic spindle assembly (Roig *et al.*, 2005). Nonetheless, this active form of Nek9 represents only a small fraction of the total cellular pool of Nek9, the majority of which is diffusely distributed in the cytoplasm both in interphase and mitosis (Roig *et al.*, 2002; Holland *et al.*, 2002; Pelka *et al.*, 2007).

Consistent with its localization and regulation, functionally, Nek9 appears to be involved in regulation of mitotic progression, specifically in the assembly of the mitotic spindle. Overexpression of both wild-type and catalytically-inactive Nek9 has been reported to prevent normal progression through mitosis and results in apoptosis in cultured human cells (Roig *et al.*, 2002). However, RNAi depletion of Nek9 in human cells, or immunodepletion of Nek9 from *Xenopus* egg extracts, did not appear to disrupt cell cycle progression, suggesting that Nek9 is not essential for mitotic entry in the same way that NIMA is (Pelka *et al.*, 2007; Roig *et al.*, 2005), although, microinjection of anti-Nek9 antibodies into interphase cells prevented entry into mitosis. When antibody microinjection was performed on prophase cells, cells exhibited defective mitoses, with spindle abnormalities, chromosome alignment and/or separation defects, and/or prometaphase arrest, dependant upon the cell line used (Roig *et al.*, 2002). Similarly, immunodepletion of *X*Nek9 from *Xenopus* egg extracts impaired spindle formation resulting in delayed spindle assembly or the assembly of disorganized mitotic spindles. Similar abnormalities were observed in spindle aster assembly induced by Ran-GTP. Both phenotypes could be rescued by addition of purified recombinant XNek9 (Roig *et al.*, 2005). This then, implicates Nek9 in regulation of both centrosomal and acentrosomal pathways of spindle assembly. There is evidence to suggest that Nek9 can interact through its RCC1-like domain with the Ran GTPase (Roig *et al.*, 2005). As described earlier, this is a major contributor to spindle formation by stabilization of microtubules in the vicinity of chromosomes (Weise *et al.*, 2001; Wilde *et al.*, 2001). Thus, it is possible that Nek9 may regulate spindle formation via Ran-GTPase. However, whether Nek9 can function as an active GEF has not been tested, and it is worth noting that the RCC1-like domain of Nek9 lacks key residues which would make it an active GEF. Moreover, Nek9 activity does not appear to be altered as a result of its interaction

with the Ran GTPase, thus it is unclear exactly how Nek9 and Ran can cooperate in spindle formation (Holland *et al.*, 2002; Roig *et al.*, 2002).

One of the original studies which identified Nek9, also identified a putative Nek9 interacting partner, Bicaudal-D2 (BicD2), which co-chromatographed with Nek9 activity (Holland *et al.*, 2002). BicD2 is a coiled-coil protein implicated in dyenin-mediated microtubule-dependent transport and, more recently, microtubule anchoring at the centrosome (Fumoto *et al.*, 2006). Thus, this interaction, like the interaction of Nek9 with γ-TuRC components may provide a mechanism through which Nek9 directly regulates microtubule nucleation and anchoring.

Finally, the second original Nek9 study identified it as a result of its strong interaction with Nek6, suggesting a functional relationship between these protein kinases which extends to the closely-related Nek7 (Roig *et al.*, 2002; Belham *et al.*, 2003). Studies involving a variety of Nek6 and Nek9 mutants have demonstrated that for this interaction to occur Nek6, but not Nek9, must be active. Moreover, Nek9 is capable of activatory phosphorylation of Nek6 and Nek7 *in vitro* and co-expression of wild-type but not catalytically-inactive Nek9 with Nek6 or Nek7 resulted in increased kinase activity and slowed electrophoretic mobility of Nek6 and Nek7 (Belham *et al.*, 2003). This, along with functional analyses of Nek6 and Nek7 has led to the postulation of a putative mitotic protein kinase cascade in which Nek9 acts upstream of Nek6 and Nek7 to regulate their mitotic functions (Figure 1.10) (Belham *et al.*, 2003). The functional evidence for this proposed interaction is presented below.

1.3.4.6 Mammalian NIMA homologues implicated in mitosis: Nek6 and Nek7

Originally identified in a classic biochemical screen for kinases capable of phosphorylating and activating the hydrophobic regulatory site of the p70 ribosomal S6 kinase ($p70^{S6K}$), Nek6 and Nek7 represent a pair of small, structurally simple and closely related protein kinases (Belham *et al.*, 2001). They share 87% amino acid identity in their kinase domain and are composed simply of a NIMA-like catalytic domain with short, divergent N-terminal extensions. They lack entirely the C-terminal regulatory sequences exhibited by NIMA and, indeed, most other Neks (Kandli *et al.*, 2000; O'Connell *et al.*, 2003). Despite their initial identification as kinases capable of phosphorylating $p70^{S6K}$ (Belham *et al.* 2001) this is not thought to reflect a true physiological interaction (Lizcanzo *et al.*, 2002). In fact, the functional characterization of Nek6 and Nek7 has now focused on potential roles as mitotic protein kinases, as a result of data suggesting they act downstream of Nek9 in a NIMA-related kinase cascade involved in controlling mitotic progression (Belham *et al.*, 2003).

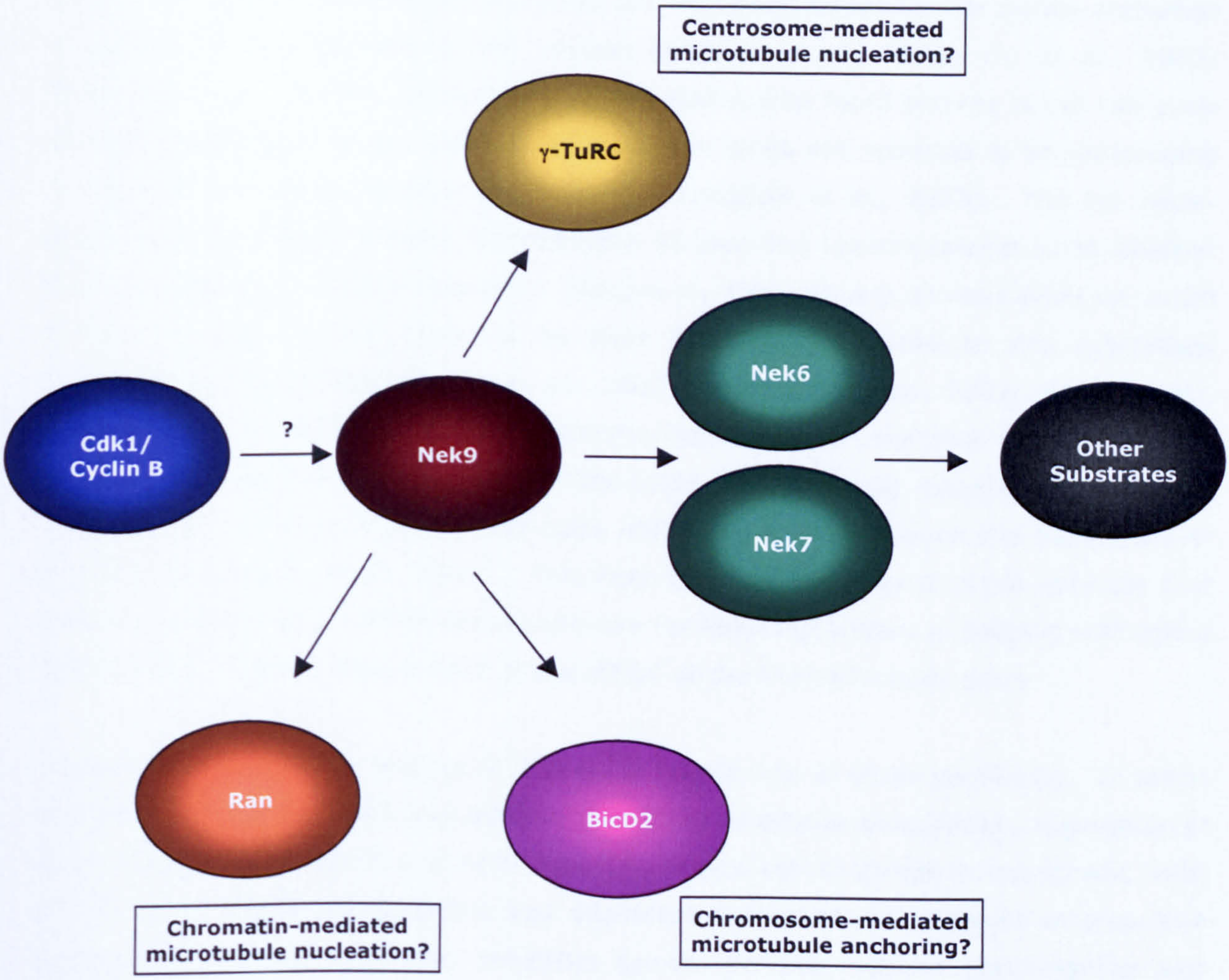


Figure 1.10 The potential Nek9-Nek6/Nek7 mitotic kinase cascade
A schematic summary of our current understanding of the interactions of Nek9 during mitosis, which include its potential interaction with Nek6 and Nek7. The white boxes suggest speculative functions mediated as a result of these interactions. Both Nek9 and Cdk1/cyclin B localize to the centrosome and Cdk1 can phosphorylate and activate Nek9 *in vitro* leading to speculation that Nek9 may be activated by cyclin B upon entry into mitosis. Once active, the interaction of Nek9 with the γ-TuRC and BicD2 may provide a mechanism by which Nek9 can regulate centrosome-mediated microtubule nucleation and anchoring. It is also attractive to speculate that the RCC1-like domain of Nek9 may mediate an interaction with Ran, thus providing a route though which Nek9 may regulate chromatin-mediated microtubule nucleation. Nek9 is proposed to activate Nek6 and Nek7 which may provide another pathway for the regulation of mitotic spindle organization and chromosome segregation, although substrates of Nek6 and Nek7 remain to be identified. Taken from O'Regan *et al.*, 2007.

Whilst many of the mitotic protein kinases discussed above have obvious cell cycle-regulated patterns of expression and localization that implicate them in regulation of mitotic progression, the situation with Nek6 and Nek7 is less well defined. There is evidence to suggest that endogenous Nek6 is activated in mitosis, although whether this is due to increased Nek6 mRNA transcription, increased translation or purely activation of protein already present is still unclear (Belham *et al.*, 2003; Yin *et al.*, 2003; Hashimoto *et al.*, 2002). Indeed, one report claims that Nek6 activity is not cell cycle regulated (Minoguchi *et al.*, 2003). Nek7 protein levels are reported to be unchanging throughout the cell cycle (Kim *et al.*, 2007; Minoguchi *et al.*, 2003). The cell cycle-dependent localization of Nek6 has not been studied and reports conflict as to whether Nek6 is cytoplasmic and/or nuclear in interphase, although one obvious point on which all reports agree is that there is no clear localization of Nek6 to any subcellular structures during interphase (Yin *et al.*, 2003; Hashimoto *et al.*, 2002; Kandli *et al.*, 2000; Chen *et al.*, 2006). However, recently, Nek7 has been reported to localize to the centrosome in both interphase and mitotic U2OS cells and may localize to the spindle midbody and midzone of late mitotic cells, although reports differ on this point (Kim *et al.*, 2007; Yissachar *et al.*, 2006). This finding is significant as it is not only the first concrete evidence for a NIMA-like mitotic role for Nek7 but it also, in keeping with many other mitotic kinases, places Nek7 at the MTOC at the time of mitotic entry.

Functional data for Nek6 and Nek7 is likewise sparse and at times conflicting. In terms of a putative role for Nek6 and Nek7 in regulation of mitotic progression, expression of catalytically-inactive Nek6 and Nek7 typically causes cell to arrest in metaphase, with normal chromosome condensation and alignment but unable to complete chromosome segregation. These cells also exhibited spindle defects, nuclear abnormalities and apoptosis (Yin *et al.*, 2003; Yissachar *et al.*, 2006; Kim *et al.*, 2007). Similarly, RNAi depletion of both endogenous Nek6 and Nek7 in HeLa cells appeared to result in the same phenotypes, with cells arresting in mitosis with abnormal or no mitotic spindles before undergoing apoptosis (Yin *et al.*, 2003; Kim *et al.*, 2007). However, as is becoming a common theme, reports differ as to the type of spindle abnormalities seen. In one report, Nek7 depleted cells were described as arresting in prometaphase with nuclear envelope breakdown, chromosome condensation and duplicated, but not separated centrosomes and no visible spindle (Kim *et al.*, 2007), whilst another report described multipolar spindles as being the most common result of RNAi depletion of Nek7 (Yissachar *et al.*, 2006). Furthermore, there is one report which claims that overexpression of either wild-type of kinase-inactive Nek6 or Nek7 had no effect on cell cycle progression (Minoguchi *et al.*, 2003). Nonetheless, the consensus phenotypes seen upon interference with Nek6 and Nek7 are reminiscent of those observed upon

interference with Nek9 (Roig *et al.*, 2005). Further to this, RNAi depletion of Nek7 was shown to reduce the microtubule nucleating capacity of cells as well as levels of γ-tubulin detectable at the centrosome. This implicates Nek7 in microtubule nucleation and specifically recruitment of γ-tubulin to the centrosome (Kim *et al.*, 2007). Thus, there is clear support for a novel mitotic kinase cascade involving Nek6, Nek7 and Nek9, although whether this functions to regulate centrosome separation, centrosome maturation or microtubule nucleation remains unclear.

The possible localization of Nek7 to the spindle midzone and midbody of late mitotic cells, along with reports that RNAi depletion of Nek7 results in multinucleate cells and the formation of multipolar spindles provides evidence that Nek7, and perhaps also Nek6, may function to regulate late mitotic events (Kim *et al.*, 2007; Yissachar *et al.*, 2006). Moreover, co-depletion of both Nek7 and the SAC component, Mad2, results in a failure in cytokinesis (Kim *et al.*, 2007). However, whether this reflects a second function for Nek7 in regulation of cytokinesis or is the result of segregation errors caused by abrogating Nek7 function at earlier stages of mitosis remains unclear. Nonetheless, the fact that abrogation of the SAC by depletion of Mad2 results in progression through metaphase in cells which have also been depleted of Nek7, suggests that the metaphase arrest seen upon interference with Nek7 alone may arise in part through the action of this surveillance system.

An important unanswered question is whether these two highly similar proteins share redundant functions or whether their divergent N-termini are sufficient to allow for functional and/or regulatory differences. Certainly, the high degree of sequence similarity shared by Nek6 and Nek7 suggests that they recently diverged from a common ancestor (Kandli *et al.*, 2000). Northern blot analysis and *in situ* hybridization assays demonstrate that the two show different tissue specific expression patterns and, in tissues that express both Nek6 and Nek7, the two genes are expressed in different cell populations. This could mean that the two proteins have largely redundant functions (Kandli *et al.*, 2000; Feige and Motro, 2002; Yissachar *et al.*, 2006). However, Nek6 and Nek7 have been shown to respond differently under conditions of serum deprivation; whilst Nek7 was activated by serum withdrawal, Nek6 kinase activity was downregulated, suggesting that these two kinases may have different roles in cellular signaling (Minoguchi *et al.*, 2003).

1.3.4.7 A common function for NIMA-related kinases?

Of all of the NIMA-related kinases identified to date, only NIM-1 from the closely related *N. crassa* has been shown to functionally complement a *nimA* temperature sensitive

mutation in *Aspergillus* and thus to be a true NIMA homologue (Pu *et al.*, 1995). Moreover, none of the others have been shown so far to be absolutely required for initiation of mitosis. This may suggest that the functions of NIMA have diverged throughout evolution, a situation which is in part reflected in the various structures of the NIMA-related kinases. Sequence similarity to NIMA is often restricted to the kinase domain of the protein; the coiled-coil and PEST regions of NIMA, essential for NIMA function, are often not similarly positioned or indeed, are completely lacking from other Neks (Osmani & Ye, 1996; Krein *et al.*, 1998).

Despite a lack of obvious direct functional homology, either between NIMA and other NIMA-related kinases, or amongst mammalian Neks themselves, an emerging theme in Nek function is the regulation of centrosomes, basal bodies and mitotic spindles. Although only four of the eleven mammalian Neks identified have so far been shown to have roles in mitotic regulation, these roles typically centre around regulation of microtubules, often at the centrosome. Moreover, many of the non-mitotic Neks appear to function at the basal body or centrosome in microtubule organization in cilia. Indeed, this is true of many NIMA-related kinases from a variety of organisms (Quarmby and Mahjoub, 2005; O'Regan *et al.*, 2007). Both mammalian and *Dictyostelium* Nek2 proteins localize to the centrosomes and NIMA and Fin1 localize to the SPB (De Souza *et al.*, 2000; Krein *et al.*, 2002). Activated Nek9 is centrosomal, and Nek7 may also be centrosomal (Roig *et al.*, 2005; Kim *et al.*, 2007; Yissachar *et al.*, 2006). Meanwhile, Nek1 and Nek8 have been implicated in the etiology of PKD and this is believed to result from a role for these kinases in ciliogenesis (Liu *et al.*, 2002; Upadhyda *et al.*, 2000). Similarly, Fa2p, from *Chlamydomonas* is localized to proximal ends of flagellar during interphase but is involved in microtubule severing prior to mitotic onset and becomes localized to spindle poles during mitosis (Table 1.1) (Mahjoub *et al.*, 2004).

It is now becoming clear that the biology of centrosomes (or basal bodies), cilia and cell cycle progression are closely linked. Whilst centrosomes function as MTOCs in dividing cells, organizing both the interphase cytoskeleton and the mitotic spindle, in differentiated cells, centrosomes migrate to the cell surface where the centrioles act as basal bodies to organize the axonemal microtubules of cilia and flagella (Dawe *et al.*, 2007). The emerging role of NIMA-related kinases in these processes is perhaps most dramatically revealed by the numbers of Nek genes present in different organisms. Non-ciliated eukaryotes, such as fungi and higher plants, have a limited number of Neks (Osmani *et al.*, 1991; Kambouris *et al.*, 1993; Krein *et al.* 1998; Bradley and Quarmby, 2005), whilst organisms which contain ciliated cells have numerous Neks (Table 1.2). Indeed, the genome of *Tetrahymena thermophilia*, an organism which possesses many

NIMA-Related Kinase		Function	Localization
***Aspergillus* NIMA**		Entry into and exit from mitosis Chromatin condensation Spindle formation Nuclear import of cyclin B Loading of cyclin B onto SPB	Spindle pole body
Chlamydomonas	**Fa2p**	Microtubule severing Regulation of the centrosome cycle?	Interphase: proximal ends of flagella Mitosis: spindle poles
	Cnk2p	Regulation of flagellar length and cell size	Ciliary axoneme
***T. brucei* NRKC**		Basal body separation and cytokinesis	Basal body
***S. pombe* Fin1**		Mitotic spindle formation Commitment to metaphase Mitotic exit	Spindle pole body and region between separating chromosomes
H. sapiens	**Nek1**	Centrosome organization and ciliogenesis?	Primary cilium, basal body and centrosome
	Nek2	Centrosome disjunction Microtubule anchoring Chromatin condensation? SAC signaling?	Centrosome, nucleus
	Nek6/Nek7	Mitotic spindle formation/function	Nek6: cytoplasm and/or nucleus Nek7: Centrosome?
	Nek8	Cytoskeletal organization Ciliary signaling	Primary cilium
	Nek9	Mitotic spindle formation and function	Centrosomes when active
	Nek11	DNA damage/replication stress response pathways	Interphase: Nucleolus Mitosis: spindle pole microtubules

Table 1.1 Functional conservation among NIMA-related kinases
Summary of the functions and localization of the best-characterized NIMA-related kinases from different organisms highlighting the conservation of functionality for regulation of MTOCs, microtubules and microtubule processes and localization to SPBs, centrosomes and basal bodies.

Organism	Number of Neks	Ciliated cells?
Homo	11	Yes
Mus	11	Yes
Ciona	8	Yes
Drosophila	2	Yes
Caenorhabditis	4	Yes
Saccharomyces	1	No
Dictyostelium	2	No
Arabidopsis	6	No
Chlamydomonas	10	Yes
Cyanidioschzyon	0	No
Trypanosoma	12	Yes
Tetrahymena	39	Yes

Table 1.2 The correlation between the presence of Neks and posession of cilia
Summary of the correlation between the possession of cilia and the number of NIMA-related kinases present within the genome. Adapted from Quarmby and Mahjoub, 2005.

distinct types of cilia, encodes 39 Neks and these are proposed to function in complex ciliary organization networks within this organism (Wloga *et al.*, 2006).

1.3.5 Mitotic protein kinases and cancer

The term cancer refers to a wide range of pathologies characterized by abnormal and uncontrolled cell division. The development and progression of a tumour typically occurs through a distinct series of transformation events in which it is thought that cells gradually acquire mutations which result in their unrestrained growth, via errors in DNA replication and/or exposure to environmental mutagens. These may include activation of one or more oncogenes, loss of tumour suppressor genes, deregulation in growth signaling pathways and deregulation of cell cycle control and control of the centrosome cycle (Brinkley and Goepfert, 1998). Deregulation of protein kinases involved in regulation of mitotic mechanics is a potential mechanism by which the genetic instability commonly associated with cancer could arise. Malignant cells are characterized by genetic instability and in particular most cancer cells are aneuploid, often containing between 60 and 90 chromosomes; indeed many individual cancers contain cells that not only possess an abnormal chromosome complement but that also differ from each other in the number of chromosomes they contain (Rajagopalan & Lengauer, 2004).

It is not yet clear whether this genetic instability is a cause or a consequence of cancer. Initially it was believed to be a consequence of deregulated growth in cancer cells whose initial malignant transformation had been brought about by mutations in oncogenes and tumour suppressor genes (Hameroff, 2004). More recently, however, this view has been revised and it is speculated that genetic instability as a result of deregulated mitosis could be a cause of malignant transformation (Duesberg *et al.*, 2000; Marx, 2002). Whatever the route, evidence is accumulating that deregulation of cell cycle kinases underlie the deregulated proliferation and aberrant cell division and consequent genetic instability commonly associated with cancer (Malumbres and Barbacid, 2001; Malumbres and Barbacid, 2007).

Indeed, many of the protein kinase families discussed above and known to be important in regulation of the mitotic machinery, have been linked to cancer progression. For example, the three Aurora genes map to chromosomal loci that are frequently altered in human tumours (Katayama *et al.*, 2003). Plk1 is overexpressed in many different cancer types with a direct correlation between levels of Plk1 and poor prognosis (Strebhardt and Ullrich, 2006). Furthermore, whilst mutations in Cdks are not frequently observed in human tumours, a significant fraction of cells carry mutations resulting in misregulation of Cdk activity, for example those that affect expression of

cyclins or of Cdk inhibitors (Malumbres and Barbacid, 2007). Among the NIMA-related kinases, Nek2 mRNA and protein expression levels are elevated in cell lines derived from a range of human tumours, as well as primary breast tumours (Hayward *et al.*, 2004; de Vos *et al.*, 2003; Wai *et al.*, 2002; Kokuryo *et al.*, 2007). Interestingly, Nek6 has also been mapped, by fluorescence in situ hybridisation analysis, to a locus at which loss of heterozygosity has been associated with several cancers (Hashimoto *et al.*, 2002). Furthermore, Nek6 has been shown to be upregulated in hepatocellular carcinoma (HCC) in association with the peptidyl-prolyl-isomerase Pin1 (Chen *et al.*, 2006). Pin1 overexpression is prevalent in a variety of cancers and Nek6 was shown to interact with Pin1 in HCC cell lines, providing a potential link between Nek6 and carcinogenesis.

Advances in our understanding of how cell cycle processes are controlled and the role that these regulatory mechanisms play in tumorigenesis are expected to lead to a paradigm shift in tumour treatment from the non-specific targeting of all proliferating cells by anti-microtubule agents such as paclitaxel, to the development of highly specific therapeutic agents designed to treat the tumour-specific lesion. One such group of treatments is small molecule mitotic kinase inhibitors which typically aim to induce aberrant mitosis in tumour cells and thus result in mitotic arrest and cell death (Jackson *et al.*, 2007). Small molecule inhibitors have been identified for Aurora-A, Aurora-B, Plk1 and Cdk1 and are being developed for potential clinical use in the future (Jackson *et al.*, 2007). The identification of Nek2 upregulation in human cancers along with its well-defined role in cell-cycle regulation, similarly makes it an attractive target for small molecule inhibitor development (Hayward *et al.*, 2004). With a better understanding of the mitotic functions of Nek6, Nek7 and Nek9, these too may provide new targets for cancer treatment.

1.4 Aims and Objectives

There are clearly more questions than answers regarding Nek6 and Nek7 function and their potential role in a putative mitotic Nek cascade. Their mitotic localization and regulation is at yet unclear, as is the requirement for their protein kinase activity for cell cycle progression. Further analyses of the cell cycle dependent regulation and localization, particularly of Nek6, are clearly needed as are more detailed analyses of the effects of interfering with Nek6 and Nek7 function. If Nek6 and Nek7 do regulate mitotic entry and specifically spindle formation and organization, more detailed analyses of these structures under such circumstances is clearly required. Moreover, no substrates of Nek6 or Nek7 have yet been identified, although mitotic spindle and centrosome components would be obvious candidates. Such analyses should not only

give insights into the functions of Nek6 and Nek7 but also help to reveal any functional differences between these two highly similar proteins. The aim of this project is to use a variety of biochemical and cell biology based assays to carry out a detailed functional characterization of Nek6 and Nek7, focusing particularly on their postulated role in mitotic regulation, as well as the identification of their substrates.

Specific experimental aims are as follows:

1. To generate, affinity purify and characterize anti-Nek6 and anti-Nek7 antibodies to be used for functional analysis of Nek6 and Nek7. Antibodies would be raised in rabbits against N-terminal specific peptides of Nek6 and Nek7 in order to maximize the chances of generating specific antibodies. Following purification, these would be used to assess cell cycle-dependent localization and regulation.

2. To investigate the effects of overexpression of wild-type and kinase-deficient versions of Nek6 and Nek7 on cell cycle progression. Immunofluorescence microscopy would be used to examine the mitotic and apoptotic indices of cultured mammalian cells expressing the proteins as well as flow cytometry analysis of cell populations transiently transfected with wild-type and catalytically-inactive versions of Nek6 and Nek7.

3. To investigate the effect of RNAi depletion of Nek6 and Nek7 on cell cycle progression by examining mitotic and apoptotic indices in depleted cell populations using flow cytometry and immunofluorescence microscopy. The generation of good, specific antibodies to Nek6 and Nek7 would be fundamental to achieving this aim.

4. To characterize the mitotic phenotypes observed upon interference with Nek6 and Nek7 function as a result of RNAi depletion or expression of kinase-deficient versions of the protein.

5. To identify potential substrates and interacting partners of Nek6 and Nek7 using Kinase Substrate Tracking and Elucidation (KESTREL) assays as well as coimmunoprecipitation and kinase assays.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Suppliers and manufacturers

All chemicals were of analytical grade purity or higher and supplied by Sigma (Poole, UK), Roche (Lewes, UK), or as stated below.

Reagent	Supplier
siRNA oligoribonucleotides SiPort NeoFX transfection reagent	Ambion (Austin, USA)
ECL plus Western blotting reagent CNBr activated sepharose Deoxyribonucleotides (dATP, dCTP, dGTP, dTTP)	Amersham Pharmacia Biotech (Buckinghamshire, UK)
Annexin V/FITC apoptosis detection kit	Bender MedSystems (Burlingame, USA)
Protein A beads Poly-prep columns	BioRad (Hemel Hempstead, UK)
Chloramphenicol Hoechst 33258	Calbiochem (Nottingham, UK)
Glacial acetic acid EDTA EGTA NaCl KCl Na_2HPO_4 KH_2PO_4	Fisher Scientific (Loughborough, UK)
Bovine serum albumin (fraction V)	Fluka (Gillingham, UK)
Super RX X-Ray film	Fuji Photo Film (Tokyo, Japan)
Ethidium Bromide GeneTailor site directed mutagenesis kit SuperScript III reverse transcriptase NI-NTA agarose Oligonucleotide primers DH5α-T1R max efficiency chemically competent bacteria Gateway cloning enzymes (LR and BP clonase) Protein G beads Penicillin/streptomycin Fetal bovine serum D-MEM with Glutamax MEM Opti-MEM Lipofectamine 2000	Invitrogen/Gibco (Paisley, UK)
GeneRuler 1 kb DNA ladder	MBI Fermentas (York, UK)
Protogel liquid acrylamide (30% w/v)	National Diagnostics (Hessle, UK)

Bacto-agar Bacto-tryptone Yeast extract	Oxoid (Basingstoke, UK)
Slide-A-Lyzer dialysis cassettes BCA protein assay reagent	Pierce (Rockford, USA)
TnT T7 quick coupled transcription/translation kit	Promega (Southampton, UK)
QIAfilter plasmid miniprep spin kit QIAfilter plsmid maxiprep kit QIAquick PCR purification kit QIAquick gel extraction kit	Qiagen (Hilden, Germany)
Restriction endonucleases Expand high fidelity DNA polymerase Rapid DNA ligation kit Shrimp alkaline phosphatase	Roche (Lewes, UK)
Nitrocellulose transfer membrane	Schleicher & Schuell (Dassel, Germany)
His_6Nek6 His_6Nek7	Upstate (Dundee, UK)
3MM chromatography paper	Whattman International (Maidstone, UK)

2.1.2 Radioisotopes

Isotope	Specific Activity	Supplier
[γ-^{32}P]-ATP	167 Tbq/mmol	PerkinElmer
[^{35}S]-methionine	43.5 Tbq/mmol	NEN Life Sciences Products

2.1.3 Vectors

Vector	Application	Supplier
pETM-11	Bacterial protein expression	EMBL
pCMV-Tag-3C	Eukaryotic protein expression	Stratagene
pFLAG-CMV2	Eukaryotic protein expression	Sigma
pcDNA-Dest53	Eukaryotic protein expression	Invitrogen

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Antibody#	Dilution ([Antibody])*	Supplier
Anti-FLAG Mouse monoclonal (M2, F3165)	1:1000 (0.5 µg/ml)	Sigma
Anti-GFP Mouse monoclonal (GFP-20, G6539)	1:500 (0.25 µg/ml)	Abcam
Anti-GFP Rabbit polyclonal (ab6556)	1:1000 (0.1 µg/ml)	Abcam
Anti-FLAG Rabbit polyclonal (F7425)	1:500	Sigma
Anti-Myc Mouse monoclonal (9B11, 2276)	1:1000	Cell Signalling Technology
Anti-α-tubulin Mouse monoclonal (B-5-1-2, T5168)	1:2000 (0.3 µg/ml)	Sigma
Anti-γ-tubulin Mouse monoclonal (GTU-88, T6557)	1:2000 (0.15 µg/ml)	Sigma
Anti-α-tubulin Rabbit polyclonal (Ab4074)	1:200	Abcam
Anti-γ-tubulin Rabbit polyclonal (T3559)	1:500	Sigma
Anti-HSP70 Mouse monoclonal (3A3: sc-32239)	1:200 (2 µg/ml)	Cell Signalling Technology
Anti-Cortactin A Rabbit polyclonal (H-191: sc-11408)	1:200 (2 µg/ml)	Cell Signalling Technology
Anti-cleaved caspase 3 Rabbit polyclonal (ASP175)	1:100	Cell Signalling Technology
Anti-Nek6 Rabbit polyclonal	1:200 (2 µg/ml)	This work
Anti-Nek7 Rabbit polyclonal	1:200 (2 µg/ml)	This work

* Where known, final antibody concentrations are stated in brackets after the working dilution

#Catalogue numbers and clone detalts (where appropriate) are stated in brackets underneath the antibody name

2.1.4.2 Secondary antibodies

Antibody	Dilution ([Antibody])*	Supplier
Anti-mouse alkaline phosphatase conjugate	1:7500 (0.1 µg/ml)	Promega
Anti-rabbit alkaline phosphatase conjugate	1:7500 (0.1 µg/ml)	Promega
Anti-mouse horseradish peroxidase conjugate	1:1000	Sigma
Anti-rabbit horseradish peroxidase conjugate	1:1000	Sigma
Anti-mouse Alexa 488 nm	1:200 (10 µg/ml)	Invitrogen
Anti-rabbit Alexa 488 nm	1:200 (10 µg/ml)	Invitrogen
Anti-mouse Alexa 594 nm	1:200 (10 µg/ml)	Invitrogen
Anti-rabbit Alexa 594 nm	1:200 (10µg/ml)	Invitrogen

* Where known, final antibody concentrations are stated in brackets after the working dilution

2.1.5 Bacterial strains

Strain	Genotype	Supplier
DH5α library efficiency *E. coli*	Φ80d *lacZ*ΔM15, *Rec*A1, *end*A1, A1, *gyr*A96, *thi*-1, *hsd*R17(r_K-m_K^+), *sup*E44, *rel*A1, *deo*R, [Δ*lacZYA*-argF]U169	Invitrogen
DH5α-T1^R max efficiency *E. coli*	F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K-, m_{K+}) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A	Invitrogen
BL21 star (DE3) *E. coli*	F- ompT hsdS$_B$ (r_B-m_B-) gal dcm rne131 (DE3)	Invitrogen

2.2 Molecular Biology Techniques

2.2.1 Cloning

The typical cloning procedure followed involved amplification of insert DNA by polymerase chain reaction (PCR), restriction digestion of purified PCR products and destination vector and ligation of the two under appropriate conditions as outlined below. A PCR screening approach was then employed to identify colonies likely to contain plasmid with insert DNA, after which DNA was prepared from bacterial cultures in the appropriate manner. The specific details of each stage are described below.

2.2.1.1 Oligonucleotide design

Oligonucleotides for PCR-based cloning were designed to be 25-30 bp in length with an AT:CG ratio of 50%, thus ensuring an annealing temperature of around 55-60°C. To incorporate appropriate restriction enzyme sites for cloning, additional bases were added to the 5' end of the oligonucleotide sequences with additional bases added in to ensure the maintenance of the correct reading frame upon insertion into the vector, as necessary.

2.2.1.2 Polymerase Chain Reaction

To obtain insert DNA for cloning, PCR amplification was carried out using Expand high fidelity proof-reading DNA polymerase (Roche, Lewes, UK). A standard reaction mixture was 50 µl in volume and typically contained 0.1-1 µg template DNA, 1 mM forward primer, 1 mM reverse primer, 10x PCR amplification buffer, 0.1-0.5 mM $MgCl_2$, 0.4 mM dNTPs, 1 U DNA polymerase and the appropriate volume of water to complete reaction volume. Annealing temperatures and elongation times suitable for each set of primers and template, respectively, were employed as appropriate, however the basic PCR reaction consisted of one cycle of denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at the appropriate temperature for 30 sec and elongation at 72°C for the appropriate extension time (1 min per kb), followed by one cycle of extension at 72°C for 10 min. PCR products were then analyzed by agarose gel electrophoresis to assess yield.

2.2.1.3 Agarose gel electrophoresis

DNA was combined with loading buffer (50% v/v glycerol, 100 mM EDTA, 0.3% v/v bromophenol blue) in a 1:5 ratio and resolved by electrophoresis on a 1% (w/v) agarose gel made by dissolving agarose in 1x TBE (89 mM Tris-HCl, 89 mM Boric acid, 1 mM EDTA pH 8.0) supplemented with ethidium bromide (0.5 µg/ml). Electrophoresis was carried out at 80 V for 45 min after which resolved DNA was analyzed by UV transillumination (302 nM) and images captured using a Gene Genuis CDC gel documentation system (Syngene, Cambridge, UK).

2.2.1.4 Purification of PCR products for cloning

Upon verification that the PCR reaction had been successful and sufficient levels of a product of the appropriate size obtained, PCR products were purified for cloning using a PCR purification kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Purified DNA was eluted in 50 µl of sterile water and product yield and DNA concentration assessed by agarose gel electrophoresis as described above.

2.2.1.5 Restriction enzyme digest

Purified insert DNA and destination vectors were digested with the appropriate restriction enzymes using suitable buffer conditions and temperature, as specified by the manufacturer. Typically, 2 µg of DNA was incubated with 5 U of restriction endonuclease and appropriate buffer at 37°C for 2-4 h, before total reaction volumes were electrophoresed in a 1% agarose gel as described in section 2.2.1.3. to allow purification of digested DNA by gel extraction.

2.2.1.6 Extraction of DNA from agarose gels

Following agarose gel electrophoresis of the DNA to be purified, gels were visualized by UV transillumination and the band representing the DNA to be purified was excised. DNA was extracted using QIAquick gel extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. DNA was eluted in 30 µl of sterile water and DNA yield was assessed by agarose gel electrophoresis in order to assess DNA concentrations for ligation.

2.2.1.7 DNA ligation

Ligation reactions were carried out using a rapid DNA ligation kit (Roche, Lewes, UK). Typically a vector:insert molar ratio of 1:3 was employed in a standard reaction mix of 75 ng vector DNA, 150 ng insert DNA and 5 U DNA ligase diluted in the appropriate volume of ligation buffer. Reactions were incubated for 5-15 min at room temperature.

2.2.1.8 Bacterial transformation

For cloning, 100 ng of ligated DNA was added to 50 µl of chemically competent DH5α, which had been defrosted on ice, and the two mixed by gently tapping the side of the tube. The mix was incubated on ice of 30 min, heat shocked at 42°C for 1 min to induce plasmid DNA uptake, and returned to the ice for a further 2 min. 450 µl of pre-warmed Luria Bertani (LB) media (tryptone, 10 g/l, yeast extract 5 g/l, NaCl 5 g/l pH 7.0) was added to the cells and the transformation mix was incubated at 37°, 225 rpm for 1 h. Following incubation, 200 µl of the cell suspension was spread onto LB agar plates containing the appropriate antibiotic for selection (tryptone 10 g/l, yeast extract 5g/l, NaCl 5 g/l, agar 2% w/v, pH 7.0 plus, ampicillin 100 µg/ml, chloramphenicol 34 µg/ml or kanamycin 50 µg/ml as appropriate). Plates were incubated for 12-14 h at 37°C. Colonies were then picked for insert screening by PCR and plasmid preparation.

2.2.1.9 DNA insert verification

PCR screening of colony DNA was used to verify the presence of appropriate DNA insert using Taq DNA polymerase (Invitrogen, Paisley, UK). A standard reaction mixture was 50 µl in volume and typically contained, 1 mM forward primer, 1 mM reverse primer, 1x PCR amplification buffer plus $MgCl_2$, 0-5 mM $MgCl_2$, 0.4 mM dNTPs, 1 U DNA polymerase and the appropriate volume of water to complete reaction volume. Colonies to be screened were picked and cells added to the PCR reaction mix. PCR reaction cycles were as described above in section 2.2.1.2. Colonies which proved positive by PCR screen were then grown up for DNA isolation and further insert verification by DNA sequencing.

2.2.1.10 Isolation of plasmid DNA by DNA miniprep

Individual bacterial colonies were picked off LB agar plates, inoculated into 5 ml LB containing the appropriate antibiotic and incubated at 37°C 225 rpm for 12-15 h. Following incubation, cells were recovered by centrifugation (2500 g, 10 min) and DNA was isolated using a Qiagen plasmid miniprep kit according to the manufacturer's instructions. DNA was eluted into 50 µl of sterile water.

2.2.1.11 DNA sequencing

Automated DNA sequencing was employed to verify the presence of the appropriate insert and the correct reading frame. This was carried out by Cogenics inc. (Takely, Essex).

2.2.2 Isolation of plasmid DNA by DNA maxiprep

For isolation of large quantities of high quality, pure plasmid DNA for transfection, DNA plasmid maxipreps were used. Starter cultures were made by inoculating 5 ml LB containing the appropriate antibiotic and incubating for 8 h at 37°C, 225 rpm. These were used to inoculate 100 ml LB + antibiotic at a dilution of 1:1000 which were likewise grown at 37°C 225 rpm for 12-15 h. Cells were then harvested by centrifugation at 6000x g for 15 min at 4°C before plasmid DNA was isolated using a QIAfilter plasmid maxiprep kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Isolated plasmid DNA was resuspended in 250 µl water and DNA concentration was determined by measuring OD_{260} after which DNA was diluted to a final concentration of 1 µg/µl.

2.2.3 Site-directed mutagenesis

2.2.3.1 Oligonucleotide design

Oligonucleotides for site directed mutagenesis were 30 bp in length. The forward primer consisted of an overlapping region of 18-21 bases 5′ of the mutation site, the mutation site, and an extended region of 12 bp 3′ of the mutation site. The reverse primer consisted of region overlapping the forward primer from the mutation site to the 5′ end of the forward primer and extended beyond this for a further 6-12 bp.

2.2.3.2 Site-directed mutagenesis reaction

To induce base codon changes in plasmid DNA, the Genetailor site-directed mutagenesis system (Invitrogen) was used according to the manufacturer's instructions. In brief, 100 ng of target plasmid was methylated by incubation with 4 U of DNA methylase in appropriate buffer conditions at 37°C for 1 h. Methylated DNA was then amplified by PCR with appropriate mutagenesis primers at a final concentration of 12.5 ng in a reaction mix containing 10 µM each forward and reverse primer, 10 mM dNTPs and 2.5 U Expand DNA polymerase in appropriate buffer conditions. The PCR amplification cycle consisted of denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and elongation at 68°C for 1 min/kb template DNA, and a final elongation step of 68°C for 10 min. Amplification was confirmed by agarose gel electrophoresis, following which plasmid DNA was transformed into DH5α-T1^{R} and transformants containing plasmid DNA selected for on appropriate selective media. Individual transformants were selected, plasmid DNA was isolated and the mutation was verified by DNA sequencing.

2.3 Protein Analysis Techniques

2.3.1 SDS-PAGE

Protein gels were resolved using the Mini Protean 2 polyacrylamide gel electrophoresis (PAGE) system (Bio-Rad) with a discontinuous Tris-HCl buffer system. This comprised a stacking gel (1.5 M Tris-HCl pH 6.8, 0.4% SDS (w/v), 3.9% (v/v) acrylamide) and a resolving gel (1.5 M Tris-HCl pH 6.8, 0.4% SDS, 5-18% (v/v) acrylamide with an SDS running buffer (0.1% SDS (w/v), 0.3% Tris (w/v), 1.44% glycine (w/v)). Gel polymerization was brought about by addition of 0.8% or 2.4% (v/v) TEMED and 0.015% or 0.045% (v/v) APS for stacking and resolving gels, respectively. The percentage of acrylamide in the resolving gel varied between 5-18% according to the size of the proteins to be resolved (upper and lower size ranges ~120-200 for 5% and ~10-30 for 18%). Samples to be resolved were mixed with 5x Laemmli buffer in appropriate proportions to give a final Laemmli buffer concentration of 1x.

Electrophoresis was carried out at a standard of 180 V for 1 h. Gels were then further analysed by Coomassie Blue staining or Western blotting.

2.3.2 Coomassie Blue staining

To directly visualize protein content following electrophoresis, the stacking gel was discarded and gels were soaked in Coomassie Brilliant Blue solution (0.25% (w/v) Coomassie Brilliant Blue, 40% (v/v) IMS, 10% (v/v) acetic acid) for a minimum of 30 min. The Coomassie Blue solution was then removed and protein bands were visualized by repeated washes in destain solution (25% (v/v) IMS, 7.5% (v/v) acetic acid) to remove background gel staining, until gel bands became clearly visible. Appropriate bands were then excised for mass spectrometry or gels were dried onto 3 MM paper (Whatmann) under a vacuum at 80°C as appropriate. Radiolabelled proteins were visualized by autoradiography where necessary.

2.3.3 Western blotting

For immunodetection of proteins, resolved proteins were transferred to nitrocellulose by semi-dry electrophoretic blotting. Briefly, the resolving gel, ProTran nitrocellulose membrane (Schleicher and Schuell), and 3MM chromatography paper were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol) before the gel was placed on the membrane and in turn sandwiched between the buffer-soaked blotting paper. Transfer was carried out in a Hoefer semi-phor blotting apparatus (Pharmacia) for 1 h at 1 mA/cm^2 membrane. Following transfer, equality of loading was visualized using ponceau red solution (0.1% (w/v) Ponceau S, 5% (v/v) acetic acid) and the position of size markers and lanes marked. Prior to immunoblotting, membranes were incubated in 5% (w/v) non-fat milk power in 1x PBS-Tween (PBS-T; 137 mM NaCl, 2.7 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1% (v/v) Tween-20) for 30 min to block non-specific antibody binding. The blocked membrane was then incubated with primary antibody at the appropriate dilution in 5% non-fat milk powder/ PBS-T for 1 h at room temperature or overnight at 4°C. Membranes were then washed three time in PBS-T to remove unbound antibody before being incubated with the alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibody for a further hour at room temperature. Membranes were then washed a further three times to remove unbound secondary antibody and membranes were either washed and developed in AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl pH 9.5, 1.65 mg/ml NBT, 3.3 mg/ml BCIP) and proteins visualized directly or in ECL Plus Western blotting detection solution (Amersham Pharmacia), and proteins visualized by autoradiography.

2.3.4 *In vitro* translation

The TNT T7 quick coupled transcription/translation system was used to generate proteins for microtubule sedimentation assays. Briefly, 1 µg plasmid DNA was incubated with 1 µg ^{35}S-labelled methionine and 8 µl T7 quickmix (Promega) for 90 min at 30°C. The *in vitro* translated protein was then used as appropriate.

2.3.5 Immunoprecipitation

Immunoprecipitation experiments were carried out on whole cell lysates (see section 2.5.5 for cell lysis) using the appropriate rabbit polyclonal or mouse monoclonal antibody bound to protein A beads or protein G sepharose, respectively. Briefly, 500 µl cell lysate in NEB buffer (50 mM HEPES-KOH pH 7.4, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 100 mM NaCl, 5 mM KCl, 0.1% (v/v) NP-40, 30 µg/ml RNase A, 30 µg/ml DNase I, 10 µg/ml leupeptin, 10 µg/ml bestatin, 10 µg/ml pepstatin, 1 mM PMSF, 20 mM β-glycerophosphate, 20 mM NaF) was incubated with 10 µl of washed beads rotating at 4°C for 1 h to pre-clear the lysate of any proteins that nonspecifically bound to the beads. The pre-cleared supernatant was removed and incubated on ice for 1 h with the appropriate antibody and then rotating at 4°C for 1 h with 40 µl washed beads which had been previously blocked by incubation with control cell lysate (45 min at 4°C). Beads were then prepared for further analysis by washing 3x in NEB buffer.

2.3.6 *In vitro* kinase assays

Kinase assays were carried out using either 5-10 µl of washed immune complex beads, prepared as described above, or 0.1 µg of purified kinase. Briefly, proteins were incubated with 5 µg of the appropriate substrate and 1 µCi of [γ-^{32}P]-ATP in 40 µl kinase buffer (50 mM Hepes-KOH pH 7.4, 5 mM $MnCl_2$, 5 mM β-glycerophosphate, 5 mM NaF, 4 µM ATP, 1 mM DTT) for 30 min at 30°C. Reactions were stopped by addition of 50 µl 3x Laemmli buffer and analysed by SDS-PAGE and autoradiography. Substrate phosphorylation was quantified by scintillation counting of protein bands excised from dried gels. Excised bands were immersed in 3 ml Optiphase HiSafe 2 liquid scintillant (Wallace-Perkin Elmer) and amount of ^{32}P incorporation determined by quantification in a LS6500 scintillation analyzer (Beckman Coulter).

2.4 Recombinant Protein Expression and Purification

2.4.1 Recombinant protein expression in *E. coli*

For recombinant protein expression, Hsp70 and Cortactin A DNA sequences were amplified by PCR and inserted into the pETM-11 bacterial expression vector as NcoI/HindIII and NcoI/NotI fragments, respectively, as described in section 2.2.1.

Plasmids were transformed into *E. coli* BL21(DE3) (Invitrogen) and individual colonies were used to inoculate cultures of LB supplemented with the appropriate antibiotic. Cultures were grown at 37°C, 225 rpm until an A_{600} of ~0.6 was reached. The temperature was then adjusted to 22°C and expression was induced for 18 h using 100 μM isopropyl 1-thio-β-D-galactopyranoside (IPTG). Cells were then collected by centrifugation and the recombinant protein purified.

2.4.2 Recombinant protein purification

Following harvesting by centrifugation, recombinant-protein expressing cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) supplemented with 10 mM imidazole and lysed by incubation on ice for 30 min with lysozyme (1 mg/ml), followed by sonication on ice (MSE Soniprep 150, 10 mm probe, amplitude 12 μm) using 6 cycles of 10 s sonication at 200-300W followed by 10 s cooling. The lysate was then clarified by centrifugation at 15000 g for 1 h at 4°C and the clarified lysate loaded onto a nickel column and subjected to two rounds of washing with lysis buffer supplemented with 20 mM imidazole. Bound protein was then eluted in lysis buffer supplemented with 250 mM imidazole in several 500 μl fractions. Protein recovery was determined by SDS-PAGE and Coomassie Blue analysis of aliquots of each fraction and those containing highest protein levels were pooled and dialysed against 50 mM Hepes.KOH pH 7.4.

2.4.3 Quantification of protein concentration

Total protein content of solutions was determined using the bicinchoninic acid (BCA) assay (Pierce), which exploits the quantitative colorimetric interaction of reduced copper sulphate, bicinchoninic acid and peptide bonds. Typically, 5 μl of the protein solution (or appropriate dilutions of the protein solution) were incubated with 1 ml of BCA assay reagent at 37°C for 30 min. Following a cooling period, A_{562} was measured and protein concentration determined by comparison to absorbance of BSA standards.

2.5 Mammalian Cell Culture Techniques

2.5.1 Maintenance of human cell lines

All cell lines were maintained in a humidified 5% CO_2 atmosphere at 37°C and passaged upon reaching ~80% confluency. To passage, growth media was aspirated off adherent cell populations, cells were washed in 1x PBS and harvested by incubation in 1x PBS supplemented with 0.5 mM EDTA. Cells were then seeded into fresh, pre-warmed growth media at the appropriate density. HEK 293 and HeLa cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v)

heat inactivated foetal bovine serum (FBS, Invitrogen), penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively, Invitrogen) and 2 mM Glutamine (Invitrogen).

2.5.2 Storage of human cell lines

For long-term storage, cell lines were cryopreserved in liquid nitrogen. Briefly, cells were washed and harvested using 1x PBS and 1x PBS supplemented with 0.5 M EDTA before being collected by centrifugation (1100 rpm, 5 min). After a second wash, cells were resuspended in FBS supplemented with 10% (v/v) DMSO and transferred to cryotubes for freezing in steps (-20°C o/n; -80°C o/n; liquid nitrogen).

2.5.3 Generation of stable cell lines

Stable cell lines were generated by introducing the appropriate construct into a 10 cm dish of HeLa cells via transfection with Lipofectamine 2000 as described in section 2.5.4. After 24 h, cells were washed and incubated in selective media containing Geneticin (0.4 mg/ml, Invitrogen) until foci containing ~50-100 cells were detected (~3-4 wk). Culture dishes were then washed in 1x PBS before foci were picked using paper cloning discs (Sigma). Each disc was soaked in 1x PBS supplemented with 0.5 mM EDTA and then incubated over a single focus for 5-10 min at 37°C. Discs were then transferred to individual wells of a 24-well plate and cells grown in selective media to allow attachment to the culture vessel. Cloning discs were then removed and individual clones expanded in selective media. Cells were maintained in selective media as described above(section 2.5.1). Expression of the plasmid was determined by immunofluorescence microscopy of fixed cells and SDS-PAGE and Western blotting of whole cell lysates.

2.5.4 Transient transfection of mammalian cells

Transient transfection of cultured mammalian cells to induce expression of appropriate recombinant proteins was achieved using Lipofectamine 2000, a cationic, lipid based transfection reagent (Invitrogen). Cells were seeded at a density of $1x10^5$ cells/cm^2 in appropriate culture vessels 24 h prior to transfection. Plasmid DNA and lipofectamine were mixed in a 1:3 ratio in Opti-MEM according to the manufacturer's instructions and added dropwise to cells whose normal growth media had been replaced with Opti-MEM medium without FBS or Pen/Strep. Following a 5 h incubation period (5% CO_2, 37°C), transfection media was replaced with the appropriate complete media and cells were allowed to express recombinant protein for the appropriate length of time before transfected cells were processed as required.

2.5.5 Preparation of cell protein extracts

Whole cell lysates were prepared for SDS-PAGE and Western blotting or immunoprecipitation in NEB lysis buffer. Briefly, cells were washed in 1x PBS, harvested by incubation with 1x PBS supplemented with 0.5 mM EDTA and collected by centrifugation (1100 rpm, 5 min). Cells were then washed and lysed in a volume of NEB buffer appropriate to the number of cells collected (~100 µl/3 cm cell growth). Lysis was carried out on ice for 30 min after which lysates were passed 10 times through a 27G needle and centrifuged at 13000 g for 10 min at 4°C to remove insoluble material. Supernatants were then either used directly as required or stored at -80°C.

2.5.6 Preparation of cell RNA extracts

To prepare RNA for RT-PCR determination of RNAi efficiency, cells were homogenised with Tri reagent (Sigma). Tri reagent was added directly to washed cells adhering to the culture vessel and the subsequent homogenate collected. RNA isolation was performed according to the manufacturer's instructions. Briefly, the homogenate was separated into aqueous and organic phases by addition of bromochloropropane and centrifugation. RNA, partitioned to the aqueous phase was then precipitated with isopropopanol before washing with ethanol and solubilisation in RNase-free water. Total RNA concentration was calculated by measuring OD_{260} and samples were stored at -80°C.

2.5.7 Cell synchronization

Cells were cultured in the appropriate complete growth medium to a suitable (usually ~60-70%) confluency. M-phase arrested cells were obtained by 16 h treatment with 50 ng/ml nocodazole; rounded mitotic cells were collected by gently pipetting off the floating population. To prepare G1 cells, a mitotic population was collected, as above, pelleted by centrifugation, washed three times in 1x PBS before being replaced into fresh medium for 6 h. Synchronization was confirmed by flow cytometry.

2.5.8 Flow cytometric analysis

To determine cell cycle distributions, cell populations for analysis were harvested as appropriate (mitotic shake off for M-Phase cells or treatment with 1x PBS-0.5 mM EDTA for all other cell populations), pelleted by centrifugation and washed in 1x PBS before being resuspended in 1 ml 70% ice-cold ethanol to fix cells. Cells were maintained in ethanol at 4°C for a minimum of 30 min before being stained with propidium iodide. Briefly, cells were washed twice in 1x PBS to completely remove all traces of ethanol, and resuspended in 1x PBS supplemented with 200 µg/ml RNase A and 20 µg/ml propidium iodide. Cells were stained in the dark at 4°C for a minimum of 4 h. Cells

were then analysed via flow cytometry, using a FACScan II instrument and CellQuest Pro software (BD Biosciences).

2.5.9 Apoptosis assays

Apoptosis was determined by AnnexinV/FITC binding (Bender MedSystems) or by immunofluorescence analysis of Caspase 3 cleavage. For annexin V assays, cells were harvested by incubation with 1x PBS supplemented with 0.5 mM EDTA before being resuspended in 10 ml of the appropriate, pre-warmed, complete growth medium and allowed to recover at 37°C in a humidified 5% CO_2 atmosphere for 1 h. Cells were then stained with FITC conjugated Annexin V in annexin binding buffer (10 mM Hepes.KOH pH 7.4, 140 mM NaCl, 1.5 mM $CaCl_2$) for 10 min at room temperature before being washed in annexin binding buffer and resuspended in binding buffer supplemented with 1 µg/ml propidium iodide. Cells were analysed using a FACScan II instrument and CellQuest Pro software (BD Biosciences).

For analysis of Caspase 3 cleavage, immunofluorescence was carried out using an antibody specific to the cleaved form of Caspase 3, essentially as described in section 2.6 with the exception that 1x PBS was replaced with 1x TBS (20 mM Tris-HCL pH 7.6, 140 mM NaCl) in all steps. Incubation with the primary antibody was carried out overnight at 4°C.

2.5.10 Nek6 and Nek7 depletion by RNA interference

siRNA oligonucleotides specific to Nek6 (GAUCGAGCAGUGUGACUACdTdT) or Nek7 (CTCCGACAGTTAGTTAATATTdTdT) were obtained from Ambion (Austin, U.S.A.) and transfected into HeLa cells using Siport-NeoFX transfection reagent (Ambion) according to the manufacturer's instructions. Briefly, cells were seeded at a density of ~$1x10^4/cm^2$ in complete growth medium and concomitantly reverse transfected with the appropriate duplexes (50 nM final concentration) and appropriate volume of transfection reagent. 72 hours after transfection, cells were either fixed for immunocytochemistry or prepared for Western blot or flow cytometry analysis.

2.5.11Time-lapse imaging

For live cell microscopy, cells were grown on glass-bottomed dishes in CO_2-independent medium without glutamine or phenol red (Invitrogen). The culture dish was mounted on the microscope stage regulated at 37°C by a TC-202A temperature controller (Harvard Apparatus, Holliston, MA, supplied by Digitimer, Welwyn Garden City, United Kingdom). Images were captured at 60 s intervals with a TE300 inverted microscope (Nikon, Kingston upon Thames, United Kingdom) using an ORCA ER CCD camera (Hamamatsu,

Hamamatsu, Japan) using Openlab 5 software (Version 5.5.0, Improvision, Coventry, United Kingdom) and images were processed using Adobe Photoshop or converted to QuickTime movies.

2.6 Indirect Immunofluorescence Microscopy

Cells grown on acid-etched coverslips were fixed and permeabilised using ice-cold methanol before processing for immunofluorescence microscopy. Briefly, cells were maintained at -20°C in methanol for a minimum of 6 minutes before being rehydrated by three 5 min washes in 1x PBS. Non-specific antibody binding was blocked by incubation for 10 min in 1x PBS supplemented with 1% (w/v) BSA. Coverslips were incubated with an appropriate volume (100-250 µl) of primary antibody solution diluted to a suitable concentration in 1x PBS supplemented with 3% (w/v) BSA. Incubation with primary antibodies was for a minimum of 1 h at room temperature or o/n at 4°C. Coverslips were then subjected to three 5 min washes in 1x PBS to remove unbound antibody before incubation with appropriate secondary antibodies diluted in 1x PBS supplemented with 3 % (w/v) BSA and 0.3 µg/ml Hoechst 33258 in the dark for 1 h at room temperature. Coverslips were then subjected to a third round of washes before being mounted on glass microscope slides in mountant (80% (v/v) glycerol, 35 (w/v) n-propyl gallate). Images were obtained with a TE300 inverted microscope (Nikon, Kingston upon Thames, United Kingdom) using an ORCA ER CCD camera (Hamamatsu, Hamamatsu, Japan) using Openlab 5 software (Improvision, Coventry, United Kingdom) and images were processed using Adobe Photoshop 7 and where necessary quantified using Openlab 5 or Image J software (Section 2.8.2).

2.7 Antibody Generation and Purification

2.7.1 Antibody generation

For production of anti-Nek6 and anti-Nek7 antibodies, rabbits were immunised with peptides corresponding to the N-terminal regions of Nek6 or Nek7 proteins, conjugated to keyhole limpet haemocyanin (KLH) via N-terminal cysteines. Peptide synthesis, conjugation and immunizations were carried out by Cambridge Research Biochemicals. Rabbit bleeds were screened for reactivity against recombinant and endogenous Nek6 and Nek7 by Western blotting of HeLa cell lysates and immunofluorescence microscopy of methanol-fixed HeLa cells as described in sections 2.3.3 and 2.6 respectively.

2.7.2 Affinity purification of antibodies

Antibodies were affinity purified using the Nek6 or Nek7 peptides against which the antibodies were raised, covalently bound to a CNBr activated sepharose column, made

according to the manufacturer's instructions (Amersham). Briefly, 20 mg of peptide was coupled to 2 ml of swollen CNBr activated sepharose beads by incubation in coupling buffer (0.5 M Na_2PO_4 pH 7.5) after which 5 ml of the final bleed antisera (diluted 1:5 in 10 mM Tris.HCl pH 7.5) was purified over the peptide column. Columns were washed extensively with 10 mM Tris-HCl pH 7.5 followed by 10 mM Tris-HCl pH 7.5, 500 mM NaCl and specific antibodies were eluted under acidic conditions with 100 mM glycine pH 2.5 into tubes containing neutralising quantities of 1 M Tris-HCl pH 8.0. Following dialysis of fractions containing significant levels of antibody (as assessed by ponceau staining and dot blot analysis) against 1x PBS supplemented with 0.02% NaN_3, reactivity and specificity of affinity purified antibodies was assessed by Western blot, and immunofluorescence microscopy, as described above (sections 2.3.3 and 2.6, respectively).

2.8 Miscellaneous Techniques

2.8.1 Microtubule binding assays

Iv vitro translated proteins were generated using the TnT-coupled transcription/translation kit in the presence of [^{35}S]-methionine as described above. 2 µl of the ^{35}S-labelled IVT protein was added to microtubule stabilization buffer (80 mM K.PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 25 mM NaCl, 20 µM taxol, 100 mM GTP, 50 mM AMP-PNP) and centrifuged at 35000 rpm at 20°C for 30 min. The supernatant was collected, purified bovine tubulin (Cytoskeleton Inc.) added to a final concentration of 0.7 µg/ml, and samples incubated for 30 min at 30°C. Following incubation, samples were again centrifuged at 35000 rpm for 30 min at 20°C on a 20% sucrose cushion. The supernatant was carefully removed and added to one third volume of 3x Laemmli buffer. The pellet was then carefully washed with microtubule stabilization buffer prior to resuspension in 3x Laemmli buffer. Equal volumes of the samples were subjected to SDS-PAGE, Coomassie Blue staining and autoradiography as described.

2.8.2 Data analysis and quantification techniques

2.8.2.1 Quantification of centrosome intensities

The relative abundance of protein at the centrosome was quantified using the relevant immunofluorescence images, captured and processed as described above and Openlab 5 software (Improvision). All images to be measured for a given experiment were captured under identical conditions and processed identically. Briefly, a region of interest of the appropriate size (the average size of one centrosome) was selected and saved. Total pixel intensity within this region was calculated and background intensity subtracted for each image to be analysed.

2.8.2.2 Quantification of spindle intensities

Spindle intensity measurements were carried out in one of two ways. Firstly, the intensity of the whole spindle area was measured using Openlab 5 software (Improvision) as described above for centrosome intensity but with a region of interest appropriate for the mitotic spindle area selected. Alternatively, the mean pixel intensity across the mitotic spindle was measured using ImageJ software (v64). Briefly, for each cell analysed, a line was drawn across the mitotic spindle, from spindle pole to spindle pole and the pixel intensity measured at regular intervals along this line. As before, all images to be measured for a given experiment were captured under identical conditions and processed identically.

2.8.2.3 Quantification of Western blot band intensities

For comparative quantification of protein band intensities on Western blots, ImageJ software was used. Briefly, the nitrocellulose membrane or autoradiograph was scanned using an Epson 2600 scanner and Adobe Photoshop 7 software (Adobe). ImageJ was used to select a region of interest (to encompass the largest band to be analysed) and pixel intensity within this region of interest calculated for each band to be analysed, with background intensity subtracted.

2.8.3 KESTREL analysis

All KESTREL analysis work was carried out by Dr Axel Knebel, Kinasource Ltd (Dundee).

600 confluent 15 cm dishes of HEK293 cells were collected, washed in PBS and lysed in 50 mM Tris.HCl, pH 7.5, 5% glycerol, 14 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Pefabloc. Insoluble material was sedimented by centrifugation at 15000 g for 25 min at 4°C. The supernatant was filtered and degassed through a Stericup vacuum filter and then desalted and buffer exchanged into MOPS-LSB (30 mM MOPS pH 7.0, 5% glycerol, 7 mM 2-mercaptoethanol, 0.03% Brij) by chromatography over a 480 ml Sephadex G25 fine column. The desalted extract was applied to a 25 ml heparin sepharose HP column and the column washed in 125 ml MOPS-LSB, developed over 400 ml to 1.2 M NaCl in MOPS-LSB and 40 fractions collected. 20 µl of each fraction were diluted 1:10 in KESTREL test buffer (50 mM Tris.HCl pH 7.5, 7 mM 2-mercaptoethanol, 1 mM EGTA, 10 µg/ml leupeptin, 1 mM Pefabloc) and incubated for 5 min with 3 mM $MnCl_2$ and 1 kBq/ml [γ^{32}P]-ATP in the absence or presence of 1 µg/ml active recombinant Nek2, Nek6, Nek7 or Nek9 kinase (Invitrogen). Reaction products were analysed by SDS-PAGE before electrotransfer to Immobilon P (Millipore, Watford, U.K.) and subsequent autoradiography. Heparin

fractions 16-18 contained potential Nek6 substrates and so were pooled, desalted, chromatographed with a 10 ml gradient to 1 M NaCl on a 1 ml Source 15Q column and analysed for potential Nek6 substrates as above. Substrate containing Q fractions 16-18 were pooled, chromatographed on a 120 ml Superdex 200 column and analysed as above. Superdex 200 fractions 3-7 and 15-20 were separately pooled, concentrated by filtration and incubated with either 3 mM $MnCl_2$ or 10 mM Mg-acetate in the presence of [γ^{32}P]-ATP, with or without 1 µg/ml Nek6. The reactions were denatured, alkylated with 50 mM iodoacetamide, separated by SDS-PAGE and the gels stained with colloidal Coomassie (Invitrogen) and subsequently analysed by autoradiography. Protein bands that were visible with Coomassie staining and radiolabelled in the presence of Nek6 were excised from the gels, destained, digested with trypsin and subjected to Mass Spectrometry Fingerprinting (University of Dundee, U.K.).

2.8.4 RT-PCR

RNA was prepared as described in section 2.5.6 and used for first strand cDNA synthesis with Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Briefly, 1 µg RNA was used in a reaction containing 2.5 µM oligo$(dt)_{20}$, o.5 mM dNTPs, 5 mM DTT, 2 U Recombinant RNase inhibitor and 10 U Superscript III reverse transcriptase. RNA was denatured by incubation at 65°C for 5 min after which first strand synthesis was carried out at 50°C for 60 min. The reaction was inactivated by heating at 70°C for 15 min.

10% of the first strand reaction was then used in the PCR reaction with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 mM each forward and reverse primer and 1 U *Taq* DNA polymerase (Invitrogen). Annealing temperatures and elongation times suitable for the primers and template used, respectively, were employed as appropriate, however the basic PCR reaction consisted of one cycle of denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 2 min, annealing at the appropriate temperature for 30 sec and elongation at 68°C for the appropriate extension time (1 min per Kb), followed by one cycle of extension at 68°C for 10 min. PCR products were then analyzed by agarose gel electrophoresis to assess yield as described in section 2.2.1.3.

Chapter 3

Generation of Anti-Peptide Antibodies against Human Nek6 and Nek7

3.1 Introduction

Nek6 and Nek7 are thought to cooperate with Nek9 to regulate mitotic spindle formation and/or organization in a signaling cascade where Nek9 acts upstream of Nek6 and Nek7. Nek6 coprecipitated with and was activated by Nek9 with both kinases exhibiting maximal activity in mitosis (Roig *et al.*, 2002; Belham *et al.*, 2003). RNAi depletion of Nek6 or Nek7 as well as overexpression of kinase-inactive Nek6 triggered apoptosis, possibly as a result of a preceding mitotic arrest (Yin *et al.*, 2003), whilst microinjection of anti-Nek9 antibodies or expression of truncated fragments of Nek9 causes mitotic spindle abnormalities and errors in chromosome alignment and segregation (Roig *et al.*, 2002). Furthermore, depletion of Nek9 from *Xenopus* egg extracts interfered with spindle assembly *in vitro*, while a fraction of Nek9 localised to centrosomes and interacted with the γ-TuRC MT nucleation complex (Roig *et al.*, 2005). Nonetheless much remains to be determined about the downstream targets, mode of regulation and possible function in spindle assembly, as well as the basic biochemistry of these kinases.

Such analyses require the availability of highly specific antibodies to Nek6 and Nek7 in order to be able to carry out the cell biology and biochemical assays required for functional characterization. Thus, antibodies for applications including Western blotting, immunofluorescence microscopy and immunoprecipitation experiments were essential, but not available commercially.

To this end, antibodies specific for human Nek6 and Nek7 were raised, affinity-purified and characterized to provide, along with constructs containing Nek6 and Nek7 cDNAs in mammalian and bacterial expression vectors, the molecular tools required for the further characterization of Nek6 and Nek7 both *in vivo* and *in vitro*.

3.2 Results

3.2.1 Generation of antisera against human Nek6 and Nek7 N-terminal peptides

Nek6 and Nek7 are the shortest of the human Neks, encoding little more than the NIMA-like catalytic domain which is reasonably conserved between the different Neks. They are furthermore highly similar to one another, sharing 87% identity with the only significant difference between the two proteins being confined to the short ~30 residue non-catalytic sequence at their extreme N-terminus (Figure 3.1A). For this reason, peptides designed to correspond to the N-terminal amino acid sequences of Nek6 and Nek7 were used as antigens for immunization of rabbits for antibody generation (Figure 3.1B). These peptides were synthesized with an N-terminal cysteine and coupled to KLH for immunization. Two rabbits were immunized for each antigen and each rabbit was subjected to four cycles of immunization and bleeding. Peptide synthesis, coupling and rabbit immunization were carried out by Cambridge Research Biochemicals.

Reactivity of the rabbit bleeds was tested by Western blot analysis using asynchronous HeLa epithelial whole cell lysates (Figure 3.2) and by immunofluorescence microscopy of methanol fixed HeLa cells (Figures 3.3 & 3.4).

Western blot analysis of the Nek6-N antisera from both rabbits (SG2331 and SG2332) used at a 1:1000 dilution, revealed a major double band at approximately 36 kDa in the post-immunisation antisera, which was absent from the pre-immune sera and increased in intensity from bleeds one to five (Figure 3.2A). The predicted molecular weight of Nek6 is 35.7 kDa. There was very little other reactivity in these antisera, suggesting a strong positive response to the immunizations, although rabbit SG2332 did detect a third band of approximately 35 kDa, raising the possibility of an alternative version of the Nek6 protein perhaps as a result of alternative splicing or protein modification.

Immunofluorescence microscopy analysis with the Nek6-N antisera at a 1:250 dilution and anti-γ-tubulin antibodies to detect centrosomes, demonstrated firstly and significantly that neither preimmune sera obviously stained the centrosome (Figure 3.3A & B). This is an important consideration due to the fact that many rabbits have sera which naturally cross-react with centrosomes and many mitotic kinases and specifically NIMA-related kinases are centrosomal in localization; NIMA itself localizes to the SPB (De Souza *et al.*, 2000), Nek2 is centrosomal (Fry *et al.*, 1998) and a phosphorylated version of Nek9 has recently been shown to localize to the centrosome in mitosis (Roig *et al.*, 2005). Furthermore, although there have been no reports of Nek6 being centrosomal, Nek7 has been reported to localize to the centrosome in both interphase

A

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hNek6   MAGQPGHMPHGGSSNNLCHTLGPVHPPDPQRHPNTLSFRCSLADFQIEKKIGRGQF   56
hNek7   ----------MDEQSQGMQGPPVPQFQPQKALRPDMGYNTLANFRIEKKIGRGQF   45

hNek6   SEVYKATCLLDRKTVALKKVQIFEMMDAKARQDCVKEIGLLKQLNHPNIIKYLDSF  112
hNek7   SEVYRAACLLDGVPVALKKVQIFDLMDAKARADCIKEIDLLKQLNHPNVIKYYASF  101

hNek6   IEDNELNIVLELADAGDLSQMIKYFKKQKRLIPERTVWKYFVQLCSAVEHMHSRRVM  169
hNek7   IEDNELNIVLELADAGDLSRMIKHFKKQKRLIPERTVWKYFVQLCSALEHMHSRRVM  158

hNek6   HRDIKPANVFITATGVVKLGDLGLGRFFSSETTAAHSLVGTPYYMSP            216
hNek7   HRDIKPANVFITATGVVKLGDLGLGRFFSSKTTAAHSLVGTPYYMSP            205

hNek6   ERIHENGYNFKSIWSLGCLLYEMAALQSPFYGDKMNLFSLCQKIEQCDYPPLPGEHY  273
hNek7   ERIHENGYNFKSIWSLGCLLYEMAALQSPFYGDKMNLYSLCKKIEQCDYPPLPSDHY  262

hNek6   SEKLRELVSMCICPDPHQRPDIGYVHQVAKQMHIWMSS                     313
hNek7   SEELRQLVNMCINPDPEKRPDVTYVYDVAKRMHACTAS                     302
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B

hNek6-N C-^{1}MAGQPGHMPHGGSSN^{15}

hNek7-N C-^{12}PVPQFQPQKALRPDM^{28}

Figure 3.1 Peptide sequences used for Nek6 and Nek7 antipeptide antibody production

(**A**) Alignment of human Nek6 and Nek7 protein sequences demonstrating the high degree of amino acid identity between the two proteins. Conserved residues are shown in black and residues which differ between the two proteins are shown in blue. The region underlined represents the kinase domain of the proteins with the activation loop in larger font size. (**B**) For anti-Nek6 and -Nek7 antibody generation, peptides were designed to correspond to the N-terminal amino acid sequences of the human Nek6 (hNek6-N) and Nek7 (hNek7-N) proteins. Peptide sequences are shown along with the specific amino acid residues that they represent in the Nek6 and Nek7 sequences, the position of the cysteine used to conjugate the peptide is also shown (**C-**).

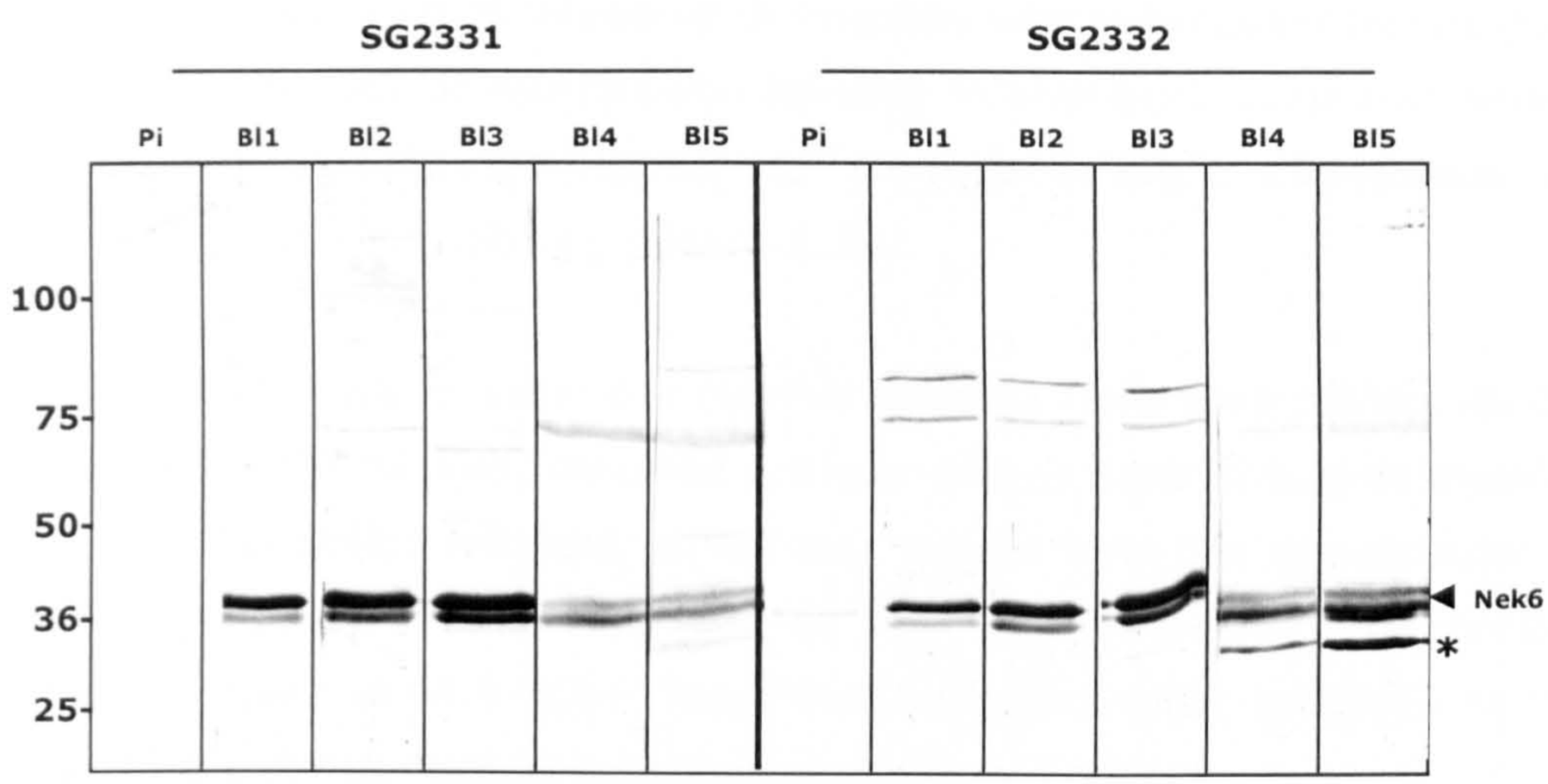

Figure 3.2 Characterization of Nek6 and Nek7 antisera by Western blot analysis
(**A**) Reactivity of anti-hNek6-N antisera taken from two different rabbits (SG2331 and SG2332) was characterized by Western blot analysis at a dilution of 1/1000 against HeLa whole cell lysates. (**B**) Reactivity of anti-hNek7-N antisera taken from two different rabbits (SG2333 and SG2334) was characterised by Western blot analysis at a dilution of 1/1000 against HeLa whole cell lysates. Pi, pre-immune sera, Bl1-5, antisera from bleeds 1-5, respectively. Molecular weights (kDa) are shown on the left of each panel. The predicted sizes of Nek6 and Nek7 are indicated on the right of each panel, and the asterisk (*) indicates a non-specific band detected by SG2332.

and mitosis (Yissachar *et al*, 2006; Kim *et al*., 2007). Thus there is a significant possibility of Nek6 and/or Nek7 being centrosomally-associated proteins. Nonetheless, immunofluorescence microscopy analysis of post immunization sera showed that these likewise did not obviously detect the centrosome in interphase cells. Instead, there was a granular cytoplasmic stain which increased in intensity with subsequent bleeds (Figure 3.3A & B). Despite the lack of centrosomal staining in anti-Nek6 unpurified antisera, antisera from rabbit SG2332 did appear to produce a slight filamentous stain reminiscent of the microtubule network (Figure 3.3B).

Similarly, Western blot analysis using the Nek7-N antisera from both rabbits (SG2333 and SG2334) at a 1:1000 dilution, revealed a major double band at approximately 35 kDa in the post-immunisation antisera, which was absent from the pre-immune sera and increased in intensity from bleeds one to five (Figure 3.2B). The predicted molecular weight of Nek7 is 34.5 kDa. There was very little other reactivity in these antisera, suggesting a strong positive response to the immunizations.

Immunofluorescence microscopy analysis with the Nek7-N antisera at a 1:250 dilution and anti-γ-tubulin antibodies to detect centrosomes, similarly demonstrated that neither preimmune sera obviously stained the centrosome, and that post immunization sera produced a granular cytoplasmic stain which increased in intensity with subsequent bleeds (Figure 3.4A & B). In this case however, there did occasionally appear to be some co-staining with the centrosome, although it was not present in all the bleeds tested and did not necessarily increase in intensity from bleeds 1-5 (Figure 3.4A & B). Nonetheless, this does raise the possibility that these antisera detect a centrosomal protein and this protein may be Nek7.

To further investigate the staining patterns seen with anti-Nek6 and anti-Nek7 antisera, final bleed antisera from each of the rabbits was analysed in more detail by immunofluorescence microscopy specifically looking at the staining patterns seen at different stages of mitosis. Both proteins are believed to have mitotic roles and thus localization to mitotic structures may be likely. Staining patterns of final bleed antisera compared to pre-immune sera, both at a 1:250 dilution, for each rabbit were analysed at different mitotic stages. Cells were co-stained with anti-γ-tubulin antibodies to assess potential colocalization with centrosomes and spindle poles (Figures 3.5 and 3.6). Analysis of this data demonstrated that for the two rabbits immunized with hNek6-N (SG2331 and SG2332) neither pre-immune serum gave a strong signal at any cell cycle stage analyzed and there was no obvious staining of centrosomes or spindle poles (Figures 3.5 A and C). The antisera for these rabbits also did not show a strong

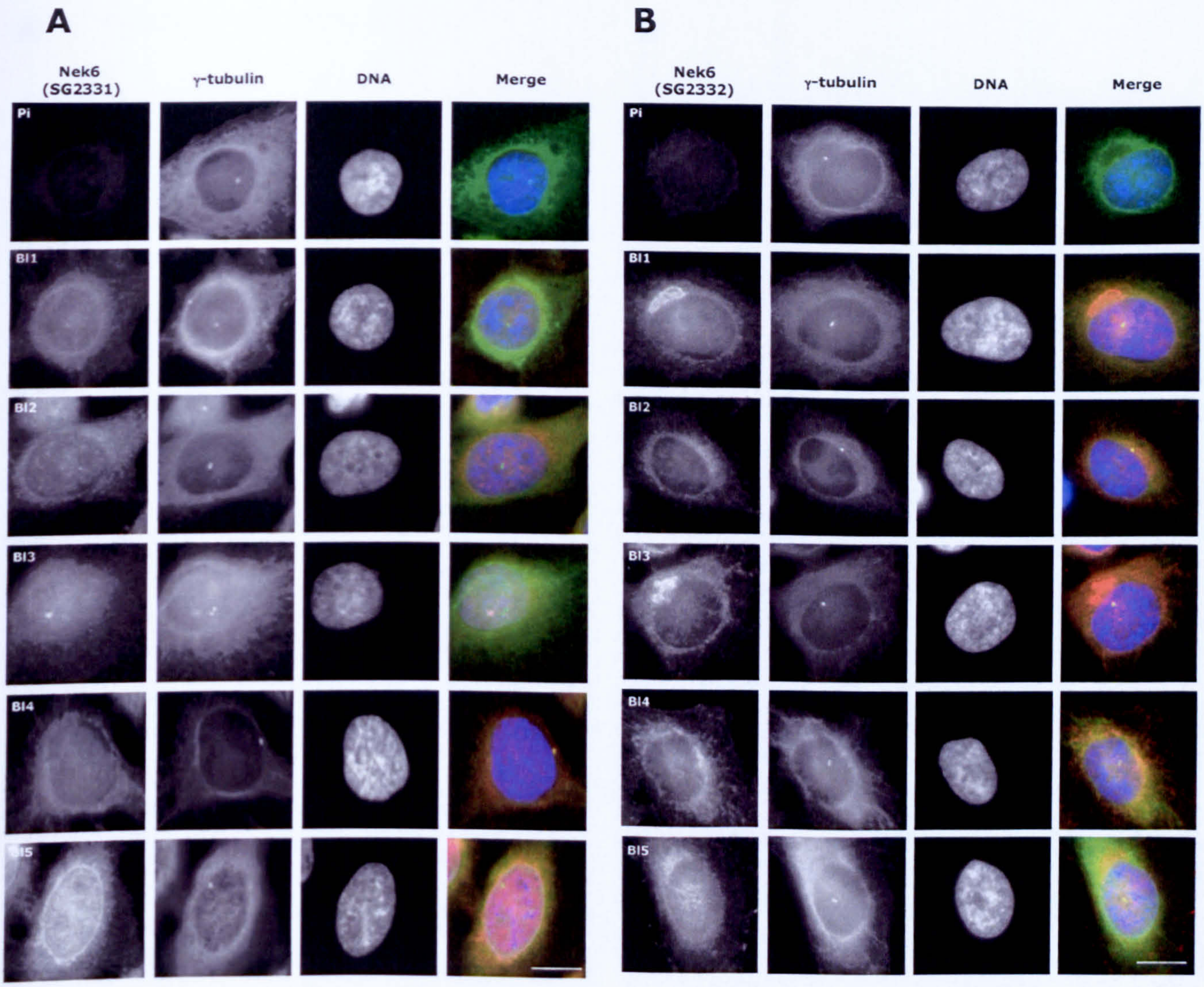

Figure 3.3 Characterization of anti-Nek6 antipeptide antisera by immunofluorescence microscopy

(**A**) & (**B**) Reactivity of rabbit antisera raised against the hNek6-N peptide was tested by indirect immunofluorescence microscopy in HeLa cells. Cells were methanol-fixed before being processed for immunofluorescence microscopy with anti-γ-tubulin antibodies (green on merge) and the appropriate rabbit antisera as indicated (red on merge). DNA was stained with Hoechst 33258 (blue). Rabbit sera were used at a dilution of 1/250. Scale bars, 10 μm. Pi, preimmune sera; Bl1-5, antisera from bleeds 1 to 5, respectively.

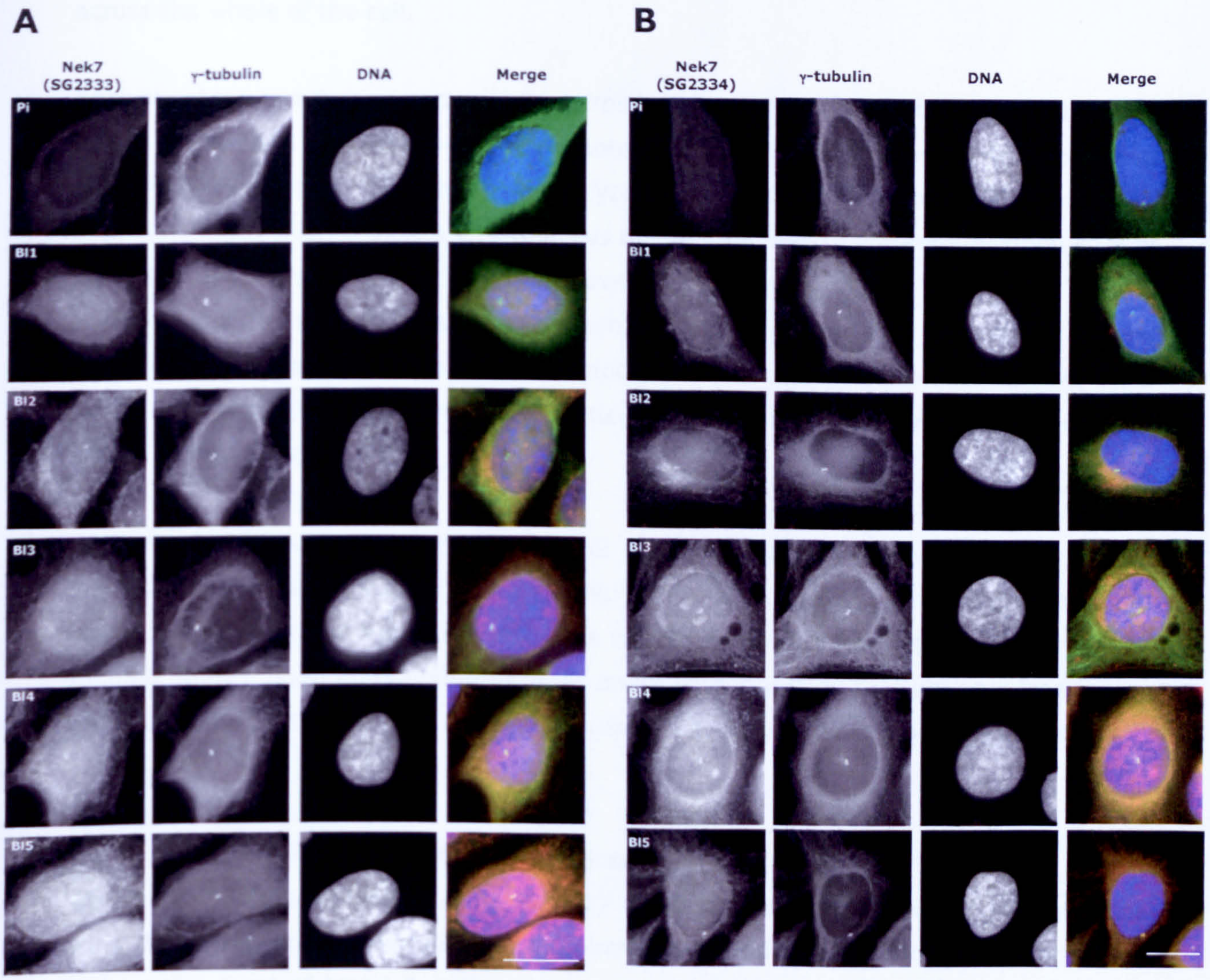

Figure 3.4 Characterization of anti-Nek7 antisera by immunofluorescence microscopy

(**A**) & (**B**) Reactivity of rabbit antisera raised against the hNek7-N peptide was tested by indirect immunofluorescence microscopy in HeLa cells. Cells were methanol-fixed before being processed for immunofluorescence microscopy with anti-γ-tubulin antibodies (green on merge) and the appropriate rabbit antisera, as indicated (red on merge). DNA was stained with Hoechst 33258 (blue). Rabbit sera were used at a dilution of 1/250. Scale bars, 10 μm. Pi, preimmune sera; BI1-5, antisera from bleeds 1 to 5, respectively.

consistent localization to any mitotic structures, although there was occasional centrosomal or spindle pole staining (Figure 3.5D, Metaphase, for example) and post immunization sera from rabbit SG2332 showed midbody staining in late mitotic cells (Figure 3.5D). However, overall the pattern at all stages of mitosis was a strong stain across the whole of the cell.

Analysis of the localization pattern of pre-immune sera from the rabbits immunized with hNek7-N (SG2333 and SG2334) also demonstrated that neither pre-immune sera gave a strong signal at any cell cycle stage analyzed with no obvious staining of centrosomes or spindle poles (Figure 3.6A & C). Analysis of the final bleed post-immunisation sera of these two rabbits showed that whilst the overriding staining pattern was, as for hNek6-N antisera, a strong diffuse staining pattern across the whole cell, there were several cases where the antisera appeared to colocalise with the centrosomal γ-tubulin stain particularly during mitosis, this was particularly apparent with antisera from rabbit SG2333 (Figure 3.6B).

Of the rabbits immunized, rabbit SG2332 showed the strongest and most specific response to the immunizations with Nek6-N, whilst rabbit SG2333 showed the strongest response to immunizations with Nek7-N as well as the strongest apparent centrosomal staining pattern upon immunofluorescence analysis, suggesting that antisera from these two rabbits would be the most suitable for use in subsequent antibody purification.

3.2.2 Affinity purification of hNek6-N and hNek7-N antisera

Nek6 antisera from rabbit SG2332 and Nek7 antisera from rabbit SG2333 were purified using the corresponding peptide antigen covalently bound to a column of CNBr activated sepharose. 5 ml of bleed 5 antisera from the appropriate rabbits was diluted 1:5 in coupling buffer was passed over the corresponding affinity column. Non-specific antibodies were removed by washing with wash buffers supplemented with 500 mM $NaCl_2$ and anti-Nek6 or -Nek7 antibodies were eluted under acidic conditions. The presence of the appropriate IgG heavy chain was confirmed by Coomassie Blue staining of aliquots of each of the fractions resolved by SDS-PAGE analysis (Figure 3.7). Reactivity of the fractions was initially determined by dot blot analysis against recombinant His_6Nek6 and His_6Nek7 proteins purchased from Upstate (Dundee).

Purified antibodies from elution fractions 2-5 and 4-8 for anti-Nek6 and anti-Nek7 antibodies, respectively, were each pooled, dialysed against 1x PBS and concentrated

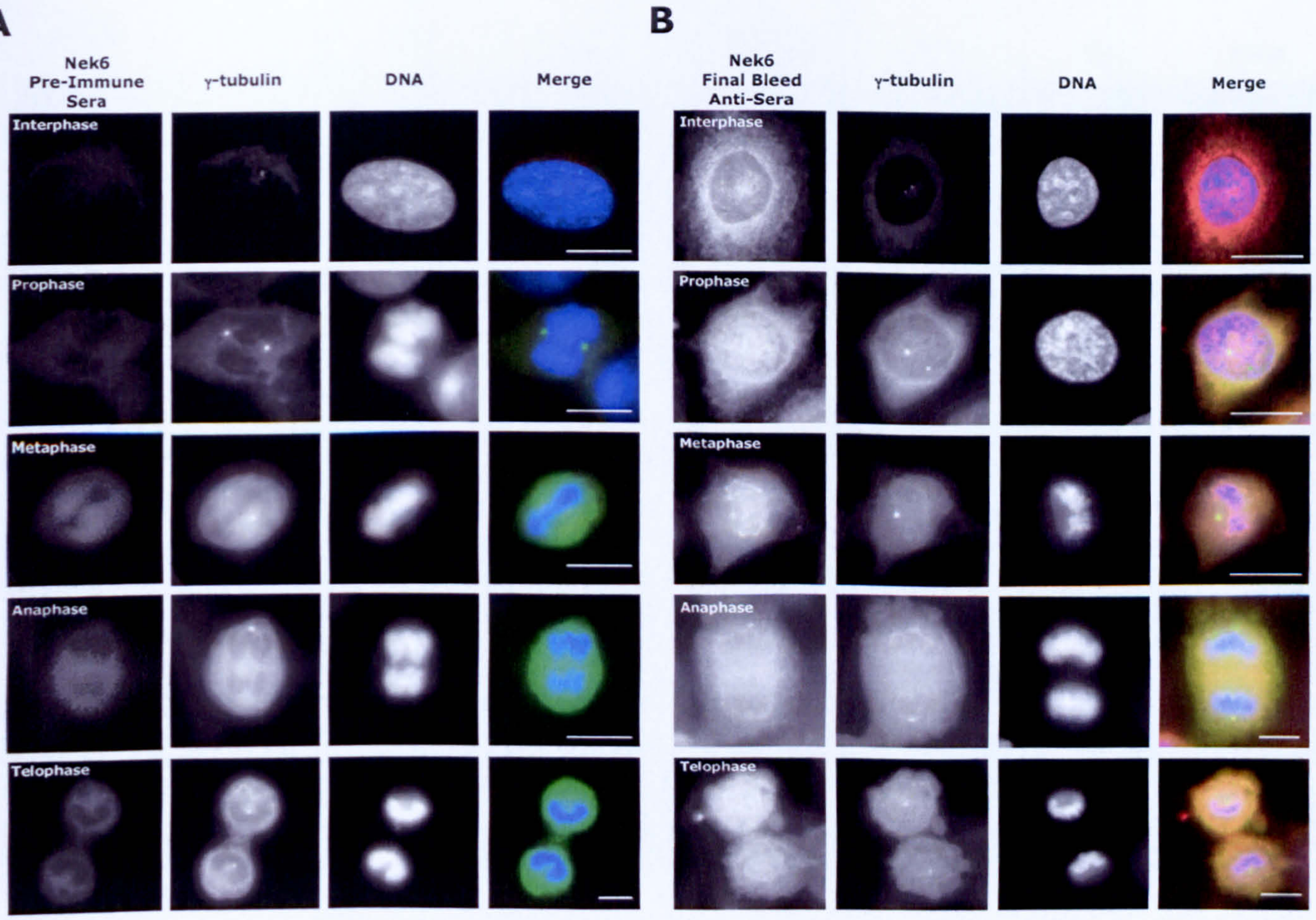

Figure 3.5 Immunofluorescence staining of mitotic cells with anti-Nek6 antisera

The reactivity of the final bleed antisera raised against hNek6-N at different stages of mitosis was characterized by immunofluorescence microscopy with methanol-fixed HeLa cells. (**A**) Cells were stained with anti-γ-tubulin antibodies (green) and pre-immune sera from rabbit SG2331 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). (**B**) Cells were processed for immunofluorescence microscopy with anti-γ-tubulin antibodies (green) and anti-hNek6-N final bleed antisera from rabbit SG2331 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). The different cell cycle stages are indicated. Scale bars, 10 μm.

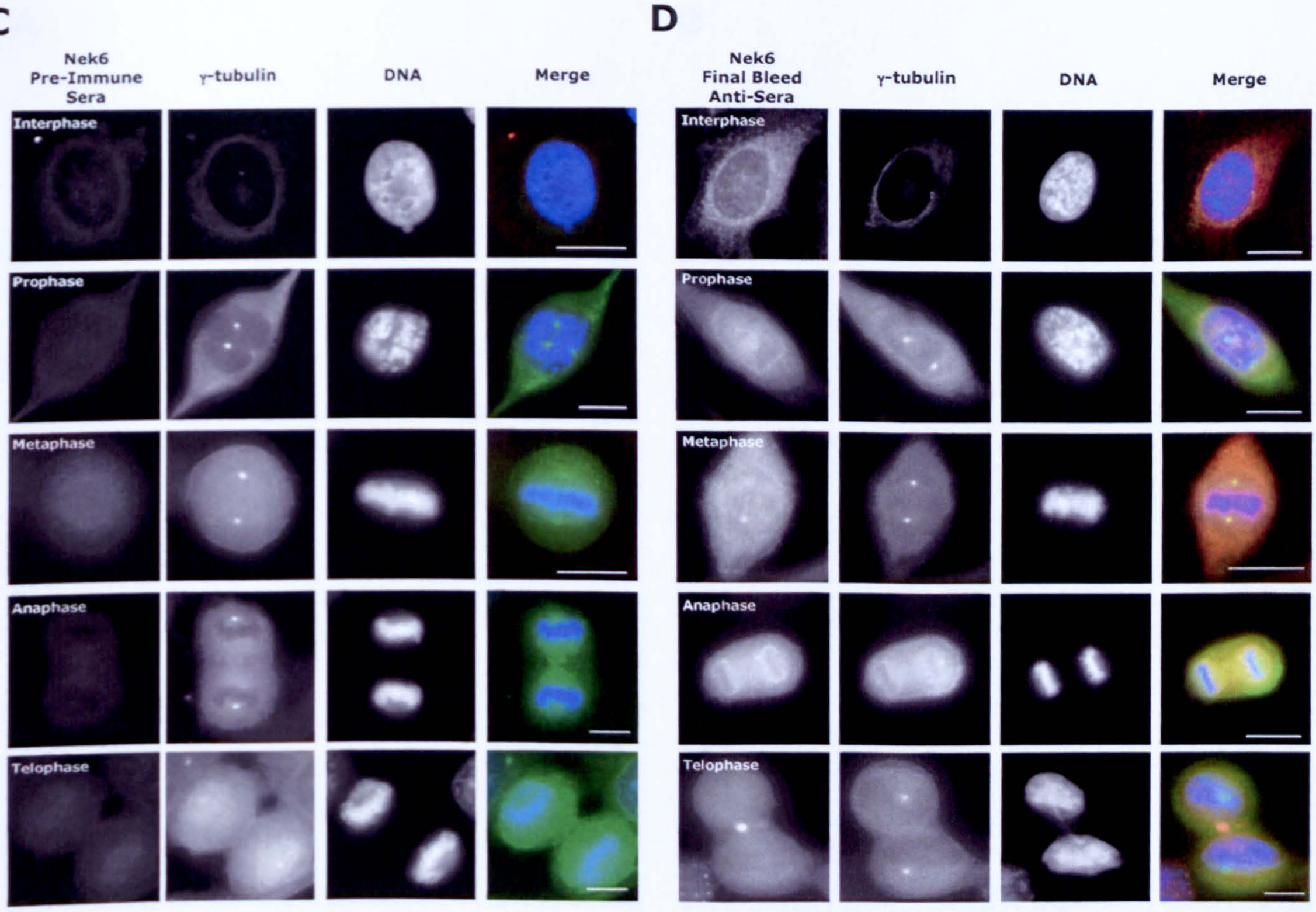

Figure 3.5 continued. Immunofluorescence staining of mitotic cells with anti-Nek6 antisera

The reactivity of the final bleed antisera raised against hNek6-N at different stages of mitosis was characterized by immunofluorescence microscopy with methanol-fixed HeLa cells. (**C**) Cells were stained with anti-γ-tubulin antibodies (green) and pre-immune sera from rabbit SG2332 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). (**D**) Cells were stained with anti-γ-tubulin antibodies (green) anti-hNek6-N final bleed antisera from rabbit SG2332 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). The different cell cycle stages are indicated. Scale bars, 10 μm.

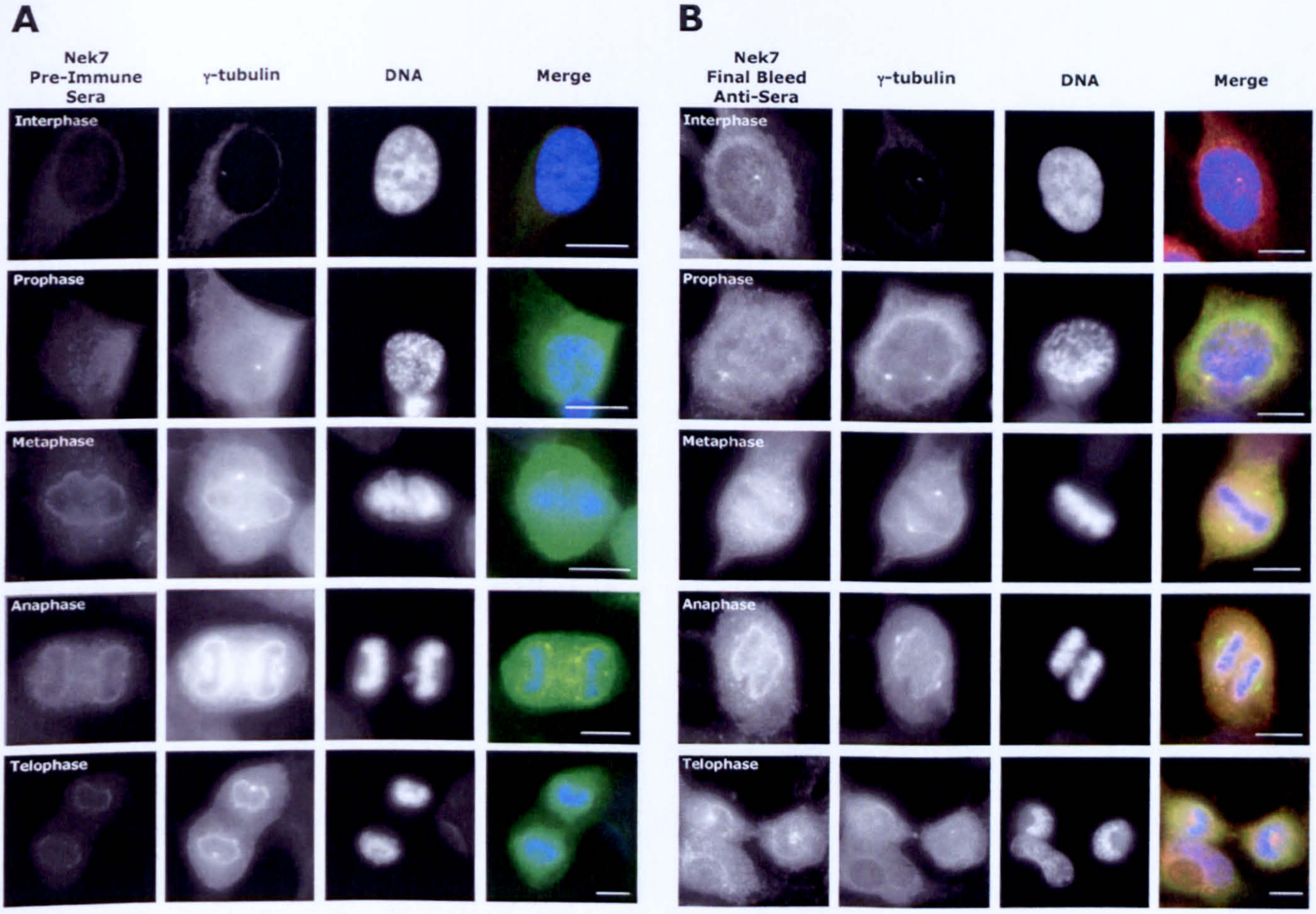

Figure 3.6 Immunofluorescence staining of mitotic cells with anti-Nek7 antisera

The reactivity of the final bleed rabbit antipeptide antisera raised against hNek7-N at different stages of mitosis was characterized by immunofluorescence microscopy with methanol-fixed HeLa cells. (**A**) Cells were stained with anti-γ-tubulin antibodies (green) and pre-immune sera from rabbit SG2333 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). (**B**) Cells were stained with anti-γ-tubulin antibodies (green) anti-hNek7-N final bleed antisera from rabbit SG2333 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). The different cell cycle stages are indicated. Scale bars, 10 μm.

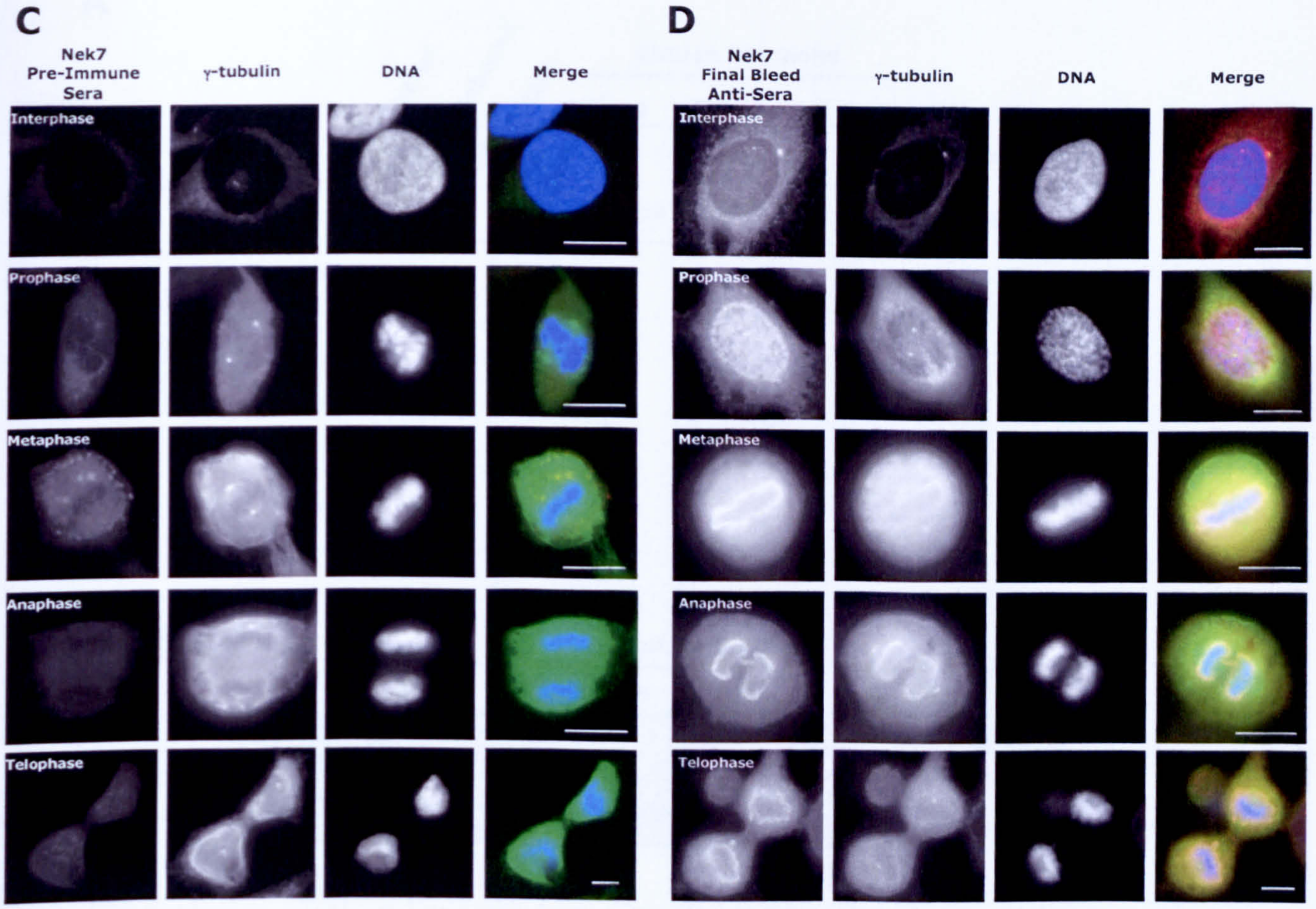

Figure 3.6 continued. Immunofluorescence staining of mitotic cells with anti-Nek7 antisera

The reactivity of the final bleed rabbit antipeptide antisera raised against hNek7-N at different stages of mitosis was characterized by immunofluorescence microscopy with methanol-fixed HeLa cells. (**C**) Cells were stained with anti-γ-tubulin antibodies (green) and pre-immune sera from rabbit SG2334 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). (**D**) Cells were stained with anti-γ-tubulin antibodies (green) and anti-hNek7-N final bleed antisera from rabbit SG2334 at a 1:250 dilution (red). DNA was stained with Hoechst 33258 (blue). The different cell cycle stages are indicated. Scale bars, 10 μm.

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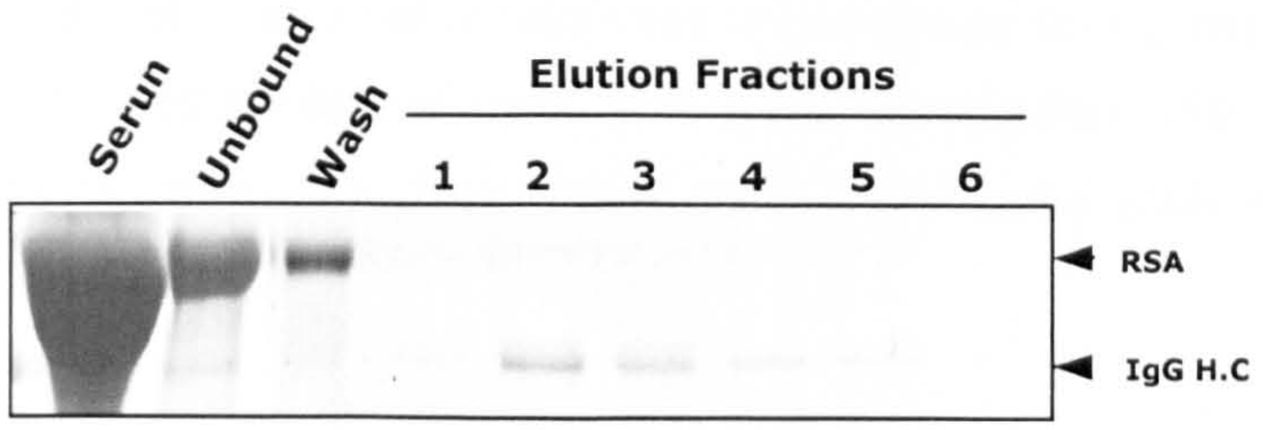

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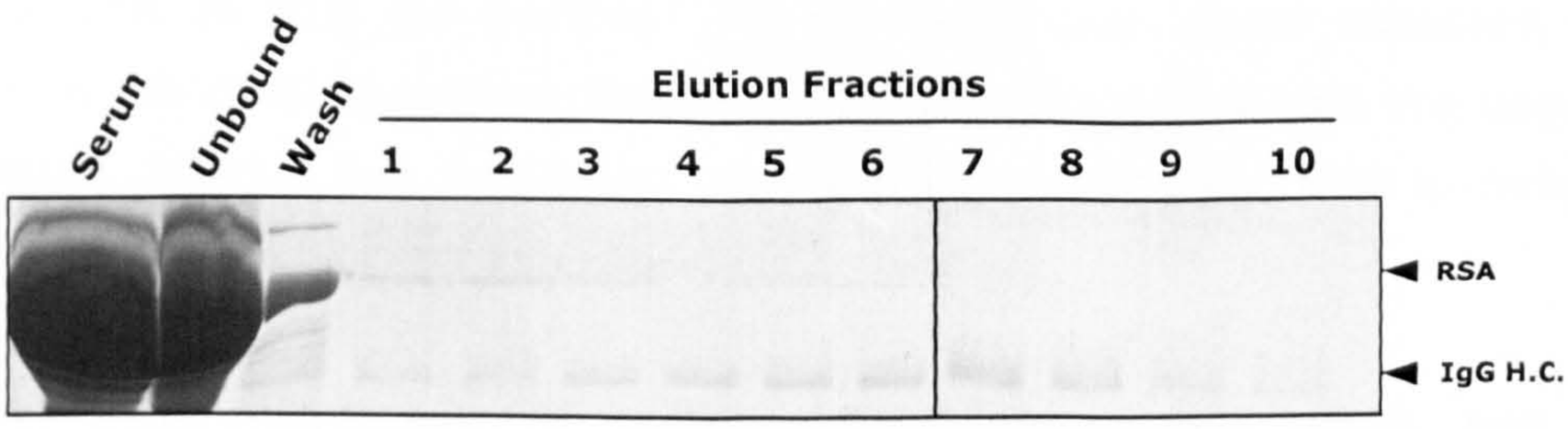


Figure 3.7 Affinity purification of anti-Nek6 and anti-Nek7 antibodies

Anti-Nek6 (SG2332) and anti-Nek7 (SG2333) antisera were purified by passing over a column of hNek6-N or hNek7-N peptide, respectively, covalently bound to CNBr activated sepharose beads and eluted using glycine pH 2.5. Purification efficiency was tested by SDS-PAGE and Coomassie Blue staining of unpurified antiserum, unbound protein at the wash as well as protein in elution fractions (E1 to E10). Fractions 1 to 5 were pooled, dialysed against PBS and concentrated. (**A**) Coomassie Blue staining of anti-Nek6 unpurified antisera and elution fractions. (**B**) Coomassie Blue staining of anti-Nek7 unpurified antisera and elution fractions. The positions of rabbit serum albumin (RSA) and the IgG heavy chain (H.C.) are indicated.

using a centrifugal membrane to give 4 ml of anti-Nek6 antibodies at a concentration of 200 µg/ml and 6 ml of anti-Nek7 antibodies at a concentration of 175 µg/ml.

3.2.3 Characterization of affinity-purified anti-Nek6 and anti-Nek7 antibodies

The reactivity and specificity of affinity-purified antibodies was tested by Western blot analysis as well as indirect immunofluorescence microscopy using asynchronous HeLa whole cell lysates from untransfected cells and cells transfected with FLAG-Nek6 and FLAG-Nek7 constructs or methanol fixed HeLa cells transfected with these constructs, respectively.

Western blotting was carried out with both antibodies at a concentration of 2 µg/ml. Control western blots using anti-FLAG antibody as well as unpurified antisera and pre-immune sera were also carried out. For the anti-Nek6 antibody, a doublet band was detected in FLAG-Nek6 transfected cell lysates, which corresponded to a doublet detected by the anti-FLAG antibody. This doublet also corresponded to the dominant doublet seen in the unpurified anti-sera control blot, but was absent from blots performed with the pre-immune sera. In untransfected cell lysates a single band was detected at around 36 kDa, the expected size for Nek6, upon longer exposure, which corresponded to the most significant band seen in the control blot with the unpurified anti-sera (Figure 3.8A). Pre incubation of the purified antibody with a competing purified His_6Nek6 protein blocked detection of this band.

Immunofluorescence microscopy of methanol-fixed HeLa cells which had been transfected with FLAG-Nek6 constructs for 24 h prior to fixation and costained with anti-FLAG antibodies and either unpurified antisera, affinity-purified antibodies, or affinity-purified antibodies which had been preincubated with competing His-Nek6 protein, was also carried out. The purified anti-Nek6 antibody appeared to specifically detect the recombinant protein, producing colocalisation with the signal produced by the FLAG antibody which disappeared upon incubation with a competing His-Nek6 protein (Figure 3.8B).

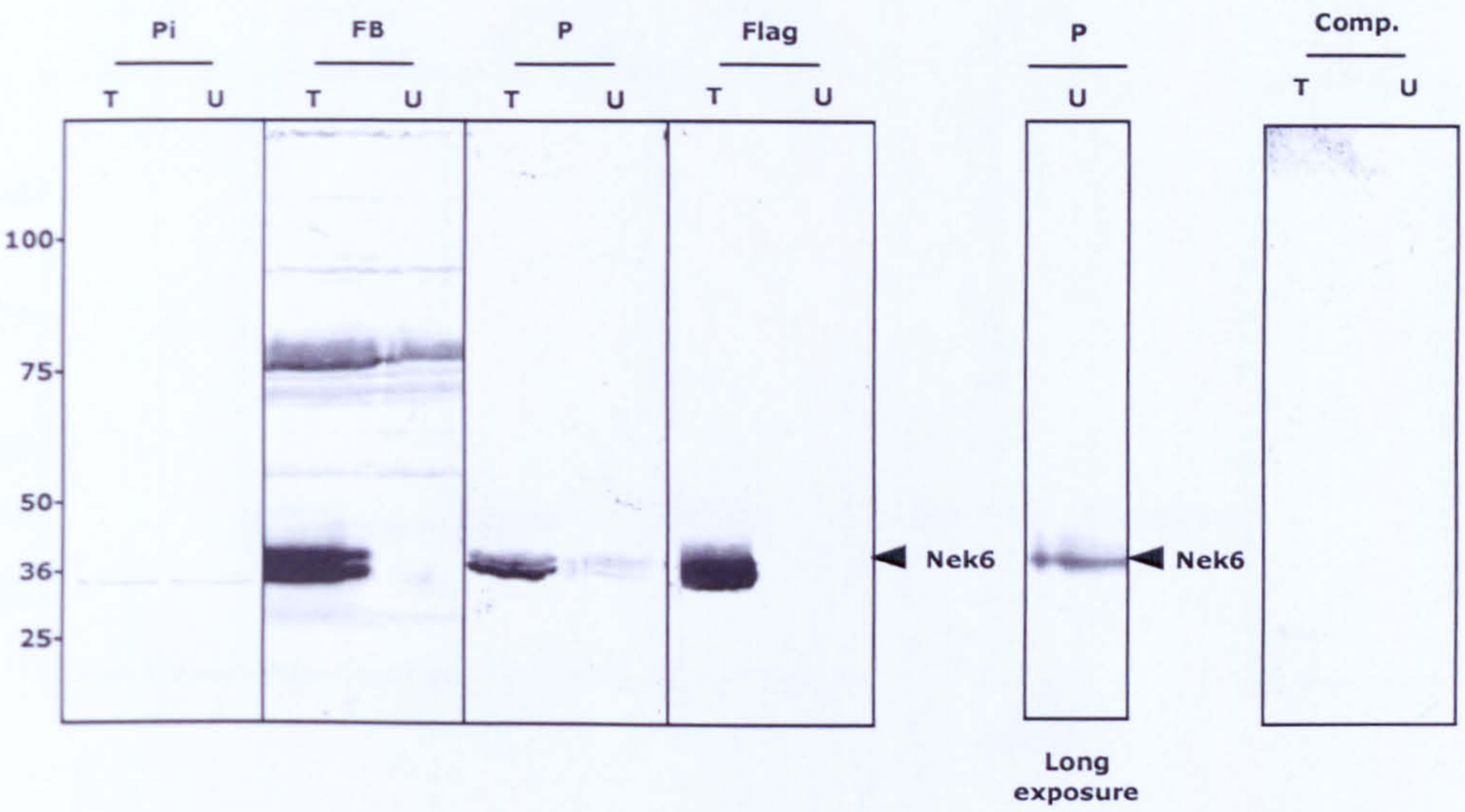
Western blot analysis to test the reactivity of affinity-purified anti-Nek7 antibodies resulted in the detection of a doublet at around 36 kDa in transfected cell lysates corresponding to the doublet detected in lysates probed with the anti-FLAG antibody and final bleed antisera but absent from pre-immune sera blots. Longer exposure resulted in the detection of a single band also at approximately 35 kDa, the expected size for Nek7, by both the affinity-purified anti-Nek7 antibodies and the final bleed antiserum

but not by the pre-immune sera or the anti-FLAG antibodies (Figure 3.9A). Pre incubation of the purified antibody with a competing purified His_6Nek7 protein blocked detection of this band

Immunofluorescence microscopy analysis as described for the Nek6 antibodies but using FLAG-Nek7 constructs, resulted in apparent co-localisation of purified anti-Nek7 antibodies with the anti-FLAG antibodies, and this signal disappeared upon pre-incubation with a competing purified His_6Nek7 protein (Figure 3.9B).

Although peptides corresponding to the divergent N-terminal regions of the Nek6 and Nek7 protein were used as antigens for antibody production, the high degree of identity shared by Nek6 and Nek7 protein sequences raised the possibility that anti-Nek6 antibodies would cross-react with Nek7 protein and vice versa. Cross-reactivity of the anti-Nek6 and anti-Nek7 antibodies with Nek7 and Nek6 proteins, respectively, was tested for by Western blot analysis of asynchronous HeLa whole cell lysates transfected with FLAG-Nek6 or FLAG-Nek7. Whilst anti-Nek6 antibodies detected a band corresponding to the FLAG-Nek6 protein in transfected lysates, it did not detect the FLAG-Nek7 protein. Likewise, anti-Nek7 antibodies whilst detecting the FLAG-Nek7 protein, were unable to detect FLAG-Nek6 (Figure 3.10). Thus the anti-Nek6 and anti-Nek7 antibodies generated appear to be specific for Nek6 and Nek7 proteins, respectively.

A



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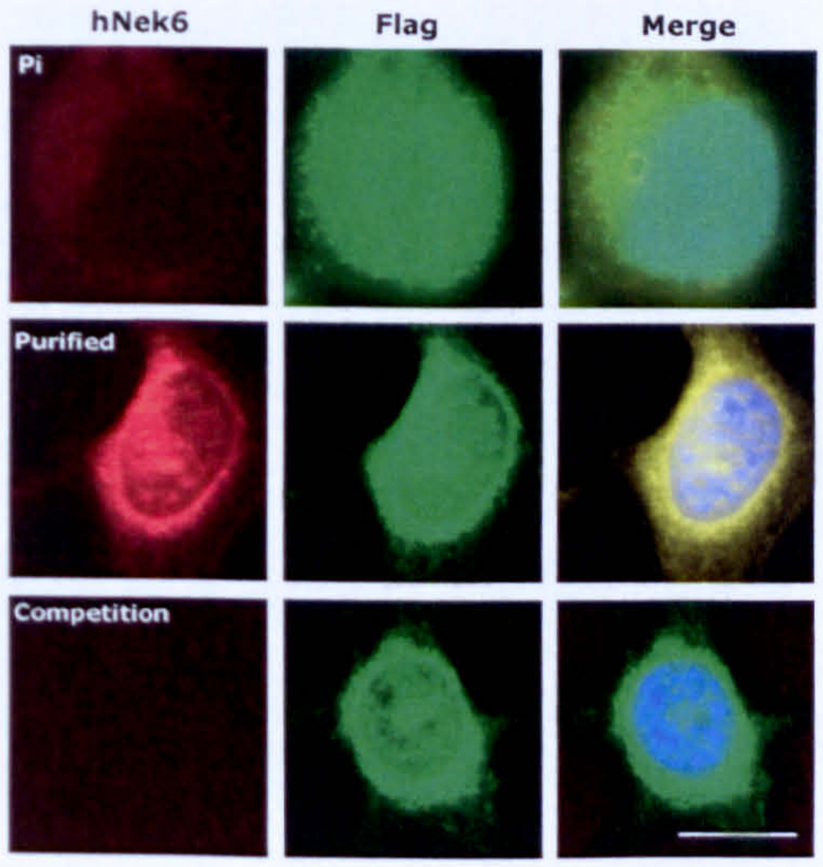

Figure 3.8 Characterization of affinity-purified anti-Nek6 antibodies

(**A**) Reactivity of purified anti-Nek6 antibody was tested by Western blot against HeLa whole cell lysates that were either untransfected (U) or transfected with Flag-Nek6 (T). Reactivity of purified antibody (P) at a working concentration of 2 µg/ml was compared to that of pre-immune sera (Pi) and unpurified final bleed antisera (FB) at a dilution of 1/1000 and anti-Flag antibody (Flag), Antibody specificity was tested by preincubating antibody with a competing His_6Nek6 antigen (comp). Molecular weights (kDa) are shown on the left. (**B**) Indirect immunofluorescence microscopy to test reactivity of purified antibodies on Flag-Nek6-transfected HeLa cells. Cells were transfected as appropriate, methanol fixed and processed for immunofluorescence microscopy. Recombinant proteins are detected with antibodies against the Flag epitope tag (green) and Nek6 at a concentration of 2 µg/ml (red); DNA is stained with Hoechst 33258 (blue). Sera are either pre-immune (Pi), affinity-purified (purified), or affinity-purified pre-incubated with the competing antigen (competition). Scale bar, 10 µm.

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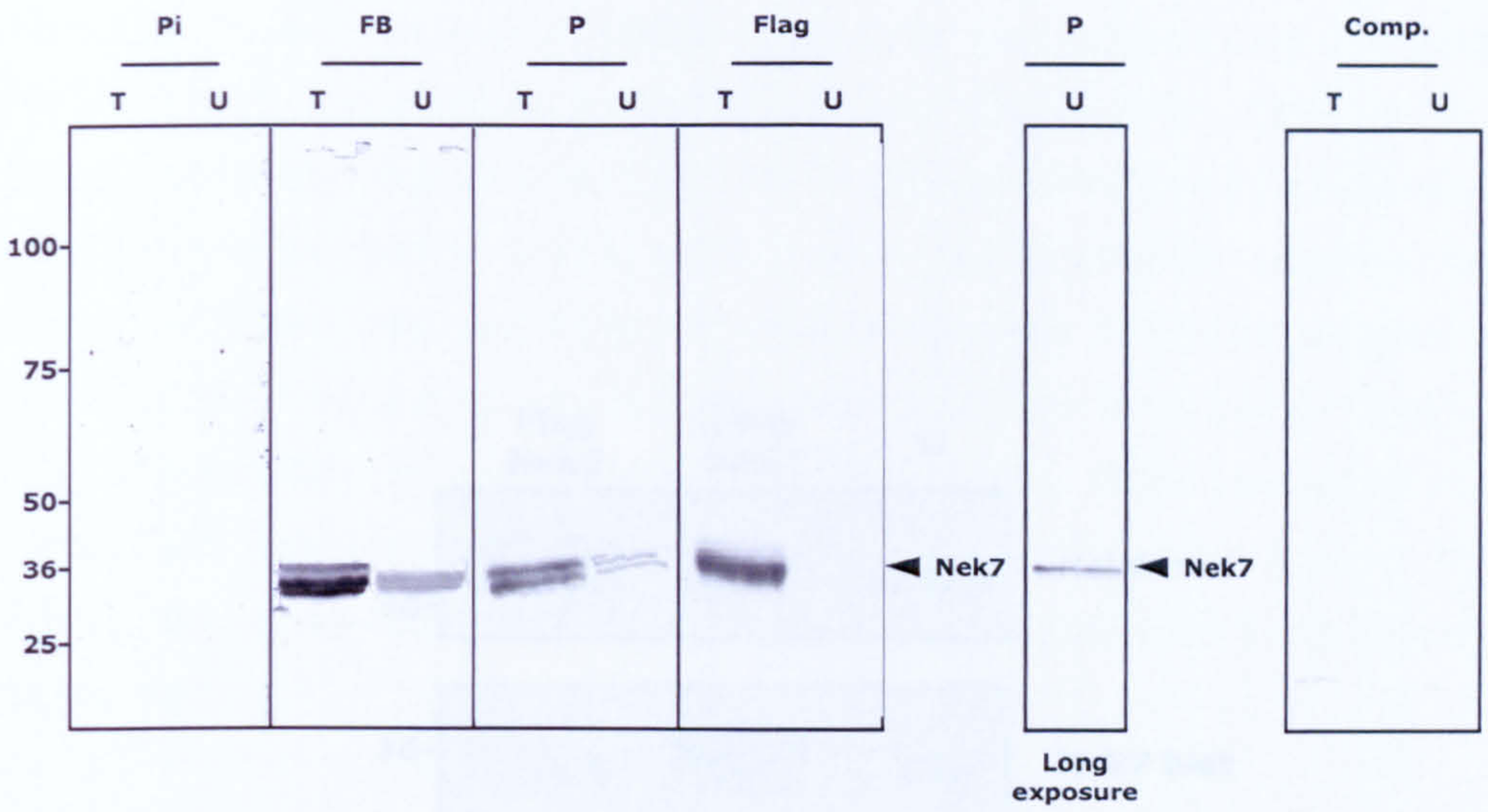

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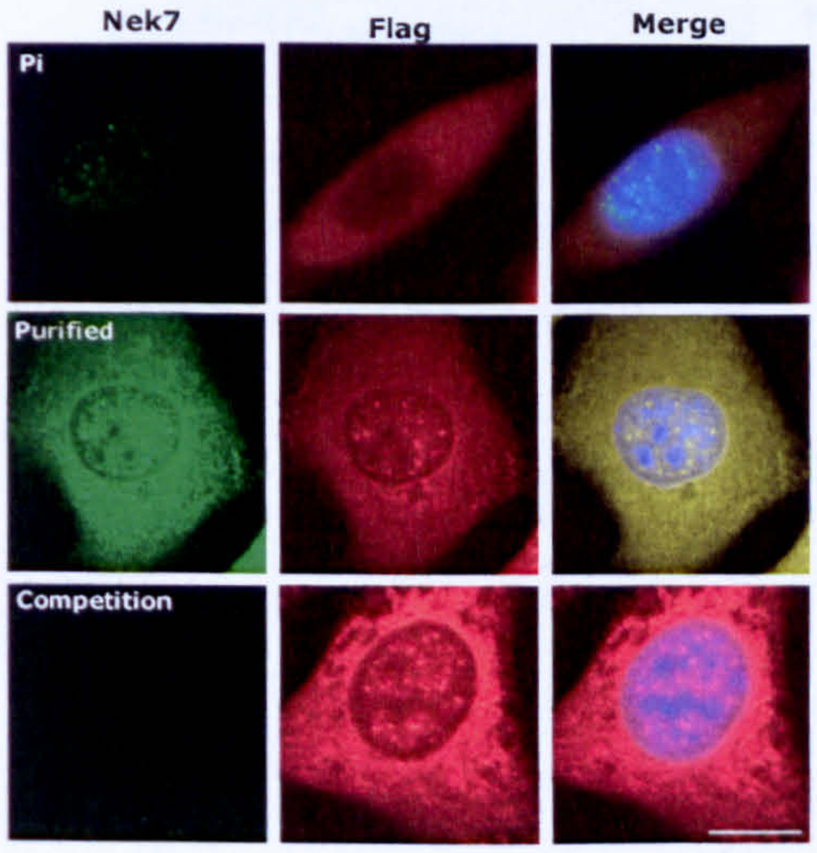


Figure 3.9 Characterization of affinity-purified anti-Nek7 antibodies

(**A**) Reactivity of purified anti-Nek7 antibody was tested by Western blot against HeLa whole cell lystates that were either untransfected (U) or transfected with Flag-Nek7 (T). Reactivity of purified antibody (P) at a working concentration of 2 µg/ml was compared to that of pre-immune sera (Pi) and final bleed antisera (FB) at a dilution of 1/1000 and anti-Flag antibody (Flag). Antibody specificity was tested by preincubating antibody with a competing His_6Nek6 antigen (comp). Molecular weights (kDa) are shown on the left. (**B**) Indirect immunofluorescence microscopy to test reactivity of purified antibodies on Flag-Nek7-transfected HeLa cells. Cells were transfected as appropriate, methanol fixed and processed for immunofluorescence microscopy. Recombinant proteins are detected with antibodies against the Flag epitope tag (red) and Nek7 at a concentration of 2 µg/ml (green); DNA is stained with Hoechst 33258 (blue). Sera are either pre-immune (Pi), affinity-purified (purified), or affinity-purified pre-incubated with the competing antigen (competition). Scale bar, 10 µm.

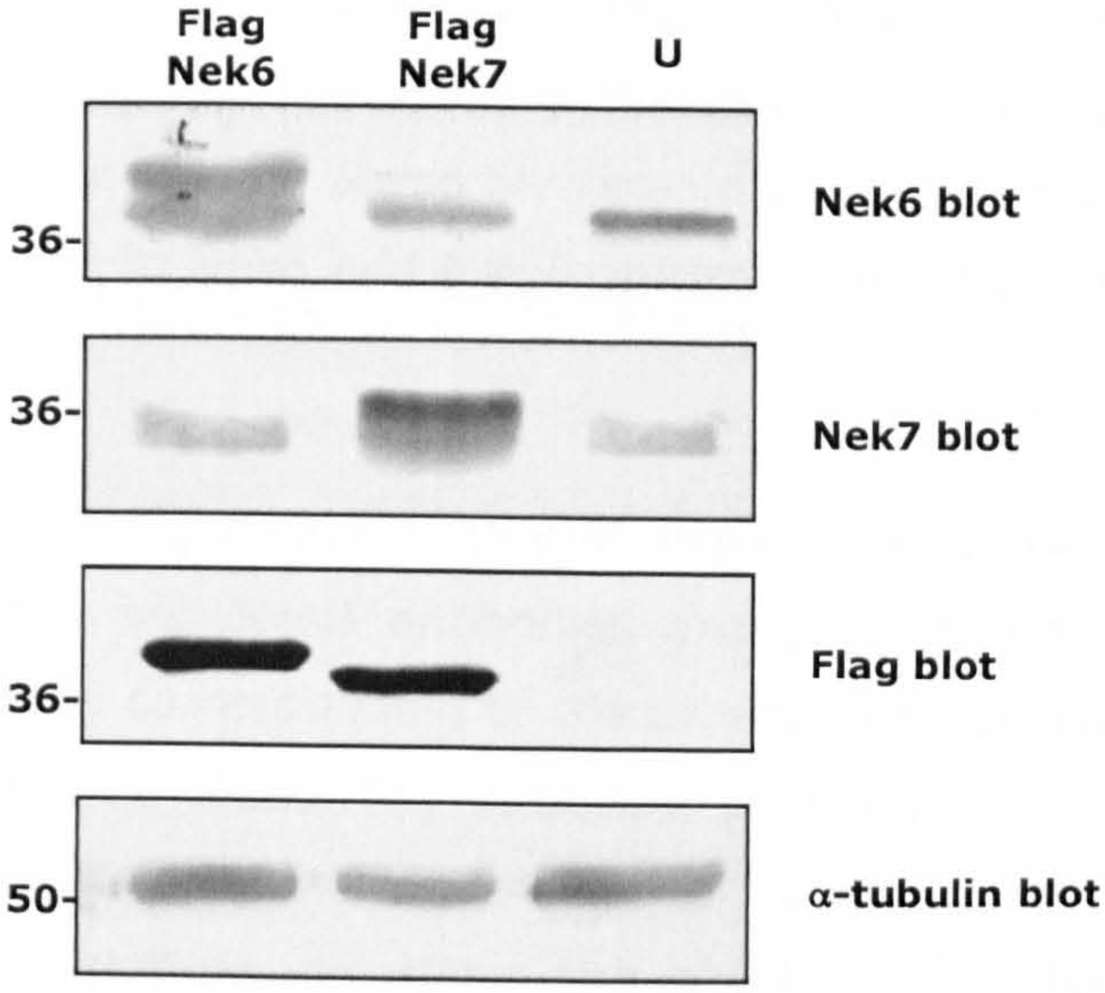


Figure 3.10 Nek6 and Nek7 affinity-purified antibodies do not detect the complementary protein

Western blot analysis of Flag-Nek6 and Flag-Nek7 transfected as well as untransfected (U) HeLa whole cell lysates with affinity-purified anti-Nek6, anti-Nek7, anti-Flag and anti-α-tubulin antibody to determine whether the Nek6 antibody recognizes recombinant Nek7 and vice versa. Anti-Flag antibodies are used to detect transfected protein and anti- α-tubulin antibodies are used as a loading control. Molecular weights (kDa) are shown on the left.

3.3 Discussion

NIMA is a serine-threonine protein kinase from the filamentous fungus *Aspergillus nidulans*, encoded by the *nimA* gene, whose activation and degradation are essential for mitotic entry and exit, respectively, in this organism (Osmani *et al.*, 1991; Pu & Osmani, 1995). Since then many mammalian NIMA homologues, NIMA-related kinases or Neks, have been identified several of which, whilst not being absolutely required for mitotic progression in the same way that NIMA is, appear to share NIMA-like mitotic roles. Of these, Nek6 and Nek7 appear to be involved, in cooperation with Nek9, in regulation of mitotic spindle organization and/or formation. However, their downstream targets, mode of action and function in spindle assembly remain to be determined. This led us to generate antibodies specific to Nek6 and Nek7 which would enable us to characterize these proteins further.

With Nek6 and Nek7 sharing a significant degree of sequence identity, the possibility of cross reaction between Nek6 and Nek7 antibodies and proteins was a concern. To circumvent this issue, peptides corresponding to the divergent N-terminal regions of the proteins were synthesized and used for antibody production. The two rabbits immunized with each peptide (hNek6-N and hNek7-N) yielded sera that recognized identical proteins by Western blotting. However, the rabbit antisera that was chosen for anti-Nek6 antibody purification also detected a further band of a similar size, raising the possibility of the existence of an alternative version of this protein. After affinity purification, each of the antibodies appeared to specifically detect recombinant FLAG-tagged Nek6 or Nek7 proteins, as appropriate, as well as a single band at either 36 or 34 kDa which corresponds to the predicted sizes of endogenous Nek6 and Nek7, respectively. The lower molecular weight band seen with the Nek6 unpurified antisera was no longer detectable after purification, even upon longer exposure with the affinity-purified antibodies. It was thus presumed that this band represented nothing more than non-specific reactivity in the unpurified antisera.

Purified antibodies also detected recombinant FLAG-Nek6 or -Nek7 proteins using indirect immunofluorescence microscopy, however there was no obvious, distinctive localization pattern of recombinant protein detectable in interphase cells. The proteins do not, for example, appear to localize to the centrosome in the same way that Nek9 or Nek2 do. However, this may have been due to the high levels of recombinant protein expression in the cells analyzed, which may have masked any specific localization patterns. Indeed, analysis of both endogenous and recombinant Flag-Nek6 and Flag-Nek7 localization in mitosis using these affinity-purified Nek6 and Nek7 antibodies and

anti-Flag antibodies suggests there is a more specific localization pattern observable in mitotic cells (see section 4.2.2 and 4.2.3).

Thus, antibodies specific to human Nek6 and Nek7 have been raised and purified. These antibodies recognize specific doublet bands corresponding to Nek6 and Nek7 proteins, respectively, and do not cross-react. The doublet bands detectable are likely to be as a result of the presence within the cell lysate of hyperphosphorylated versions of Nek6 and Nek7 resulting in their altered electrophoretic mobility. This may be as a result of phosphorylation by the related Nek9 kinase which has been shown to phosphorylate and activate Nek6 and Nek7 *in vitro* and may well perform this physiological function *in vivo*, acting in a mitotic regulatory cascade (Belham *et al.*, 2003). The specificity of these antibodies was further confirmed upon analysis of cell lysates which had been depleted for Nek6 and Nek7 via RNAi (section 5.2.5).

A primary goal had been achieved in the generation of reagents specific for Nek6 and Nek7 which would allow the further characterization of the roles of these protein kinases in regulation of mitotic progression and specifically in regulation of mitotic spindle organization.

Chapter 4

Cell Cycle-Dependent Regulation of Nek6 and Nek7

4.1 Introduction

Nek6 and Nek7 are a pair of very similar NIMA-related kinases and Nek6 has been described as being capable of interacting with and phosphorylating the p70 ribosomal S6 kinase in response to insulin or mitogen stimulation, with the assumption that the similarity between the two kinases makes it likely that this will also apply to Nek7 (Belham *et al.*, 2001). However, it was later shown that Nek7 was unable to phosphorylate p70 S6K *in vivo* (Monoguchi *et al.*, 2003) and more recent evidence suggested that this is unlikely to be the physiological role of either Nek6 or Nek7 (Lizcano *et al.*, 2002). Instead, the identification of the centrosomally-associated, mitotic Nek9 kinase as an interacting partner and potential activator of Nek6 and Nek7 (Belham *et al.*, 2003) suggests that these kinases may, like their *Aspergillus* relative, be similarly involved in regulation of mitosis.

Limited studies of Nek6 and Nek7 function have been performed, often with conflicting conclusions and many investigations have focused solely on only one of the two protein kinases. For example, it has been reported separately that endogenous Nek6 has a purely cytoplasmic localization, that it has both a cytoplasmic and nuclear localization (Hashimoto *et al.*, 2002) and that recombinant Nek6 it is localized mainly to the nucleus (Chen *et al.*, 2006).

There is evidence that Nek7 has both a general, diffuse, cytoplasmic localization and a centrosomal localization both in interphase and mitosis, although there are conflicting reports on the potential localization of the protein to the spindle midzone and midbody in late mitotic and cytokinetic cells, respectively (Kim *et al.*, 2007; Yissacher *et al.*, 2006). This centrosomal localization is in keeping with the subcellular distribution of other Nek family members involved in regulation of mitotic progression. Nek2 localises to centrosomes throughout the cell cycle and, indeed, to the midbody of mitotic cells (Fry *et al.*, 1998; Kim *et al.*, 2002) whilst a phosphorylated form of Nek9 has been shown to localize to centrosomes (Roig *et al.*, 2005). Furthermore, Nek7 was detected in extracts enriched for centrosomes via sucrose gradient centrifugation and, interestingly, these extracts were enriched for a higher molecular weight, phosphorylated and presumably, activated, form of Nek7 (Yissachar *et al.*, 2006). However, Nek7 and indeed, Nek6 were not identified as centrosome components in a proteomic characterization of the centrosome, although this screen did identify Nek2 as a centrosomal kinase (Andersen *et al.*, 2003). This may be a reflection of the low abundance of this protein or, simply the fact that Nek7 does not localize to the centrosome under the conditions used in this analysis. Indeed, this study also failed to

recognize Nek9, a low abundance centrosomal protein, where only a small fraction of the total cytosolic protein is present at the centrosome at any given time.

The murine versions of both Nek6 and Nek7 have been reported to display a diffuse cytoplasmic localization in interphase, with no obvious localization to any cellular structures or organelles, including centrosomes (Kandli *et al.*, 2000).

The potential cell cycle regulation of these protein kinases is likewise unclear and rather complicated. Whilst Nek6 and Nek7 protein levels have been reported to be constant throughout the cell cycle (Yin *et al.*, 2003; Kim *et al.*, 2007), there was an increase in the amount of Nek6 protein showing reduced electrophoretic mobility in extracts from mitotic cell populations and a concomitant increase in kinase activity and autophosphorylation (Yin *et al.*, 2003). However, Minoguchi *et al.* (2003) reported that although total Nek6 and Nek7 endogenous protein levels did not change significantly throughout the cell cycle, recombinant and endogenous Nek7, but not Nek6, kinase activity was maximal at the G2/M transition. Belham *et al.* (2003) reported a 3-fold and 4-fold increase in the relative abundance of Nek6 at the mRNA and protein level, respectively, in mitotic compared to asynchronous cell populations, accompanied by a slowing in the electrophoretic mobility of the protein, indicative of phosphorylation and a parallel increase in kinase activity.

For either kinase not to have a cell cycle-dependent pattern of regulation is, perhaps, counterintuitive in view of the reported phosphorylation and activation of Nek6 and Nek7 by the cell cycle-regulated Nek9 kinase and the postulated mitotic protein kinase cascade.

Here, the cell cycle-dependent localization of both endogenous and recombinant Nek6 and Nek7, as well as the cell cycle-dependent regulation of the abundance and activity of the endogenous protein kinases was investigated using the Nek6 and Nek7 antibodies described in chapter 3.

4.2 Results

4.2.1 Cell cycle-dependent localization of endogenous Nek6

In view of the postulated kinase cascade involving Nek6, Nek7 and Nek9 (Belham *et al.*, 2003), the centrosomal localization of Nek9, and the role Nek9 appears to have in regulation of mitotic spindle organization (Roig *et al.*, 2002; Roig *et al.*, 2005), it seems probable that Nek6 and Nek7 are likewise involved in regulation of mitotic progression. Thus, association of Nek6 and Nek7 with, and localization to, mitotic structures would also seem a distinct possibility. However, so far Nek6 has not been shown to localize to any mitotic structure and indeed whether the protein is exclusively centrosomal or shows some nuclear localization during interphase is unclear.

In order to address the issue of whether Nek6 is associated with any mitotic structures and is therefore likely to be a mitotic regulator, the localization of endogenous Nek6 at the various stages of mitosis, as well as in interphase, was examined using the affinity-purified anti-Nek6 antibodies.

HeLa cells were grown on acid-etched coverslips, fixed and permeabilised with ice-cold methanol and processed for immunofluorescence microscopy with anti-Nek6 and anti-α-or γ-tubulin antibodies. Anti-γ-tubulin antibodies were used as a marker of the centrosome and spindle poles in interphase and mitotic cells, respectively, and anti-α-tubulin antibodies were used to determine whether Nek6 associated with the cytoskeleton or mitotic spindle (Figures 4.1 and 4.2).

The most obvious conclusion from the immunofluorescence microscopy analysis was that Nek6 does not show a strong, distinct localization to any cellular structure or organelle in either interphase or mitosis. The protein is distributed throughout the interphase cytoplasm and nucleus as may be expected in view of its small size and thus capability for unrestricted diffusion through nuclear pores. This also suggests that it is not tightly associated with a protein complex that is restricted to one compartment or the other.

In mitotic cells, although Nek6 retained a diffuse, granular distribution, a stronger signal was detectable on the microtubules of the mitotic spindle in metaphase and anaphase cells and the spindle midzone and midbody in late mitotic and cytokinetic cells (Figure 4.1). Examination of cells costained with anti-Nek6 and anti-γ-tubulin antibodies revealed that Nek6 does not obviously localize to centrosomes in interphase, although there is a concentration of protein towards the spindle pole in mitosis (Figure 4.2).

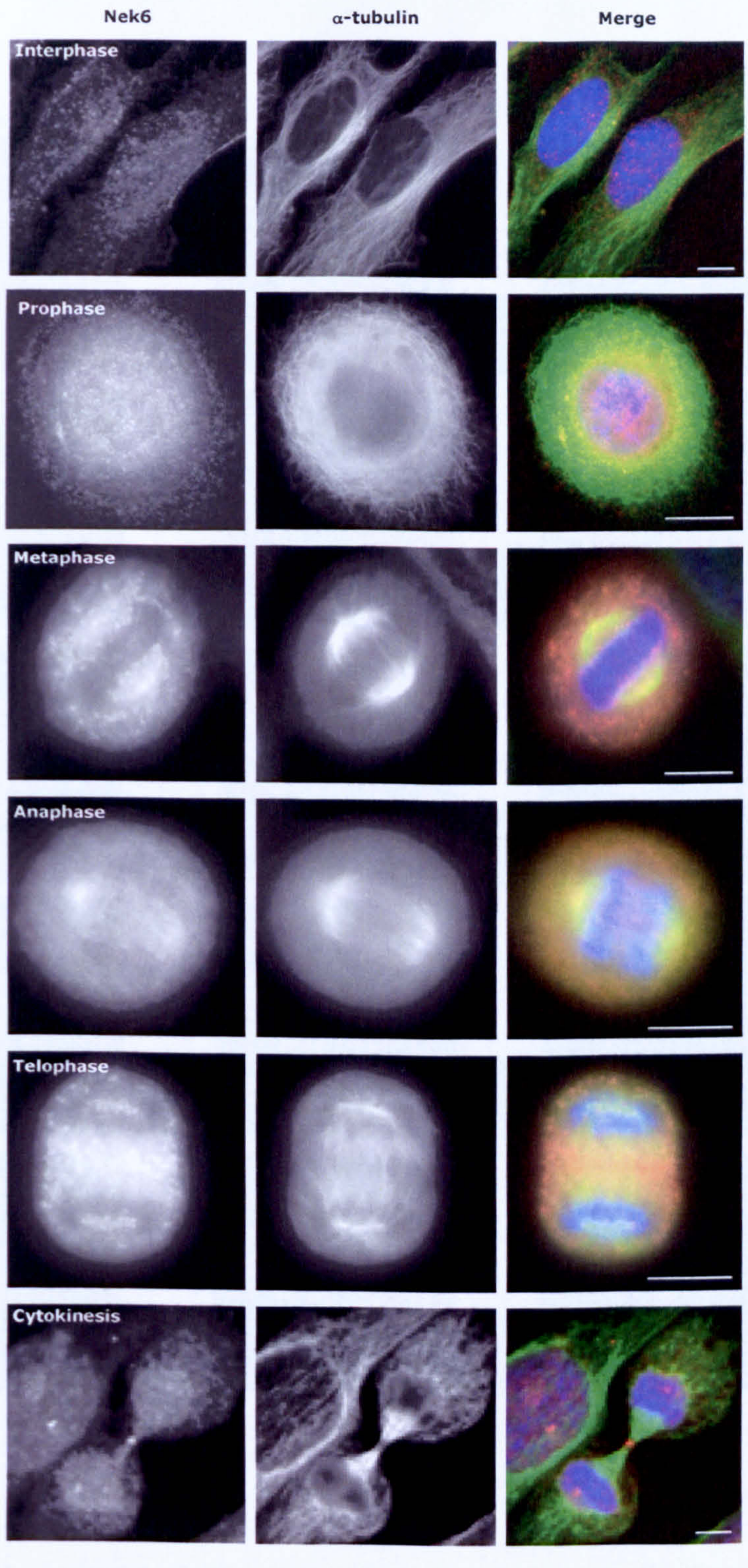


Figure 4.1 Nek6 localizes to the vicinity of the mitotic spindle in metaphase and early anaphase

HeLa cells were grown on acid-etched coverslips before being fixed and permeabilised with ice-cold methanol and processed for immunofluorescence microscopy. Cells in interphase and at different stages of mitosis were imaged as indicated. Cells were stained with anti-α-tubulin antibodies to detect the microtubule network (green on merge), and affinity purified anti-Nek6 antibodies (red on merge); DNA was stained with Hoechst 33258 (blue on merge). Scale bars, 10 μm.

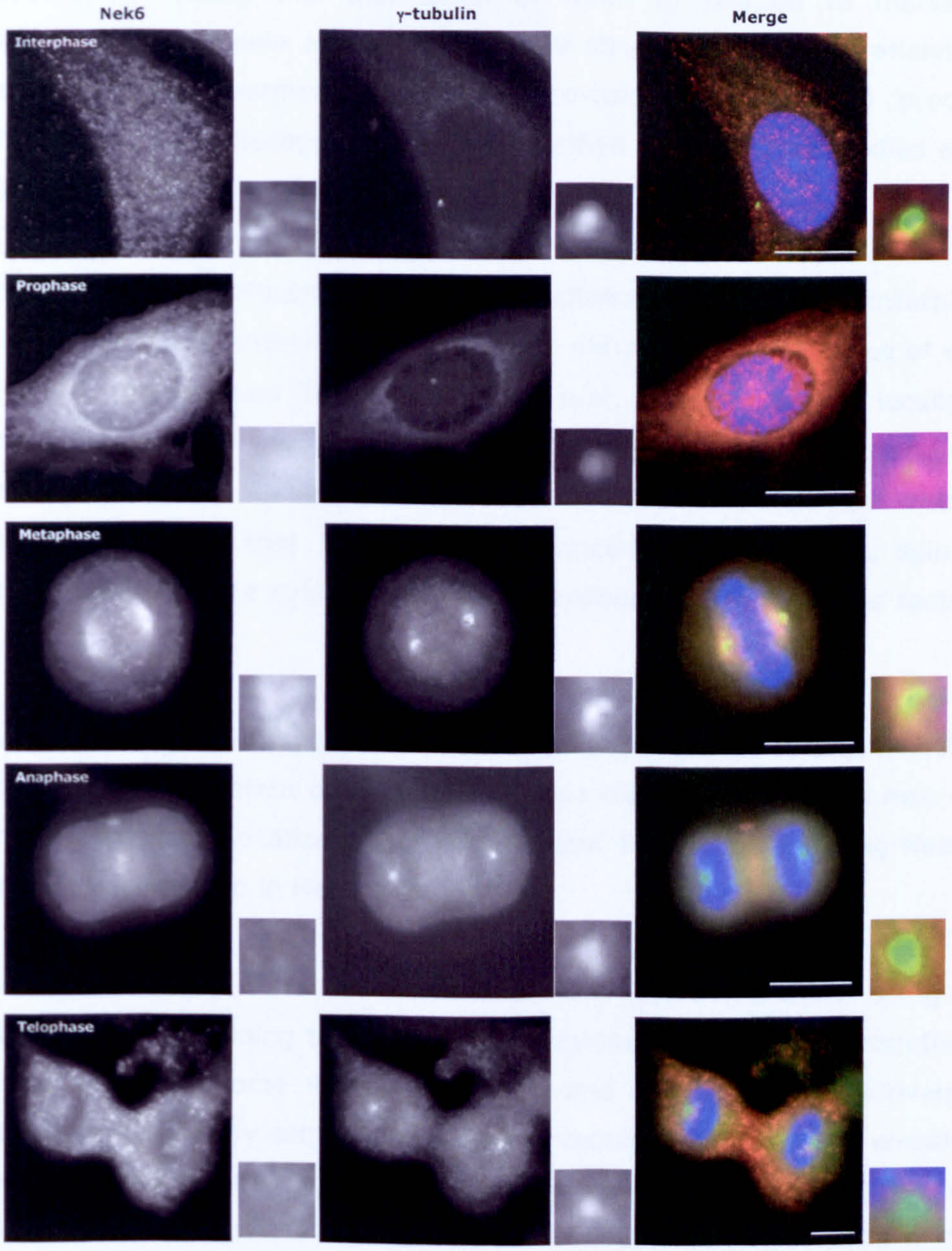


Figure 4.2 Nek6 does not localize to centrosomes or spindle poles

HeLa cells grown on acid-etched coverslips were fixed and permeabilised with ice-cold methanol before being procesed for immunofluorescence microscopy. Cells in interphase and at different stages of mitosis were imaged. Centrosomes were stained with anti-γ-tubulin antibodies to detect the centrosomes (green on merge) and affinity purified anti-Nek6 antibodies (red on merge); DNA was stained with Hoechst 33258 (blue on merge). Magnified views of the centrosomes/spindle poles are shown. Scale bars, 10 μm.

4.2.2 Cell cycle-dependent localization of endogenous Nek7

In view of the similarity of Nek6 and Nek7 in structure and their joint interaction with, and activation by, Nek9, the localization of Nek7 in relation to markers of the centrosome and spindle pole and mitotic spindle structures was also analyzed. HeLa cells were fixed and permeabilised using ice-cold methanol and processed for immunofluorescence microscopy with affinity purified anti-Nek7 antibodies and anti-α- or γ-tubulin antibodies (Figures 4.3 and 4.4).

Like Nek6, Nek7 was distributed across the cytoplasm and nucleus of interphase cells, although costaining with anti-γ-tubulin antibodies did reveal some degree of association with interphase centrosomes (Figure 4.3). However, this centrosomal localization was not invariably observed; it perhaps represents a transient or cell cycle stage dependent centrosomal association. Analysis of cells costained with anti-Nek7 and anti-α-tubulin antibodies demonstrated that Nek7 was not concentrated at mitotic spindle poles, spindle microtubules or the spindle midzone or midbody in the cell lines tested (Figure 4.4).

4.2.3 Cell cycle-dependent localization of recombinant Nek6 and Nek7

To corroborate the localization data on endogenous Nek6 and Nek7, we next examined the cell cycle-dependent localization of recombinant Flag-Nek6 and Flag-Nek7 kinases when transiently expressed in HeLa cells.

To this end, full length ESTs for Nek6 and Nek7 were identified using database searching for clones containing the appropriate sequence and obtained from the German Resource Centre for Genome Research (RZDP) and Invitrogen, respectively. These clones were then verified by sequencing in both directions and shown to encode ORFs of the correct predicted length (313 and 302) amino acid sequences for Nek6 and Nek7, respectively, with obvious ATG and stop codons. These full-length ESTs of Nek6 and Nek7 were then cloned into the pFLAG-CMV2 mammalian expression vector as NotI/XbaI fragments to generate recombinant proteins with N-terminal Flag tags which could be transiently transfected into cells.

To determine the efficiency of plasmid uptake and expression of the recombinant protein and also to confirm that the recombinant protein produced was of the expected size and therefore in frame and full-length, lysates of transfected cell populations were prepared and subjected to SDS-PAGE and Western blotting with anti-Flag antibodies. This confirmed that the proteins were expressed with a high degree of efficiency and were of the expected size for Flag-Nek6 and Flag-Nek7 proteins (Figure 4.5A). Immunoblots

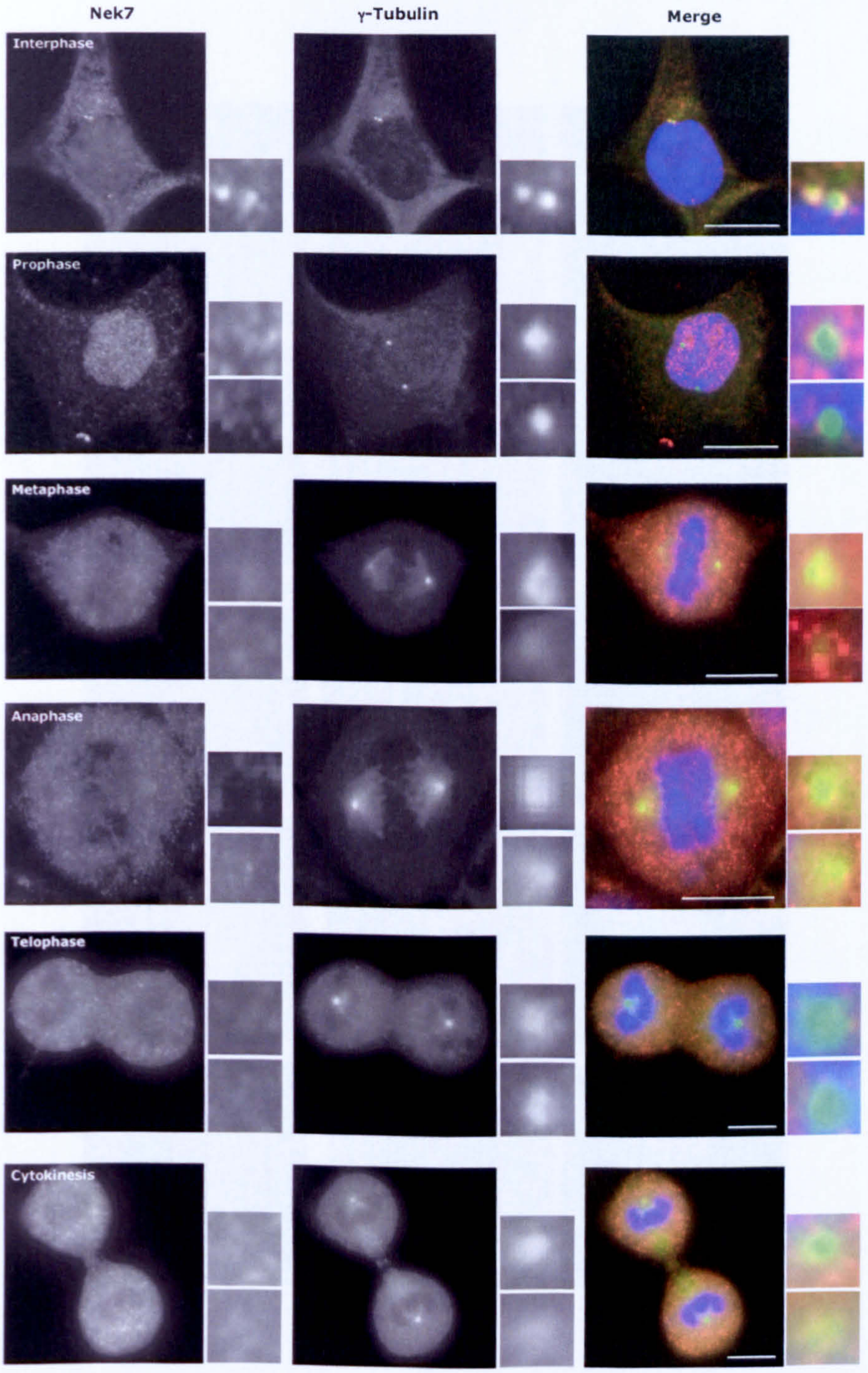


Figure 4.3 Nek7 localizes to interphase centrosomes but not mitotic spindle poles.

HeLa cells were grown on acid-etched coverslips before being fixed and permeabilised in ice-cold methanol and processed for immunofluorescence microscopy. Cells in interphase and at different stages of mitosis were imaged. Centrosomes were stained with anti-γ-tubulin antibodies to detect the centrosomes (green on merge), affinity purified anti-Nek7 antibodies (red on merge); DNA was stained with Hoechst 33258 (blue on merge). Magnified views of centrosomes/spindle poles are shown. Scale bars, 10 μm.

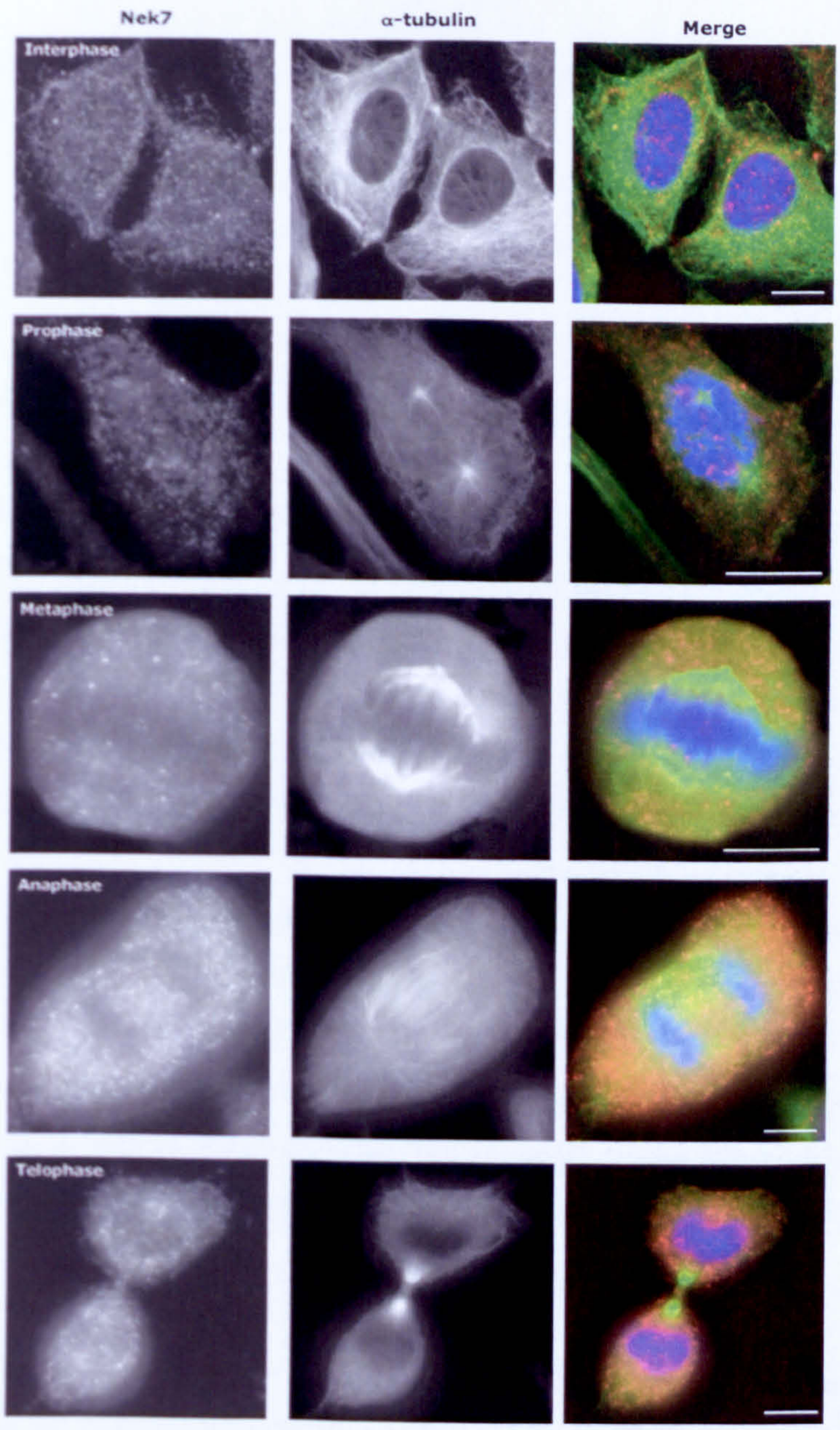


Figure 4.4 Nek7 does not localise to microtubule structures in mitotic cells
HeLa cells were grown on acid-etched coverslips before being fixed and permeabilised in ice-cold methanol and processed for immunofluorescence microscopy. Cells in interphase and at different stages of mitosis were imaged. Cells were stained with anti-α-tubulin antibodies to detect the microtubule network (green on merge), and anti-Nek7 antibodies (red on merge); DNA was stained with Hoechst 33258 (blue on merge). Scale bars, 10 μm.

with anti-Nek6 and -Nek7 antibodies further confirmed the identity of the expressed proteins.

In order to examine the localization of these recombinant proteins, the Flag-Nek6 and -Nek7 constructs were transiently transfected into HeLa cells. Cells were allowed to express the recombinant proteins for 24 h before being fixed and permeabilised using ice cold methanol and processed for immunofluorescence microscopy with anti-Flag and anti-α-tubulin or anti-γ-tubulin antibodies.

Immunofluorescence microscopic analysis of the localization of recombinant Flag-Nek6 compared to that of γ-tubulin throughout the cell cycle revealed that, like the endogenous Nek6, there is no obvious association with centrosomes either in interphase or in mitosis (Figure 4.6). However, as for the endogenous protein, there was some association of Flag-Nek6 with mitotic spindle microtubules and the midbodies of dividing cells, as revealed by costaining with anti-α-tubulin antibodies. This association was however, somewhat masked by the high background levels of cytoplasmic Flag-Nek6 resulting from high levels of expression of the recombinant protein (Figure 4.5B).

In contrast, unlike endogenous Nek7, there was never any apparent association of Flag-Nek7 with the centrosome upon immunofluorescence microscopic analysis of transfected cells co-stained with anti-Flag and anti-γ-tubulin antibodies (Figure 4.7). However, as for Flag-Nek6 this may be due to the masking effect of high levels of recombinant protein expression. There was likewise no obvious association with mitotic spindle microtubules upon analysis of transfected cells stained with anti-α-tubulin antibodies (Figure 4.8).

This data thus supports and confirms the localization pattern of endogenous Nek6 but not Nek7 proteins to microtubule structures in mitosis, but it remains unclear to what extent Nek7 may associate with interphase centrosomes.

4.2.4 Cell cycle-dependent regulation of Nek6 and Nek7 activity

The association of Nek6 with mitotic spindle structures and possibly Nek7 with centrosomes, adds further credence to the hypothesis that Nek6 and Nek7 are mitotic kinases. This also suggests that they are likely to be cell cycle-regulated and that this regulation will result in maximal protein levels and/or kinase activity at mitosis. Confusingly, published data on the cell cycle regulation of Nek6 and Nek7 variously describe the mitotic upregulation of Nek6 but not Nek7 kinase activity but not protein levels (Yin *et al.*, 2003), Nek6 but not Nek7 kinase activity, protein levels and mRNA

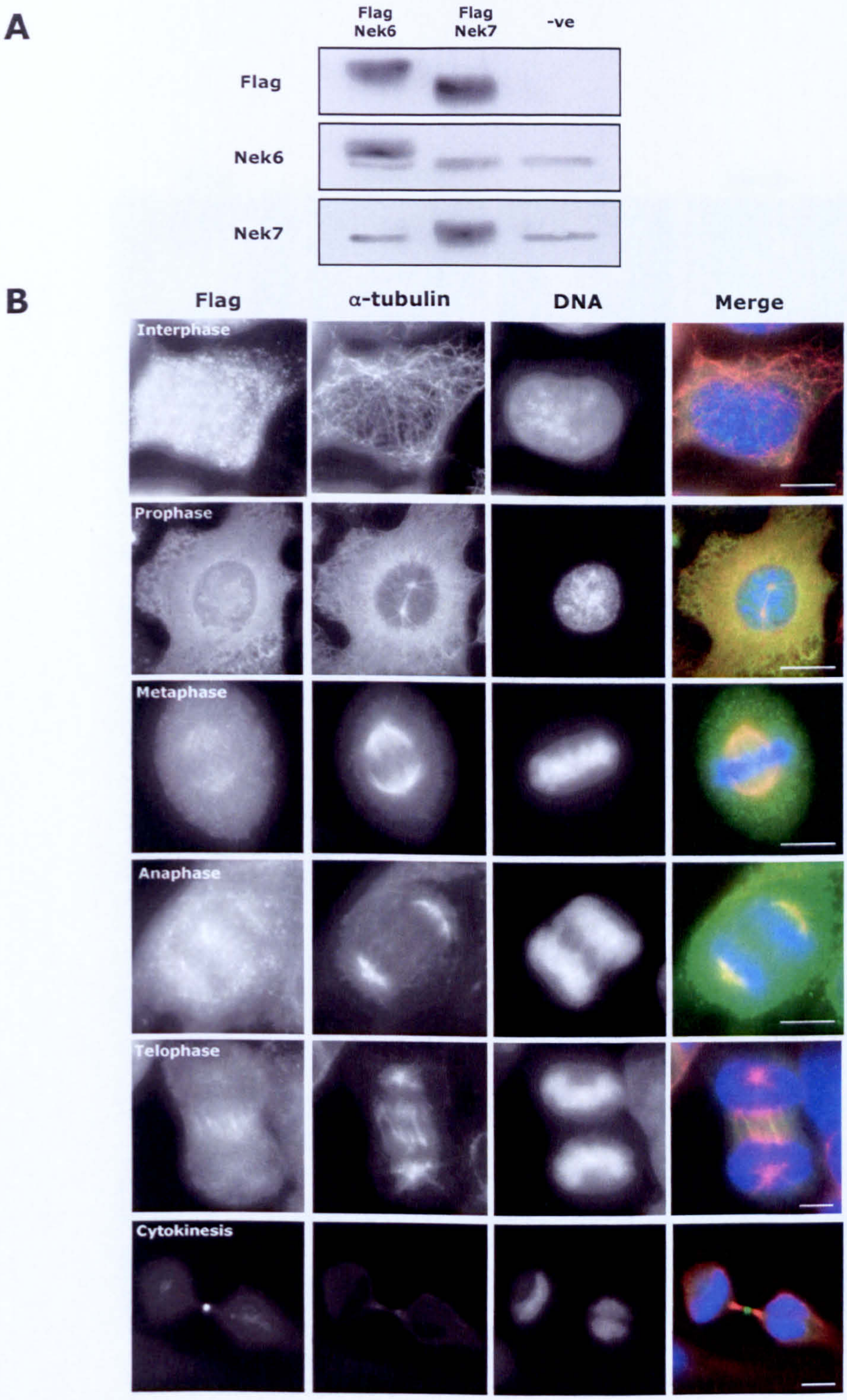


Figure 4.5 Recombinant Nek6 localizes to microtubule structures in mitosis

(**A**) HeLa cells were transiently transfected with Flag-Nek6 or Flag-Nek7 (as indicated). Control cells were untransfected (-ve). Cells were then, lysed and subjected to SDS-PAGE and Western blotting with anti-Flag, anti-Nek6 and anti-Nek7 antibodies. (**B**) HeLa cells were transiently transfected with Flag-Nek6 for 24 h before being fixed and permeabilised in ice-cold methanol and processed for imunofluorescence microscopy. Cells were immunostained with anti-α-tubulin antibodies to detect microtubule structures (red on merge). Flag-hNek6-WT was stained with anti-Flag antibodies (green on merge). DNA is stained with Hoechst 33258 (blue on merge). Cells were imaged in interphase and at various stages mitosis. Scale bars, 10 μm.

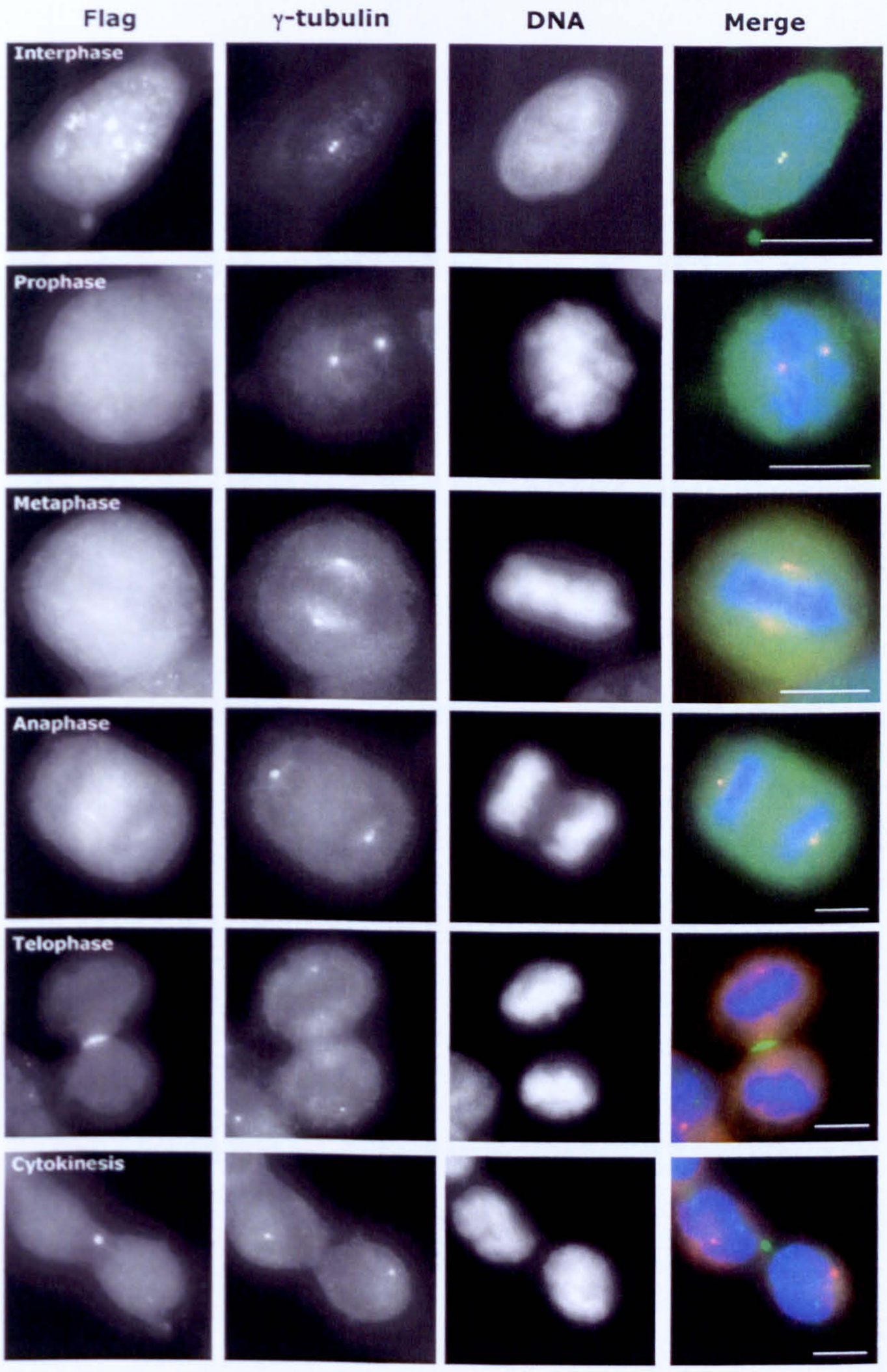


Figure 4.6 Recombinant Nek6 protein does not localize to centrosomes or spindle poles

HeLa cells were transiently transfected with Flag-Nek6 for 24 h before being fixed and permeabilised in ice-cold methanol and processed for imunofluorescence microscopy. Cells were immunostained with anti-γ-tubulin antibodies to detect centrosomes (red on merge). Flag-hNek6-WT is stained with anti-Flag antibodies (green on merge). DNA was stained with Hoechst 33258 (blue on merge). Cells were imaged in interphase and at various stages of mitosis. Scale bars, 10 μm.

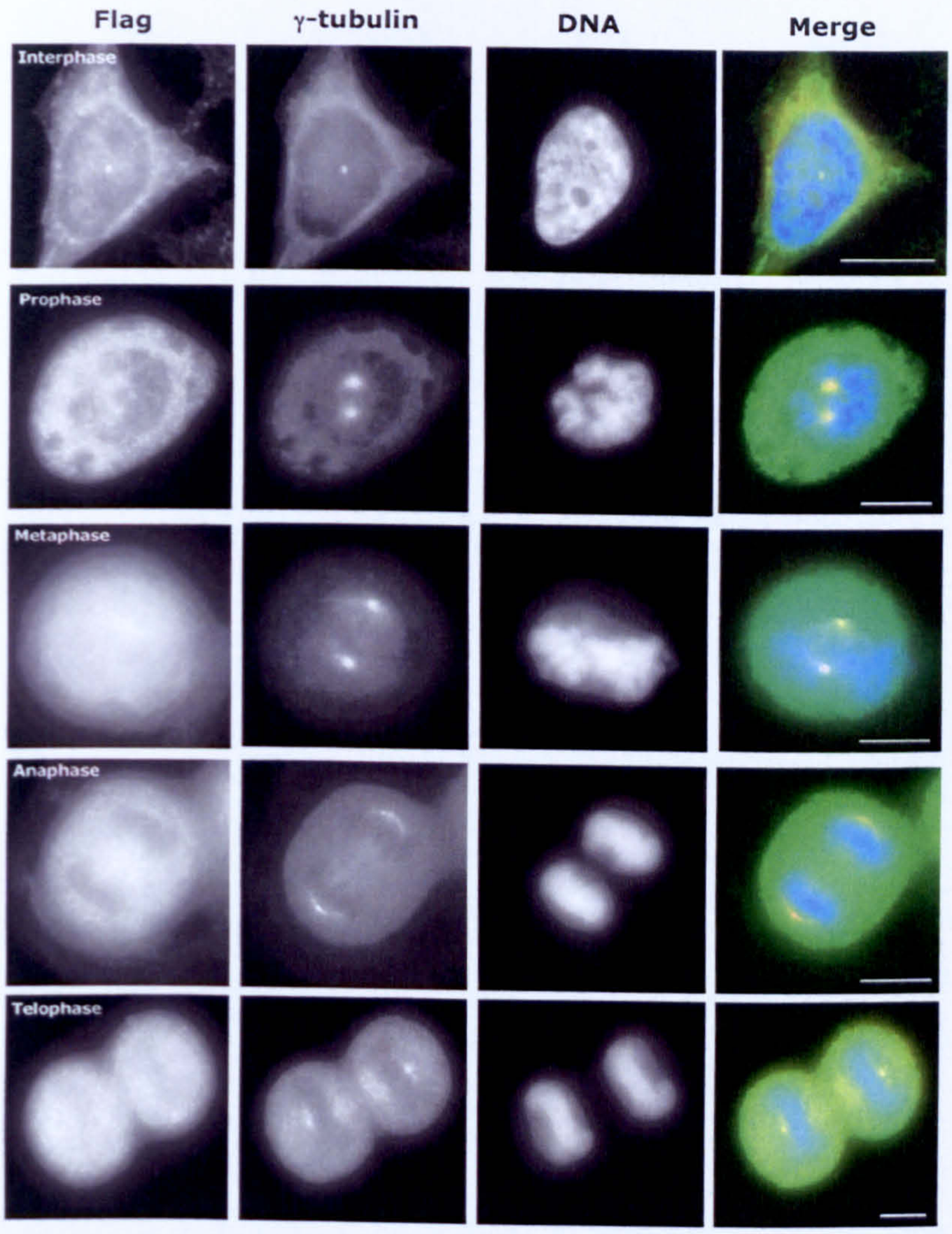


Figure 4.7 Recombinant Nek7 does not strongly associate with centrosomes or spindle poles

HeLa cells were transiently transfected with Flag-Nek7 for 24 h before being fixed and permeabilised in ice-cold methanol and processed for immunofluorescence microscopy. Cells were immunostained with anti-γ-tubulin antibodies to detect centrosomes (red on merge). Flag-Nek7 was stained with anti-FLAG antibodies (green on merge). DNA was stained with Hoechst 33258 (blue on merge). Cells were imaged in interphase and at various stages of mitosis. Scale bars, 10 μm.

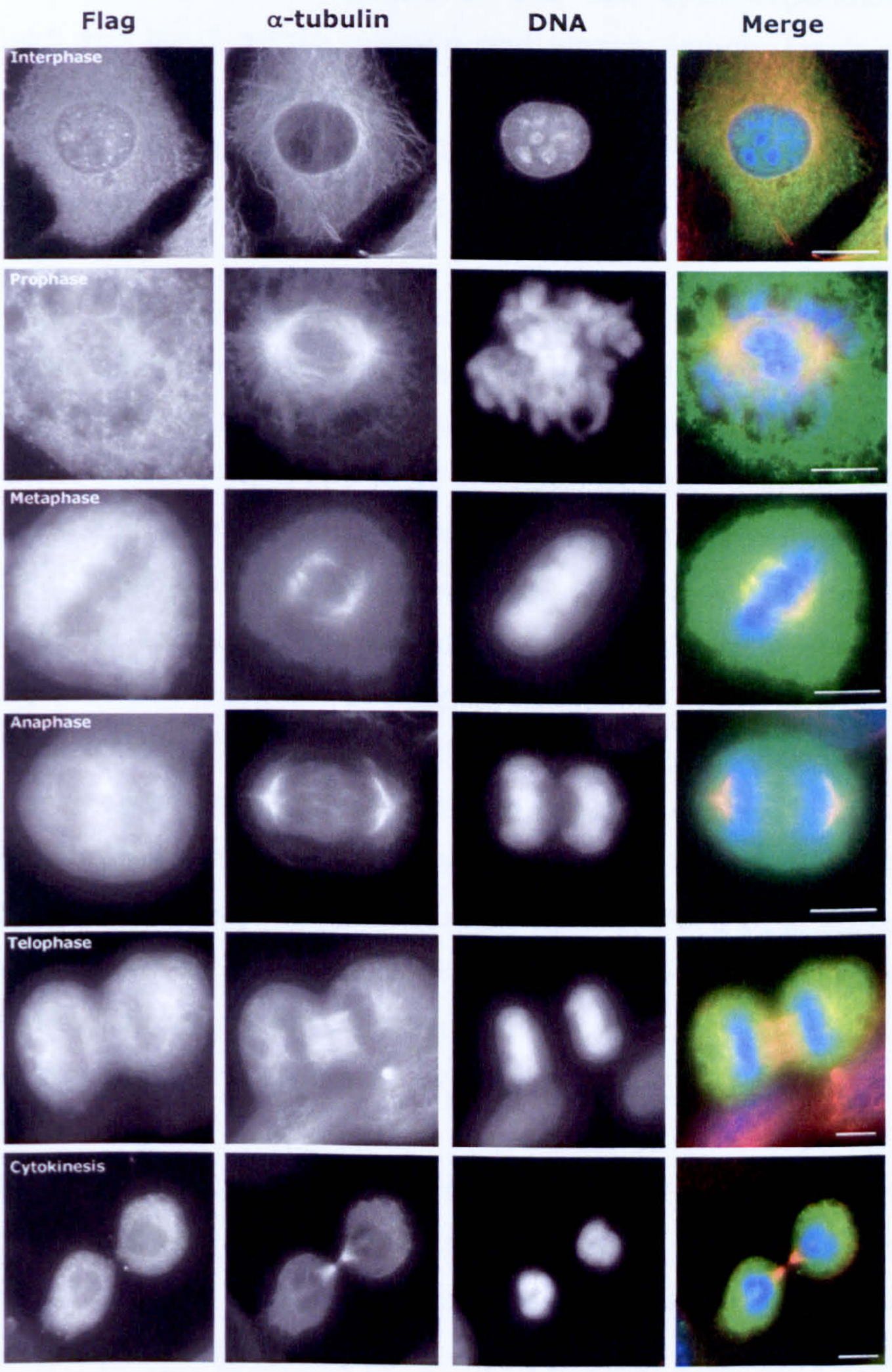


Figure 4.8 Recombinant Nek7 does not localize to microtubule structures
HeLa cells were transiently transfected with Flag-Nek7 for 24 h before being fixed and permeabilised in ice-cold methanol and processed for immunofluorescence microscopy. Cells were immunostained with anti-α-tubulin antibodies to detect microtubule structures (red on merge). Flag-Nek7 was stained with anti-Flag antibodies (green on merge). DNA was stained with Hoechst 33258 (blue on merge). Cells were imaged in interphase and at various stages of mitosis. Scale bars, 10 μm.

levels (Belham *et al.*, 2003) and, finally, Nek7 but not Nek6 kinase activity but not protein levels (Kim *et al.*, 2007).

Thus, we carried out our own analysis of the cell cycle-dependent regulation of endogenous Nek6 and Nek7 mRNA, protein and kinase activity levels using HeLa cells synchronized at various stages of the cell cycle using nocodazole and thymidine induced cell cycle arrest and washout protocols. Cell cycle synchronization was confirmed by flow cytometric analysis of the DNA content (Figure 4.9A).

To analyze the cell-cycle dependent protein expression of Nek6 and Nek7, cells were synchronized, lysed as appropriate and lysates subjected to SDS-PAGE and Western blot analysis with anti-Nek6 and anti-Nek7 antibodies. This demonstrated a clear increase in the total abundance of both Nek6 and Nek7 proteins as cells progress through the G2/M transition (Figure 4.9B). Densitometry measurements of the relative protein abundance demonstrated that there was ~40-50% more Nek6 and Nek7 in the G2 and M-phase populations compared to that in the asynchronous and G1 populations (Figure 4.9C). Western blotting with anti-α-tubulin antibodies confirmed that this change was not due to variations in the total protein content of lysates. Western blotting with anti-cyclin B2 antibodies was used to biochemically confirm cell cycle synchronization. Cyclin B2 is a well-characterized protein whose abundance is known to increase from S phase through to mitosis before being targeted for degradation by the APC/C at anaphase onset (Clute and Pines, 1999; Castro *et al.*, 2002). This protein showed the expected pattern of expression in the synchronized cell lysates: low abundance in G1 and S and increasing abundance towards the G2 and M-phases of the cell cycle (Figure 4.9B).

Along with the increase in protein content for the Nek6 and Nek7 proteins as cells progressed through G2 and M-phases of the cell cycle, there was also an apparent change in the electrophoretic mobility of the proteins. In asynchronous cell populations, Nek6 and Nek7 migrate predominantly as a single band with a less abundant, slower migrating band also apparent at low levels. In G1 and S-phase cell populations this latter band became somewhat less obvious compared to that seen in the asynchronous population. However, in populations of cells in G2 and M phase of the cell cycle, this upper, slower migrating band became far more abundant to the point where it was at least as abundant as the faster migrating form of the protein (Figure 4.9B). This suggests that Nek6 and Nek7 are cell cycle-regulated proteins which increase in abundance and are subject to phosphorylation during the G2/M transition.

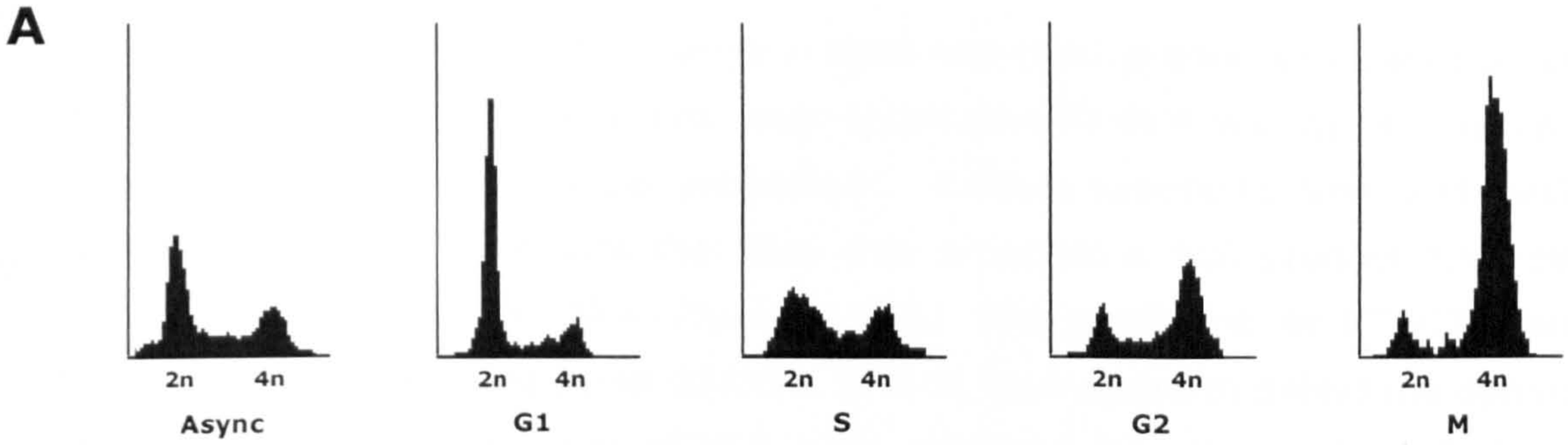


Figure 4.9 Nek6 and Nek7 protein levels are cell cycle regulated

HeLa cells were synchronized as described in Materials and Methods. (**A**) Cell synchronization was confirmed via flow cytometry analysis of cells fixed in 70% ice-cold ethanol and stained with propidium iodide. (**B**) Cells lysates were analyzed via SDS-PAGE and Western blotting with affinity-purified anti-Nek6 and anti-Nek7 antibodies at a working concentration of 2 µg/ml, anti-α-tubulin antibody as a loading control and anti-cyclin B2 antibody to confirm cell cycle synchronization. (**C**) Levels of Nek6 and Nek7 protein present in cell lysates at the different cell cycle stages was analyzed by densitometry. Protein levels are expressed as percentage of that present in asynchronous cell populations minus background. Data shown represent means (± S.D.) of three separate experiments.

In order to assess whether this change in Nek6 and Nek7 protein abundance occurred at the transcription or translation level, semi-quantative RT-PCR was carried out on mRNA extracted from synchronized cell populations. Primers specific to Nek6 and Nek7 were designed and tested to ensure that they only amplified a PCR product from plasmids encoding the appropriate cDNA (Figure 4.10A). The conditions for RT-PCR were then carefully established using serial dilutions of total RNA added to determine optimal RNA concentration and optimization of PCR cycle number. To optimize total RNA content, RT-PCR reactions were carried out on RNA derived from asynchronous HeLa cell populations using a range of RNA concentrations from 0 to 5 µg total RNA in a 35 cycle PCR reaction (Figure 4.10B). To optimize cycle number, RT-PCR reactions were carried out with Nek6 and Nek7 specific primers on 5 µg total RNA extracted from asynchronous HeLa cell populations with cycle numbers increasing from 0-35 cycles in steps of 5 cycles; aliquots were removed from the PCR reactions after the appropriate number of cycles (Figure 4.10C). Analysis of DNA yield by agarose gel electrophoresis revealed that maximum yield was obtained with 5 µg of RNA and 35 amplification cycles. Thus, in all ensuing RT-PCR experiments, 5 µg of plasmid DNA and 35 amplification cycles were used.

Following optimization, semi-quantitative RT-PCR was carried out on RNA extracted from the synchronized HeLa cell populations used for determination of protein content using Nek6 and Nek7 primers. Analysis of PCR products by agarose gel electrophoresis and visualization under UV showed that, in parallel to Nek6 and Nek7 protein abundance, Nek6 and Nek7 mRNA abundance also increased as cells approached the G2/M transition (Figure 4.11A). Quantification of the relative abundance of Nek6 and Nek7 mRNA, via densitometry measurements of PCR product abundance, demonstrated that Nek6 and Nek7 mRNA levels increased by approximately 25-30% in G2 and M-phase cell populations as compared to asynchronous, G1 and S-phase cell populations (Figure 4.11B). This is comparable to the 40-50% increase seen in Nek6 and Nek7 protein abundance. Thus, it would appear that changes in Nek6 and Nek7 protein abundance in G2/M phase are brought about at least in part by upregulated transcription.

In order to assess whether these differences in Nek6 and Nek7 protein abundance and electrophoretic mobility resulted in changes in the kinase activity of the two protein kinases, their kinase activity at G1/S and G2/M cell cycle phases was compared. The kinase activity of recombinant purified His_6Nek6 and His_6Nek7 (purchased from Upstate) on a variety of exogenous substrates was first assessed in order to identify a suitable substrate with which to test kinase activity of the endogenous protein. Nek6 has been variously reported to be unable to phosphorylate β-casein whilst strongly

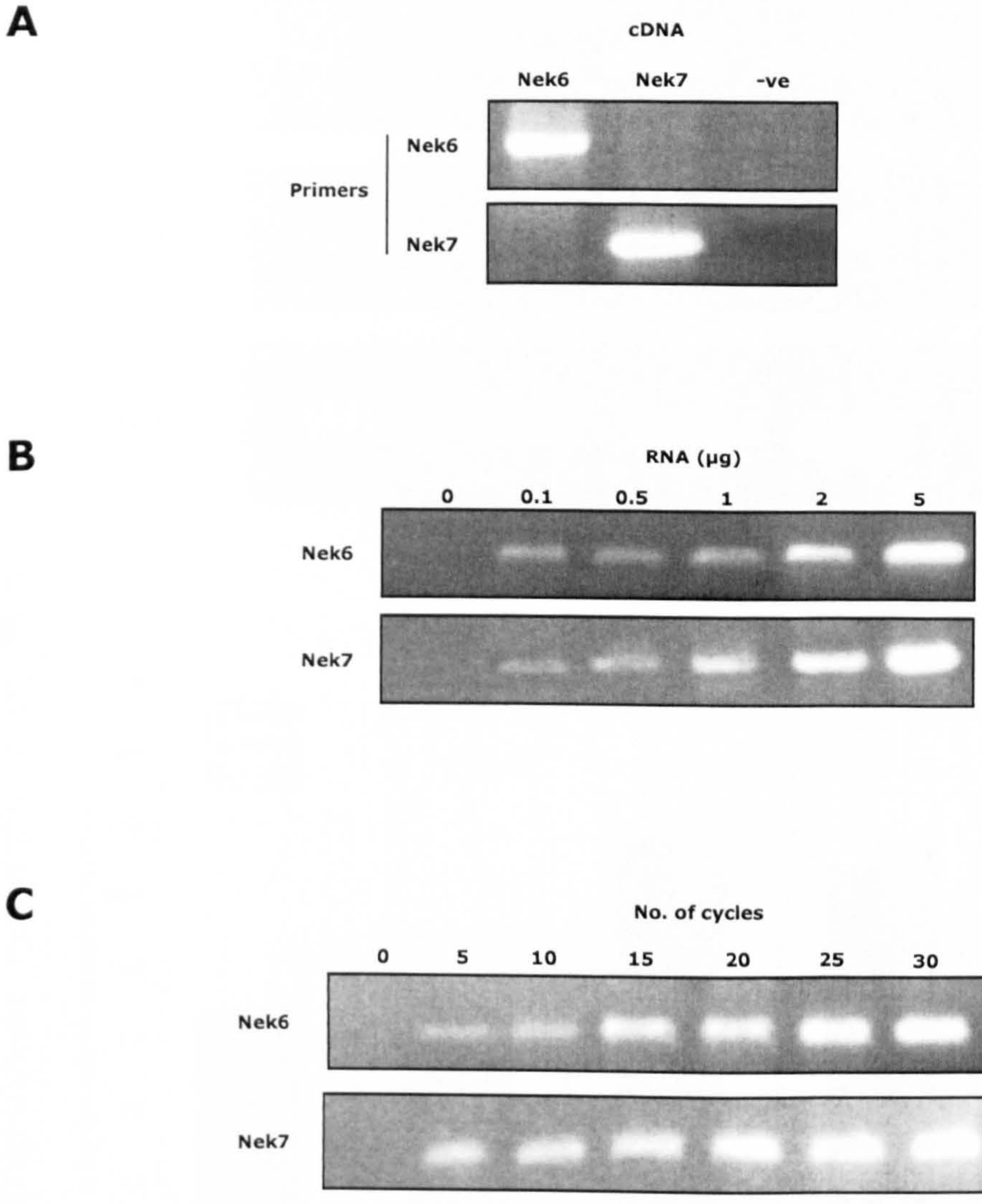
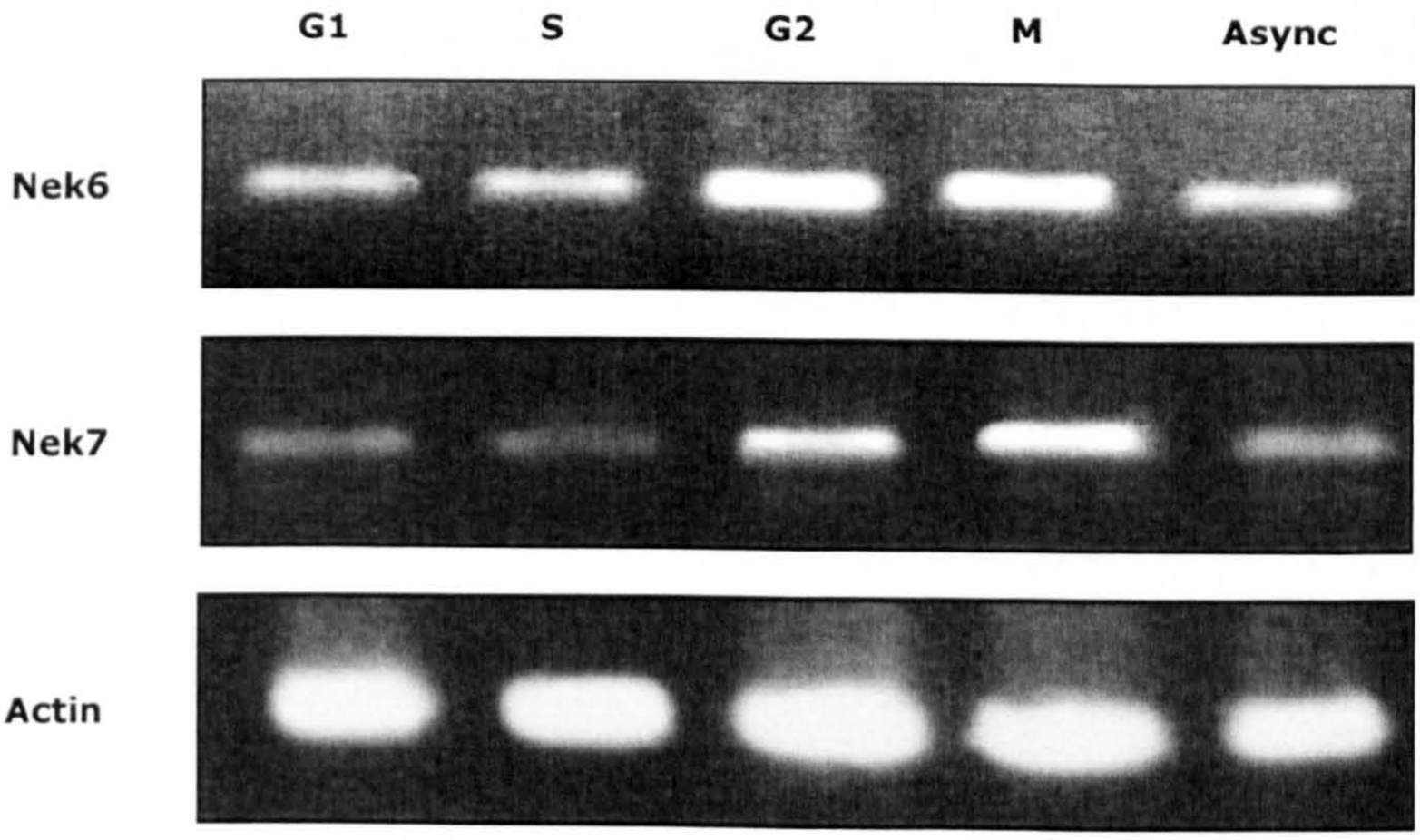


Figure 4.10 Optimization of Nek6 and Nek7 RT-PCR conditions

(**A**) PCR reactions were performed on plasmids encoding Nek6 or Nek7 cDNA using Nek6 (upper panel) or Nek7 (lower panel) specific primers which had been designed for RT-PCR, in order to assess primer specificity. (**B**) RT-PCR reactions were carried out using Nek6 or Nek7 RT-PCR primers and increasing amounts of total RNA as indicated. 35 amplification cycles were used. (**C**) RT-PCR was carried out using Nek6 and Nek7 specific primers and 5 µg total RNA for increasing numbers of cycles as indicated.

A



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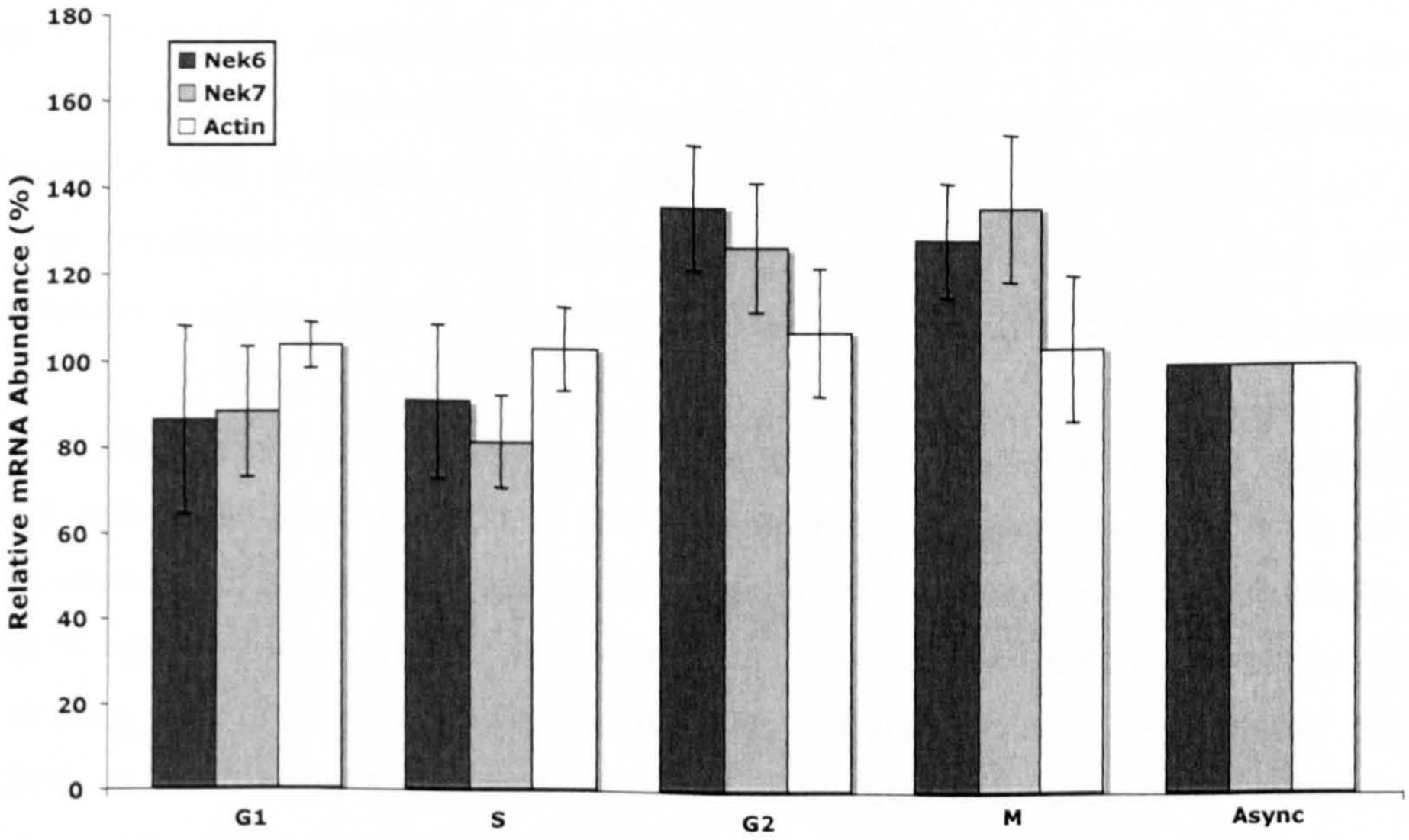

Figure 4.11 Nek6 and Nek7 mRNAs are cell cycle regulated

HeLa cells were synchronised, as indicated, as described in Materials and Methods. Async, asynchronous. (**A**) Cells were lysed using Tri reagent and RNA isolated from the lysate used to carry out RT-PCR reactions with PCR primers for Nek6, Nek7 and actin (positive control). (**B**) The relative abundance of the different PCR products was analysed via densitometry measurements using ImageJ, as described in Materials and Methods. The abundance relative to asynchronous populations, minus background is shown. Data represent mean (± S.D) of three separate experiments.

phosphorylating Histone H1 and H3 (Hashimoto *et al.*, 2002), to phosphorylate β-casein, histones and myelin basic protein all to a similar extent (Minoguchi *et al.*, 2003) and to strongly phosphorylate MBP (Belham *et al.*, 2003; Yin *et al.*, 2003). Whilst Nek7 has been shown to phosphorylate β-casein, histones and myelin basic protein all to a similar extent (Minoguchi *et al.*, 2003) and to strongly phosphorylate MBP (Belham *et al.*, 2003; Yin *et al.*, 2003). The inability of Nek6 or Nek7 to phosphorylate β-casein would seem surprising in view of the fact that most known mammalian Neks have been shown to phosphorylate β-casein *in vitro* (Osmani *et al.*, 1991; Lu *et al.*, 1993; Fry *et al.*, 1995; Tanaka and Nigg, 1999; Hashimoto *et al.*, 2002; Holland *et al.*, 2002), although Nek9, whilst still able to phosphorylate β-casein, has been shown to phosphorylate MBP and different histones more strongly (Roig *et al.*, 2001).

Thus, we sought to determine which substrate, MBP, β-casein or Histone H1, would be the best substrate for Nek6 and Nek7. To this end, His_6Nek6 and His_6Nek7 kinases were incubated in kinase buffer containing [γ-^{32}P]-ATP, with equalized concentration of either β-casein, MBP or Histone H1 as a substrate, for 30 min at 30°C in order to assess and compare different substrate phosphorylation levels. Analysis of substrate phosphorylation by SDS-PAGE, Coomassie Blue staining and autoradiography demonstrated that β-casein was the best substrate for both Nek6 and Nek7 kinases under the conditions used in this experiment (Figure 4.12A and B). Thus, all *in vitro* kinase assays involving Nek6 and Nek7 were carried out with β-casein as a substrate.

To examine the effect of cell cycle stage on endogenous Nek6 and Nek7 kinase activity, HEK 293 cells were synchronized at G1/S or M phases of the cell cycle using thymidine and nocodazole blocks, respectively, as described in Materials and Methods. Flow cytometric analysis was used to determine the DNA complement of cell populations and thus assess synchronization (Figure 4.13B). Synchronized cells were lysed and subjected to immunoprecipitation with affinity purified anti-Nek6 and anti-Nek7 antibodies. IPs using normal rabbit IgGs were carried out in parallel as a control. Successful immunoprecipitation was determined by analyzing samples of immune complexes by SDS-PAGE and Western blotting with anti-Nek6 and anti-Nek7 antibodies (Figure 4.13A), which showed that comparable levels of protein were immunoprecipitated in all cases. The remaining immune complex beads were incubated in kinase buffer containing [γ-^{32}P]-ATP and β-casein for 30 min at 30°C. Analysis of substrate phosphorylation and autophosphorylation in G1/S and M phases by SDS-PAGE, Coomassie Blue staining and autoradiography, showed that both kinases were significantly more active in M-phase compared to G1/S phase (Figure 4.13B). This

increase was shown by scintillation counting to be 2-3fold for both Nek6 and Nek7 (Figure 4.13C).

Thus, both Nek6 and Nek7 are cell cycle-regulated protein kinases with regulation occurring at the level of transcription, post-translational modification and kinase activity.

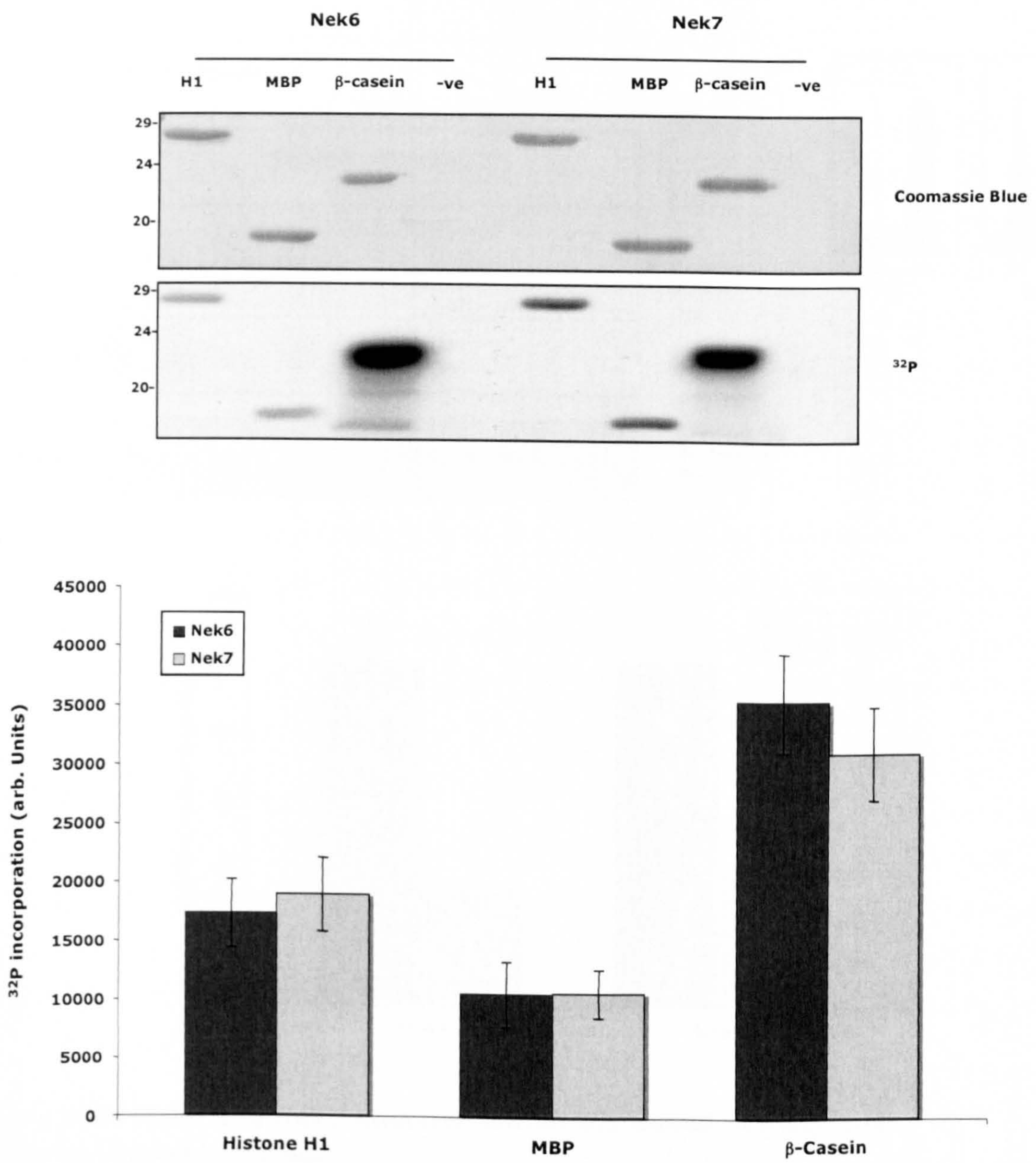

Figure 4.12 β-casein is a good *in vitro* substrate for the Nek6 and Nek7 kinases

His_6Nek6 and His_6Nek7 recombinant kinases were incubated in kinase buffer containing [γ-^{32}P]-ATP and either Histone H1, MBP or β-casein as a substrate for 30 min at 30°C. -ve, no substrate. (**A**) Samples were subjected to SDS-PAGE, Coomassie Blue staining and autoradiography. Molecular weights (kDa)are indicated on the left. (**B**) Kinase activity of Nek6 and Nek7 against the different substrates minus background values are shown. Data represent means (± S.D.) of three separate experiments.

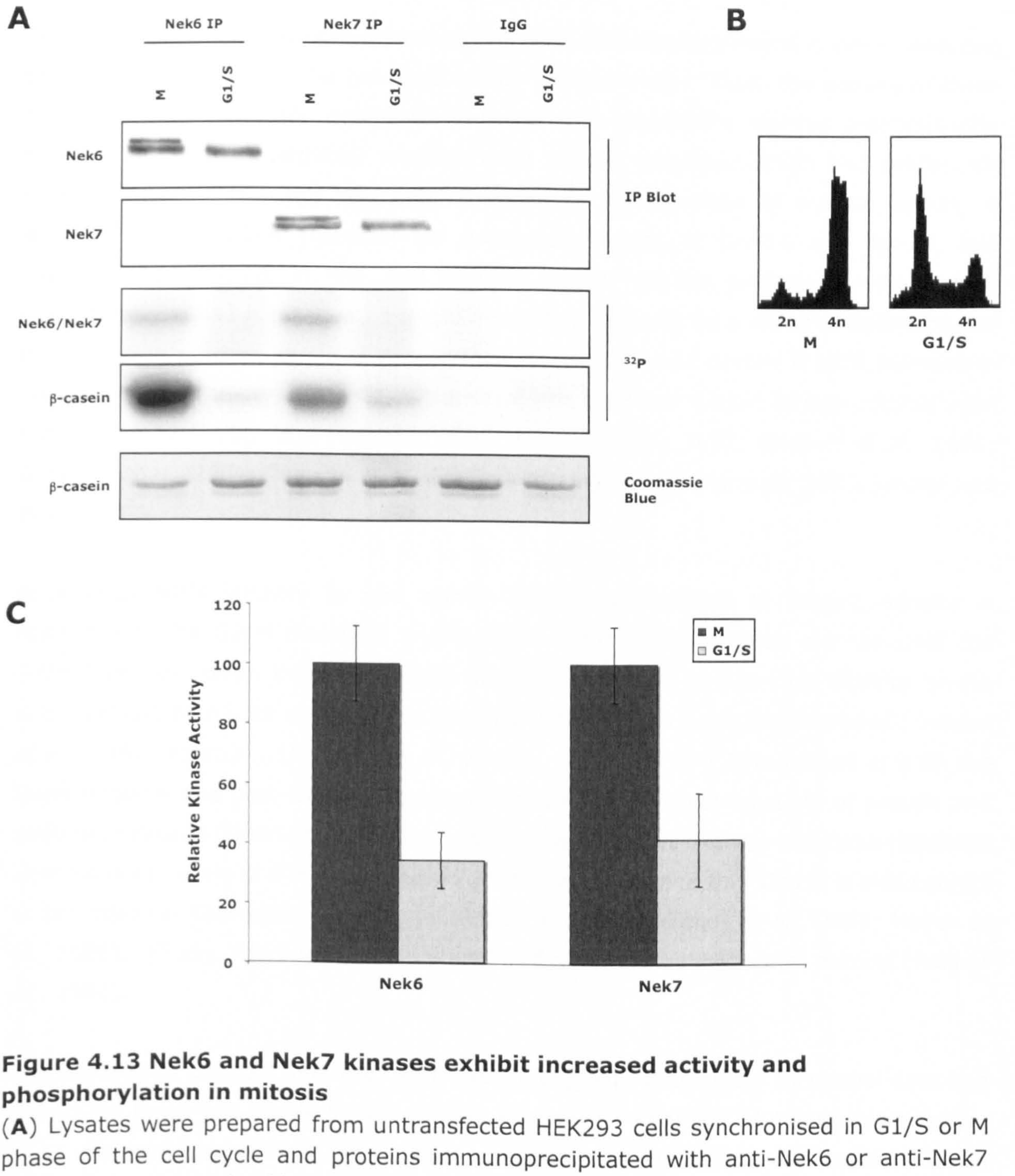

Figure 4.13 Nek6 and Nek7 kinases exhibit increased activity and phosphorylation in mitosis

(**A**) Lysates were prepared from untransfected HEK293 cells synchronised in G1/S or M phase of the cell cycle and proteins immunoprecipitated with anti-Nek6 or anti-Nek7 antibodies or control rabbit IgGs. The immunoprecipitates were subjected to Western blotting with anti-Nek6 or anti-Nek7 antibodies and used in kinase assays with β-casein as a substrate. Samples were analysed by SDS-PAGE, Coomassie Blue staining and autoradiography (^{32}P). (**B**) Cell synchronisation was confirmed via flow cytometry analysis of samples of propidium iodide stained cells which had previously been fixed and permeabilised in ice-cold 70% ethanol. (**C**) Relative activity of Nek6 and Nek7 kinases in G1/S and M phases minus the background and normalised to the amount of protein precipitated are expressed. Data is shown as mean ± S.D. of three separate experiments.

4.3 Discussion

Temporal regulation of protein kinases involved in mitotic progression is key to ensuring that this critical point in the cell cycle occurs without error. Thus, the activity of these protein kinases is usually controlled in a cell cycle-dependent manner predominantly through two post-translational modifications: protein phosphorylation and proteolysis (Nigg, 2001). Cdk1, the key mitotic kinase in orchestration of mitotic events, is activated at the G2/M transition by dephosphorylation of Thr-14 and Tyr-15, two neighbouring residues in the ATP binding pocket of the protein, by the Cdc25 phosphatase. Cdk1 is inactivated to allow exit from mitosis, as a result of destruction of its regulating partner, cyclin B (Nasmyth, 2005). Plk1 and Aurora A both increase in abundance and activity at the G2/M transition and both are subject to degradation upon mitotic exit (van Vugt and Medema, 2005; Kimura *et al.*, 1997; Bischoff *et al.*, 1998; Dutertre *et al.*, 2004; Taguchi *et al.*, 2002; Littlepage and Ruderman, 2002; Lindon and Pines, 2004).

Aspergillus NIMA activity is also tightly cell cycle-regulated to ensure activity is restricted to the G2/M transition and M-phase. NIMA mRNA levels are low until the G2/M transition when there is a rapid increase in mRNA, protein and activity levels, which remain high until mitotic exit when the NIMA protein is rapidly degraded (Osmani *et al.*, 1987; Ye *et al.*, 1995; Pu *et al.*, 1995). *S. pombe* Fin1 is regulated at both the transcriptional and post-translational level, leading to maximal amounts of protein and activity in mitosis (Krein *et al.*, 2002). Mammalian Nek2 is likewise cell cycle regulated with maximal levels at S and G2 phases of the cell cycle, and this kinase is also subject to proteosomal degradation in mitosis (Fry *et al.*, 1995; Hames *et al.*, 2001; Hayes *et al.*, 2006). Finally, Nek9 is phosphorylated and activated as cells enter mitosis (Roig *et al.*, 2001).

The association of Nek6 and Nek7 with Nek9 along with preliminary functional data had implicated these two highly similar protein kinases in regulation of mitotic progression. Thus, the exhibition of a cell cycle-dependent pattern of expression, with maximal levels at G2/M or M-Phase, by Nek6 and Nek7 would strongly suggest a role for these two protein kinases in mitosis. The data presented here suggest that both Nek6 and Nek7 are upregulated at the mRNA, protein and activity levels during G2 and M. The increase in protein levels is accompanied by a change in the electrophoretic mobility of the protein indicative of phosphorylation and this coincides with an increase in the kinase activity of both Nek6 and Nek7.

Spatial regulation of protein kinases is also essential in ensuring accurate mitosis. Typically, mitotic protein kinases are localized to the subcellular structure where they exert their effects at the appropriate time. So, consistent with its role in regulating spindle assembly, Aurora A is heavily concentrated at spindle poles in mitosis as well as along the microtubules of the mitotic spindle (Glover *et al.*, 1995; Marumoto *et al.*, 2005). Aurora B, on the other hand, with a role primarily in regulation of chromosome segregation and cytokinesis, localizes first to the chromosomes in prophase and then to the centromeres in prometaphase and metaphase, before concentrating at the central spindle at anaphase onset (Stenoien *et al.*, 2003; Adams *et al.*, 2001). Consistent with its many and varied roles in M-Phase, Plk1 displays a dynamic localization in mitosis localizing first to centrosomes in prophase, then becoming enriched at kinetochores in prometaphase and metaphase before being recruited to the central spindle in anaphase and finally accumulating at the midbody in telophase (Petronczki *et al.*, 2008; Golsteyn *et al.*, 1995; Nigg, 2001; van Vugt and Medema, 2005). NIMA localizes to spindle pole bodies, chromosomes and spindles during M-phase, consistent with its multiple roles in chromatin condensation and spindle nucleation and organization (De Souza *et al.*, 2000), whilst Fin1, whose function is also required for spindle organization and cytokinesis, is localized to the spindle pole body throughout mitosis (Krein *et al.*, 2002; Grallert and Hagan, 2002; Grallert *et al.*, 2004). A major fraction of Nek2, which appears to stimulate centrosome separation at the G2/M transition, is centrosomal (Fry *et al.*, 1998; Fletcher *et al.*, 2005; Fry *et al.*, 1998; Bahe *et al.*, 2005). Nek9, which has been implicated in both centrosome and chromatin/Ran mediated mitotic spindle formation accordingly localizes to centrosomes and chromosomes during mitosis when in a phosphorylated, active form (Roig *et al.*, 2005).

Here, examination of the subcellular distribution of endogenous and recombinant proteins by immunofluorescence microscopy has shown that whilst Nek6 is evenly distributed across the cytoplasm and nucleus of interphase cells, it appears to become associated with the mitotic spindle microtubules during metaphase, the spindle midzone during anaphase and early telophase, and the midbody during cytokinesis. There is however, no apparent centrosomal localization. The localization of Nek7 has proved more difficult to conclusively establish, although there is some evidence for a centrosomal localization which is perhaps restricted to specific periods during the cell cycle or to a small pool of the total cellular Nek7, making such a localization difficult to distinguish in a background of high cytosolic Nek7. Unlike Nek6, Nek7 does not appear to localize to the mitotic spindle microtubules at any stage of mitosis. This is an important difference between these two very similar protein kinases.

To my knowledge, this is the first evidence of a specific subcellular localization of Nek6 to a mitotic structure. Previous reports have not considered the mitotic distribution of Nek6 in any great depth and have only discussed the diffuse cytoplasmic and nuclear interphase distribution also seen in this work. Furthermore, although Nek6 has been implicated in regulation of mitotic progression and specifically the metaphase to anaphase transition there has been little progress made in establishing the extent of the role of Nek6 in mitosis.

As discussed, spatial regulation of mitotic protein kinases to target them to their place of action is common among major mitotic regulators, thus the localization of Nek6 to the mitotic spindle, spindle midzone and midbody raises the possibility of a role for Nek6 in regulation of mitotic spindle organization and also perhaps in regulation of late mitotic events and cytokinesis. In light of the interaction which has been established between Nek6 and Nek9, and the putative role of Nek9 in regulating both centrosomal and chromatin/Ran-mediated mitotic spindle organization (Roig *et al.*, 2005), it seems likely that any role for Nek6 in mitotic spindle organization may be at least in part, as a result of its interaction with Nek9. The variations in apparent subcellular distributions between Nek6 and Nek9 are noteworthy; however, only a small fraction of the total cellular pool of Nek9 is associated with centrosomes and chromosomes (Roig *et al.*, 2005), so perhaps Nek6/Nek9 complexes are not directly involved in centrosome or chromatin-mediated spindle nucleation. Likewise, only a small proportion of the total cellular pool of Nek6 forms a complex with Nek9 (Belham *et al.*, 2003).

Despite reports that Nek7 is localized to the centrosome at both interphase and mitosis, and also to the spindle midzone and midbody of late mitotic cells (Yissachar *et al.*, 2006; Kim *et al.*, 2007), we have been unable to establish a strong association between either endogenous or recombinant Nek7 and the centrosome, or indeed any other mitotic structures, although inconsistent co-localization between endogenous Nek7 and the interphase centrosome was observed. This could be accounted for by the possibility that the fraction of total cellular Nek7 which is centrosomally-associated is so small as to be beyond the limits of detection possible with the reagents available. In fact, both endogenous and recombinant Nek7 were most obviously cytoplasmic in both interphase and mitosis, thus suggesting roles perhaps beyond microtubule nucleation. For example, Nek7 could modulate the functions of mitotic regulators that do not associate directly with centrosomes, mitotic spindle or chromosomes.

It is becoming apparent that despite significant structural and functional divergence among the NIMA-related kinases, there may well be some degree of functional

conservation in regulating centrosomes, basal bodies and mitotic spindles (O'Regan *et al.*, 2007; Quarmby and Mahjoub, 2005). Indeed, along with the MTOC localization of the mitotic NIMA-related kinases, that is to say Nek2, NIMA, Fin1 and phosphorylated Nek9, other mammalian Neks, namely Nek1 and Nek8, as well as Neks from various unicellular eukaryotes including Fa2p from *Chlamydomonas,* and NRKC from *Trypanosomes* are associated with basal bodies and implicated in ciliogenesis (Krein *et al.*, 1998; Krein *et al.*, 2002; Grallert *et al.*, 2004; Roig *et al.*, 2005; Fry *et al.*, 1998; Mahjoub *et al.*, 2005; Mahjoub *et al.*, 2004; Bradley and Quarmby, 2005; Pradel *et al.*, 2006). The data presented here, adds Nek6 and Nek7 to this growing list of Neks involved in microtubule organization.

An interesting question concerning the biology of the Nek6 and Nek7 kinases is whether such structurally similar proteins have entirely redundant functions or whether the differences in their N-termini are sufficient to allow regulatory and functional differences. Although the two proteins have been shown to exhibit largely complementary patterns of tissue expression, they appear to respond differently under conditions of serum deprivation: whilst Nek6 is inhibited by serum starvation, Nek7 is apparently rapidly activated (Minoguchi *et al.*, 2003). Data concerning the localization of Nek6 and Nek7, including that presented here, highlights distinct differences in their cell cycle-dependent localization, adding weight to the possibility of differences in regulation and function between the two proteins.

Chapter 5

Nek6 and Nek7 are required for Metaphase Progression and Cytokinesis

5.1 Introduction

The establishment of Nek6 and Nek7 as cell-cycle regulated kinases with maximal activity in mitosis strongly hints at a role in regulation of mitotic progression. This is supported by their similarity to the fungal mitotic regulators, NIMA and Fin1 (Osmani *et al.*, 1987; Osmani *et al.*,1991; Grallert and Hagan, 2002; O'Connell *et al.*, 2003) and their relationship to Nek9, a protein kinase whose function has been established as important for mitotic spindle formation (Roig *et al.*, 2005; Roig *et al.*, 2001).

NIMA activity is required for the G2/M transition in *Aspergillus nidulans* and is also key in regulation of progression through mitosis with roles in chromatin condensation (possibly by phosphorylating histone H3), enabling nuclear entrance of Cdc2/cyclin B and contribution to the dramatic reorganization of the microtubule network seen during mitosis (Fry and Nigg, 1995; O'Connell *et al.*, 2003; Lu and Hunter, 1995; O'Connell *et al.*, 1994). Overexpression of NIMA not only in fungus but also in mammalian cells leads to the onset of mitotic events from any point in the cell cycle, including premature chromatin condensation and interphase microtubule depolymerisation as well as the formation of transient spindle like structures (Osmani *et al.*, 1988; Lu and Hunter, 1995). The ability of NIMA to regulate mitosis in higher organisms has hinted at the existence of NIMA homologues in metazoans. However, despite the identification of 11 mammalian Neks, functional homology to NIMA is confined to the lower orders as, like NIMA, the fission yeast homologue Fin1, contributes to multiple aspects of mitotic progression including mitotic entry, spindle formation and cytokinesis. Overexpression of the fission yeast Fin1 protein induces premature chromatin condensation and its depletion results in G2 delay, whilst Fin1 mutants cannot form a bipolar mitotic spindle and Fin1 is also believed to be involved in mitotic exit, acting upstream of the SIN pathway (Krein *et al.*, 1998; Grallert and Hagan, 2002; Grallert *et al.*, 2004). However, the *Neurospora crassa* NIM-1 protein is the only protein discovered to date capable of functionally complementing a *nimA* temperature-sensitive mutation in *A. nidulans* (Pu *et al.*, 1995).

Nonetheless, at least some of the mammalian Neks identified to date, whilst not being absolutely required for entry into and exit from mitosis, are involved in the regulation of mitotic progression. Nek2 is cell cycle-regulated with maximal expression at S and G2 phase (Fry *et al.*, 1998; Graf, 2002; Uto and Sagata, 2000; Prigent *et al.*, 2005) and is involved in the regulation of centrosome separation at the G2/M transition (Fry *et al.*, 1998; Fletcher *et al.*, 2005). Nek9 shows increased kinase activity in mitosis, when the phosphorylated, active form of the protein appears to localize to centrosomes and contribute to mitotic spindle microtubule nucleation (Roig *et al.*, 2005).

Evidence is also beginning to accumulate which implicates Nek6 and Nek7 in the regulation of mitotic progression. Although the cell cycle-dependent regulation of Nek6 and Nek7 had proved to be a controversial subject, there is some evidence that both kinases are upregulated in mitosis, at least in terms of their kinase activity. The data presented in the previous chapter supports the fact that these protein kinases are activated in mitosis. Furthermore, our data indicates that Nek6 localizes to the vicinity of mitotic spindle microtubules, the spindle midzone and the midbody of dividing cells, whilst Nek7 may have a centrosomal localization.

Limited functional studies on the effect of overexpression of kinase-deficient Nek6 or Nek7 or their depletion have been published. Whilst overexpression of kinase-dead Nek6 or RNAi depletion of the endogenous protein was reported to lead to prometaphase arrest and apoptosis (Yin *et al.*, 2003), this was disputed in a second study (Minoguchi *et al.*, 2003). RNAi depletion of Nek7 has also been reported to lead to an increase in mitotic index, with a specific increase in the numbers of prometaphase cells in which NEB had occurred and chromosomes were condensed but centrosomes were not separated. This was accompanied by an increase in apoptosis concomitant with a decrease in centrosomal-associated γ-tubulin and microtubule nucleation. Furthermore, if cells were allowed to progress beyond the SAC via co-depletion of Mad2, defects in cytokinesis were reported (Kim *et al.*, 2007). Yissachar *et al.* (2006) also reported that depletion of Nek7 results in an increase in mitotic index, apoptosis, multipolar spindle formation and the appearance of multinucleated cells.

This chapter describes the systematic functional analysis of the effects of interfering with Nek6 and Nek7 both by expression of kinase-deficient mutants and RNAi depletion. This has led to a clearer insight into the proposed mitotic roles of these two protein kinases.

5.2 Results

5.2.1 Generation of kinase-deficient mutants of Nek6 and Nek7

It has been reported that mutation of two lysine residues in the ATP binding pocket of Nek6, K74 and K75, to methionine results in a protein which, on expression in HeLa cells, causes mitotic arrest and apoptosis. However, it was not shown which of these sites was critical (Yin *et al.*, 2003). Similarly it was reported that mutation of the conserved glycine residue in kinase sub-domain I of Nek7, G43, to arginine resulted in a kinase which induces a moderate increase in apoptosis when expressed in HeLa cells. However, no direct measurement of the kinase activity of this mutant was carried out (Yissachar *et al.*, 2006). It was separately reported that expression of Nek6 and Nek7 with mutations in the ATP binding pocket did not interfere with cell cycle progression in either NIH-3T3 or HeLa cells (Minoguchi *et al.*, 2003).

It has also been reported that Nek6 kinase activity is dependent upon phosphorylation of two residues within the activation loop of the kinase, T202 and S206 (Belham *et al.*, 2003). The activation loop is a region of 20-25 amino acids whose beginning and end are typically marked by conserved DFG and APE motifs (DLG and SPE in Nek6 and Nek7), respectively (Johnson *et al.*, 1996). Phosphorylation of residues within this region is often important for protein kinase activity, acting to alter the conformation of the kinase such that either the active form is stabilized or the inactive form destabilized. Mutation of S206 alone or T202 and S206 in combination resulted in an inactive Nek6 kinase, whilst mutation of T202 alone resulted in a kinase which retained approximately 25% activity. This led to the conclusion that S206 is absolutely required for Nek6 activity and T202 phosphorylation probably occurs as a secondary event which further augments Nek6 activity (Belham *et al.*, 2003). It appears that these phosphorylation events may be carried out *in vivo* by Nek9.

In order to systematically determine whether mutants with differing levels of kinase activity are capable of inducing mitotic arrest and apoptosis, we generated a series of point mutations within each kinase. Firstly, the lysine residues within the ATP-binding pocket of Nek6, K74 and K75, and the equivalent residues in Nek7, K63 and K64, were mutated to methionine, to produce each single mutant as well as the KK/MM double mutant. Secondly, the two putative phosphorylation sites within the activation loop of Nek6, T202 and S206, and the equivalent residues in Nek7, T191 and S195, were mutated to alanine (Figure 5.1).

A

```
hNek6   MAGQPGHMPHGGSSNNLCHTLGPVHPPDPQRHPNTLSFRCSLADFQIEKKIGRGQF   56
hNek7   ------------MDEQSQGMQGPPVPQFQPQKALRPDMGYNTLANFRIEKKIGRGQF   45

hNek6   SEVYKATCLLDRKTVALKKVQIFEMMDAKARQDCVKEIGLLKQLNHPNIIKYLDSF   112
hNek7   SEVYRAACLLDGVPVALKKVQIFDLMDAKARADCIKEIDLLKQLNHPNVIKYYASF   101

hNek6   IEDNELNIVLELADAGDLSQMIKYFKKQKRLIPERTVWKYFVQLCSAVEHMHSRRV   168
hNek7   IEDNELNIVLELADAGDLSRMIKHFKKQKRLIPERTVWKYFVQLCSALEHMHSRRV   157

hNek6   MHRDIKPANVFITATGVVKLGDLGLGRFFSSETTAAHSLVGTPYYMSPERIHENGY   222
hNek7   MHRDIKPANVFITATGVVKLGDLGLGRFFSSKTTAAHSLVGTPYYMSPERIHENGY   213

hNek6   NFKSIWSLGCLLYEMAALQSPFYGDKMNLFSLCQKIEQCDYPPLPGEHYSEKLREL   280
hNek7   NFKSIWSLGCLLYEMAALQSPFYGDKMNLYSLCKKIEQCDYPPLPSDHYSEELRQL   269

hNek6   VSMCICPDPHQRPDIGYVHQVAKQMHIWMSS                            313
hNek7   VNMCINPDPEKRPDVTYVYDVAKRMHACTAS                            302
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B

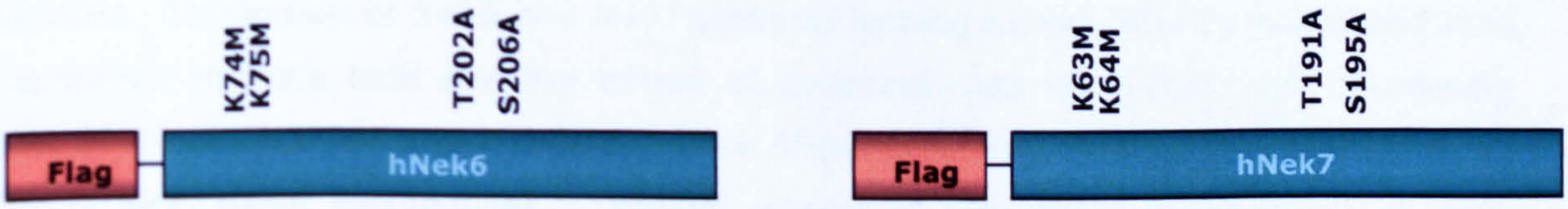


Figure 5.1 Nek6 and Nek7 protein sequences and mutations

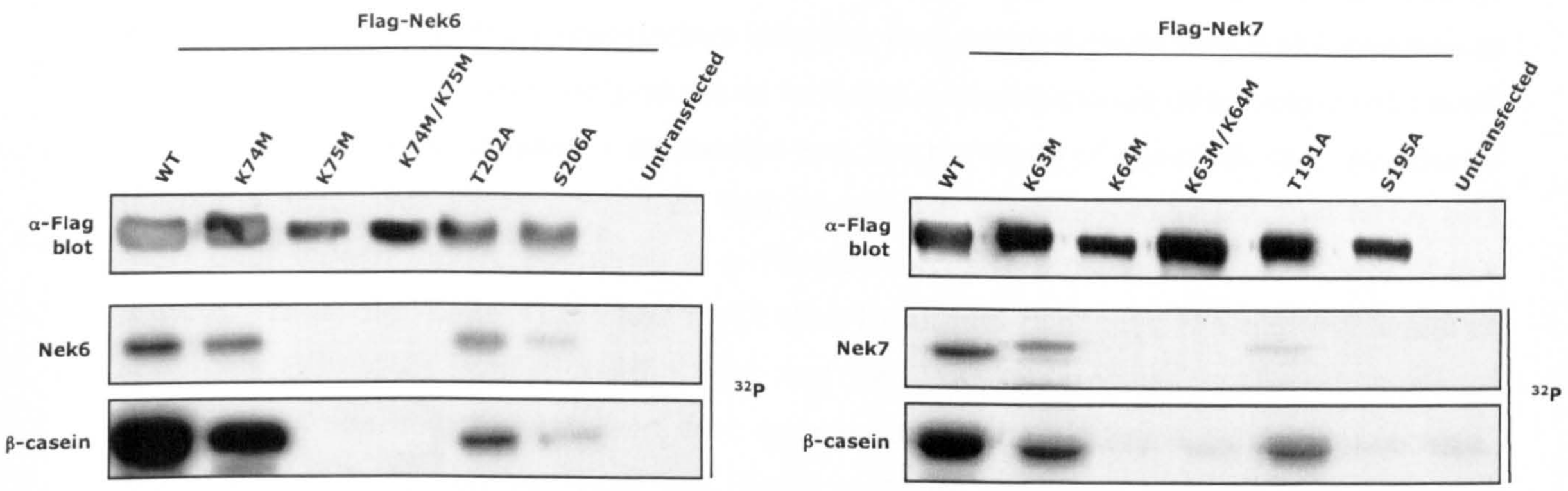
(**A**) Alignment of the Nek6 and Nek7 protein sequences. Two lysine residues in the kinase domain of the two proteins were mutated to methionine, singly and together, to determine which was the most important for kinase activity. The serine and threonine residues in the activation loop of the two proteins most likely phosphorylated by Nek9 were mutated to alanine. The residues mutated are indicated in red, the kinase domain is underlined whilst the activation loop has a double underline. (**B**) Schematic diagram of the Flag-tagged Nek6 and Nek7 recombinant proteins highlighting the position of the mutations.

In order to compare the relative activity of these mutants, N-terminally Flag-tagged constructs were expressed in HEK 293 cells for 24 h, cells were lysed and recombinant proteins immunoprecipitated with anti-Flag antibodies before immune complexes were used in kinase assays with β-casein as substrate (Figure 5.2A). Whilst mutation of K75 in Nek6 and K64 in Nek7 resulted in almost complete loss of kinase activity, with the proteins having <5% residual activity, mutation of K74 in Nek6 and the equivalent K63 in Nek7 had very little effect on kinase activity, with mutant proteins retaining >80% activity. The KK/MM double mutants were inactive, as expected. Mutation of the activation loop sites produced kinases with varying levels of residual activity: the Nek6 T202A and Nek7 T191A mutant kinases retained 30-40% activity, whilst the Nek6 S206A and Nek7 S195A mutations in Nek6 and Nek7, respectively, produced kinases with 15-20% activity compared to that of the WT kinase (Figure 5.2B). This is consistent with the finding that the Nek6 S206 residue is the more critical phosphorylation site for Nek6 activity compared to T202 (Belham *et al.*, 2003).

5.2.2 Expression of kinase-inactive Nek6 and Nek7 induces apoptosis

Having determined the relative activities of the Nek6 and Nek7 mutants, we then examined whether the expression of kinases with varying levels of activity induced apoptosis in HeLa cells. To this end, cells were transiently transfected with the different Flag-tagged constructs for 24 and 48 h before apoptosis was assessed by flow cytometric analysis of Annexin V/FITC stained cells. This assay exploits the ability of Annexin V to bind phosphatidylserine displayed on the surface of cells undergoing apoptosis. Expression of Nek6 and Nek7 proteins lacking kinase activity did indeed lead to apoptosis in HeLa cells and the extent of apoptosis was correlated with decreasing levels of kinase activity and increasing time (Figure 5.3A). Thus, expression of Nek6 K75M or Nek7 K64M mutants led to 30-40% apoptosis after 24 h, increasing to 45-60% after 48 h, whilst expression of WT Nek6 and Nek7 or Nek6 and Nek7 KM mutants which retained almost complete kinase activity (K74M and K75M, respectively) led to low (<20%) levels of apoptosis. This was comparable to the level seen in untransfected cells. Expression of hypomorphic mutants that retained intermediate levels of kinase activity resulted in intermediate (30-40%) levels of apoptosis after 24 h, which also increased by a further 10-20% after 48 h.

To confirm the correlation between decreasing kinase activity and increasing apoptosis, a second assay for apoptosis was employed using indirect immunofluorescence microscopy to observe cleaved caspase 3 staining. Active caspase 3 is a critical executioner of apoptosis, being largely responsible for the proteolytic cleavage of many key proteins in the apoptosis pathway. Its activation requires cleavage of the inactive



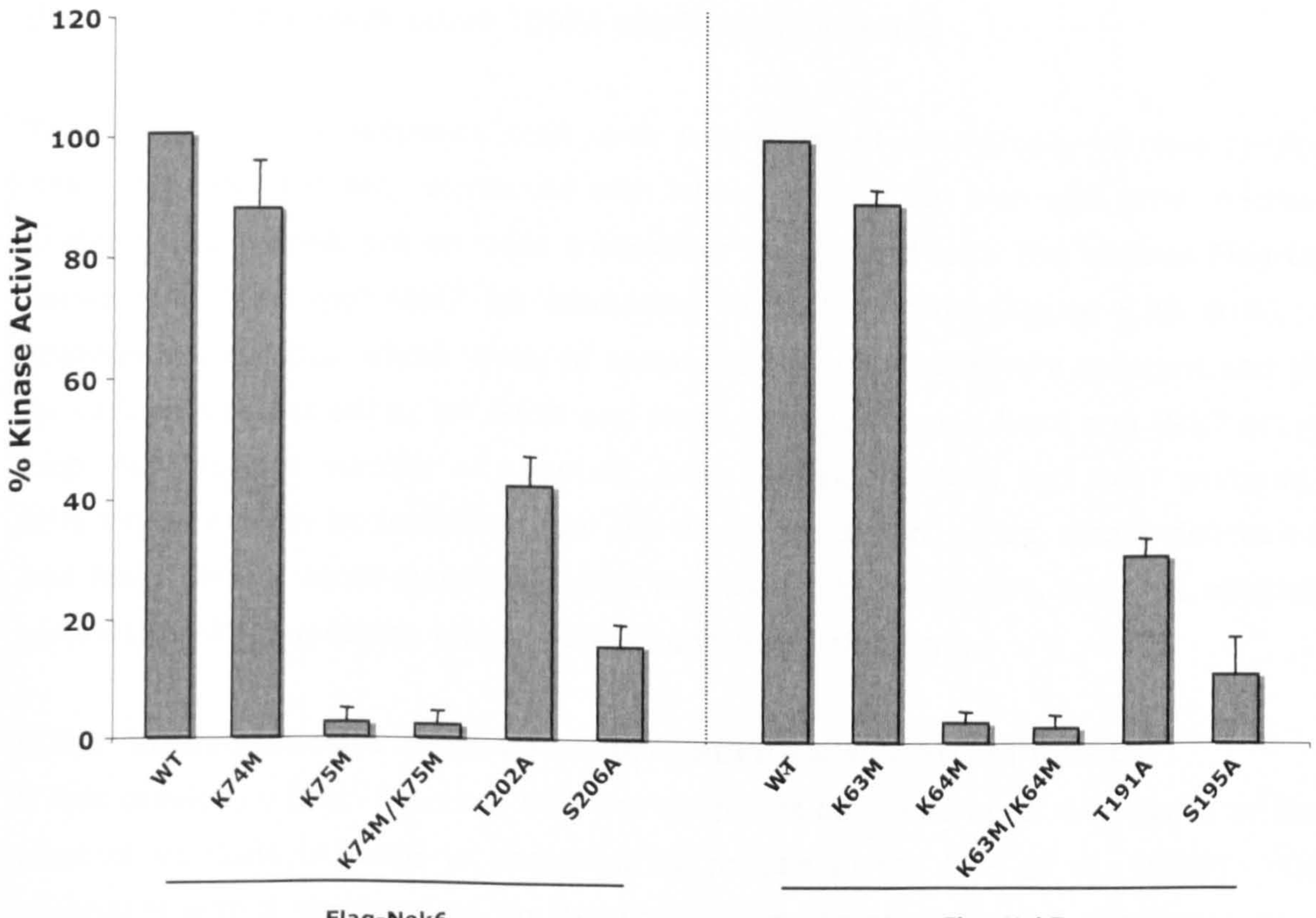


Figure 5.2 Kinase activity of Nek6 and Nek7 ATP binding and activation loop mutants

(**A**) HEK 293 cells were transiently transfected with Flag-tagged Nek6 and Nek7 constructs, as indicated, for 24 h before cells were lysed and subjected to immunoprecipitation with anti-Flag antibodies. The amount of kinase precipitated was determined by Western blot with anti-Flag antibodies and the immunoprecipitates used for kinase assays with β-casein as a substrate. Autoradiographs (^{32}P) of the Nek6/7 proteins and β-casein are shown. (**B**) Activity of the different Nek6 and Nek7 constructs is expressed as a percentage of wild-type activity normalised to the amount of precipitated protein. Data shown represents the mean ± S.D. of three separate experiments.

zymogen into activated p17 and p12 fragments, which can be specifically detected with a cleaved caspase 3 antibody (Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995). HeLa cells were transiently transfected with the Flag-tagged Nek6 and Nek7 constructs for 48 h before being fixed and processed for immunofluorescence microscopy with anti-Flag and anti-cleaved caspase 3 antibodies and the numbers of apoptotic cells measured (Figure 5.3B). This assay confirmed that expression of catalytically-inactive Nek6 and Nek7 does indeed induce apoptosis in a manner correlated to the level of activity of the protein. Thus, the Nek6 K75M and Nek7 K64M mutants produced the highest levels of apoptosis (50-60%), whilst Nek6 K74M and Nek7 K63M proteins produced levels of apoptosis comparable to that seen with expression of the wild-type protein. Likewise, expression of hypomorphic Nek6 and Nek7 mutants produced intermediate levels of apoptosis, although even here, the extent of apoptosis was higher with expression of the Nek6 S206A and Nek7 S195A mutants which have lower kinase activity compared to that seen with the more active T202A and T191A mutants.

To confirm that the apoptosis seen upon expression of catalytically-inactive versions of Nek6 and Nek7 not only correlated with kinase activity but also with time, microscopic analysis was carried out on cells transiently transfected with the various Flag-tagged versions of Nek6 and Nek7 for increasing lengths of time (Figure 5.4A & B). This analysis showed that whilst levels of apoptosis remained relatively constant and similar for cells expressing either WT Nek6 and Nek7 or hypomorphic Nek6 and Nek7 activation loop mutants, the number of apoptotic cells expressing Nek6 and Nek7 mutants with little kinase activity accumulated over the 48 h time period. Thus, kinase-deficient Nek6 and Nek7 clearly cause apoptosis upon expression in HeLa cells, and this apoptosis is correlated with decreasing kinase activity and increasing time.

5.2.3 Kinase-inactive Nek6 and Nek7 cause cells to arrest mitosis

It has previously been reported that the apoptosis observed upon expression of kinase-inactive versions of Nek6 is preceded by mitotic arrest (Yin *et al.*, 2003). This is consistent with a requirement for these kinase in the regulation of mitotic progression. Many kinases including Cdc2, Polo and NIMA, which have since proved to be important mitotic regulators were first described in mutants that failed to undergo a normal mitosis in model systems (Nurse *et al.*, 1976; Hartwell *et al.*, 1970; Sunkel and Glover, 1998, Llamazares *et al.*, 1991; Morris, 1975). It is likewise consistent with the cell cycle dependent expression and localization of Nek6 and Nek7 described in the previous chapter.

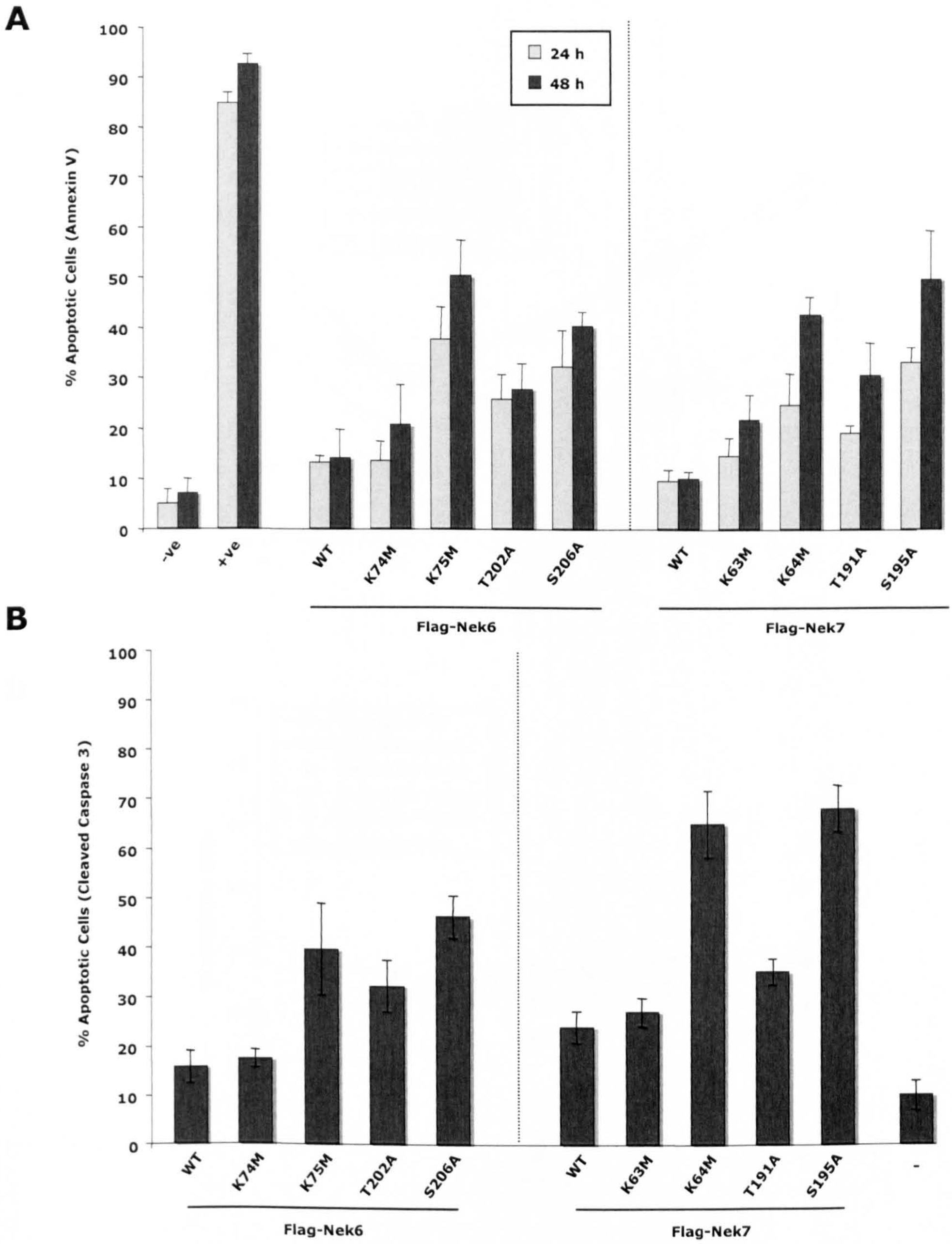
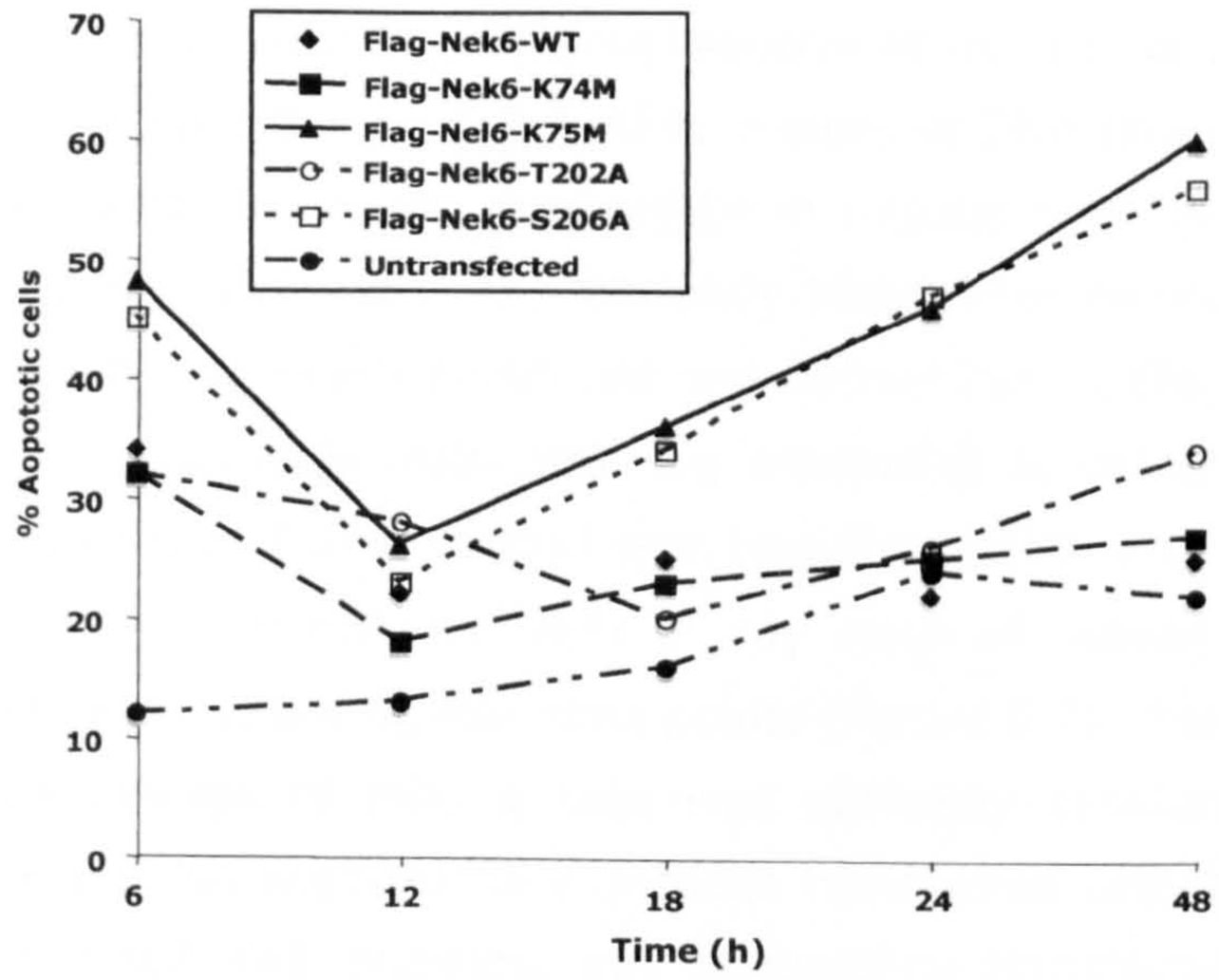


Figure 5.3 Expression of kinase-inactive Nek6 and Nek7 mutants induces apoptosis

(**A**) HeLa cells were transiently transfected with Flag-tagged Nek6 and Nek7 constructs, as indicated, for 24 and 48 h before being assayed for their ability to take up Annexin V/FITC stain via flow cytometry analysis. -ve, untransfected cells; +ve, untransfected cells treated with 1 μM Staurosporine for 6 h. (**B**) HeLa cells were transiently transfected with Flag-Nek6 and -Nek7 constructs, as indicated for 48 h before being methanol fixed and immunostained with anti-Flag antibodies to detect transfected cells and anti-cleaved caspase 3 antibodies to detect apoptotic cells. The percentages of transfected cells which were positive for cleaved caspase 3 stain is shown and data represent means (± S.D.) of counts of at least 50 cells in three separate experiments.

A



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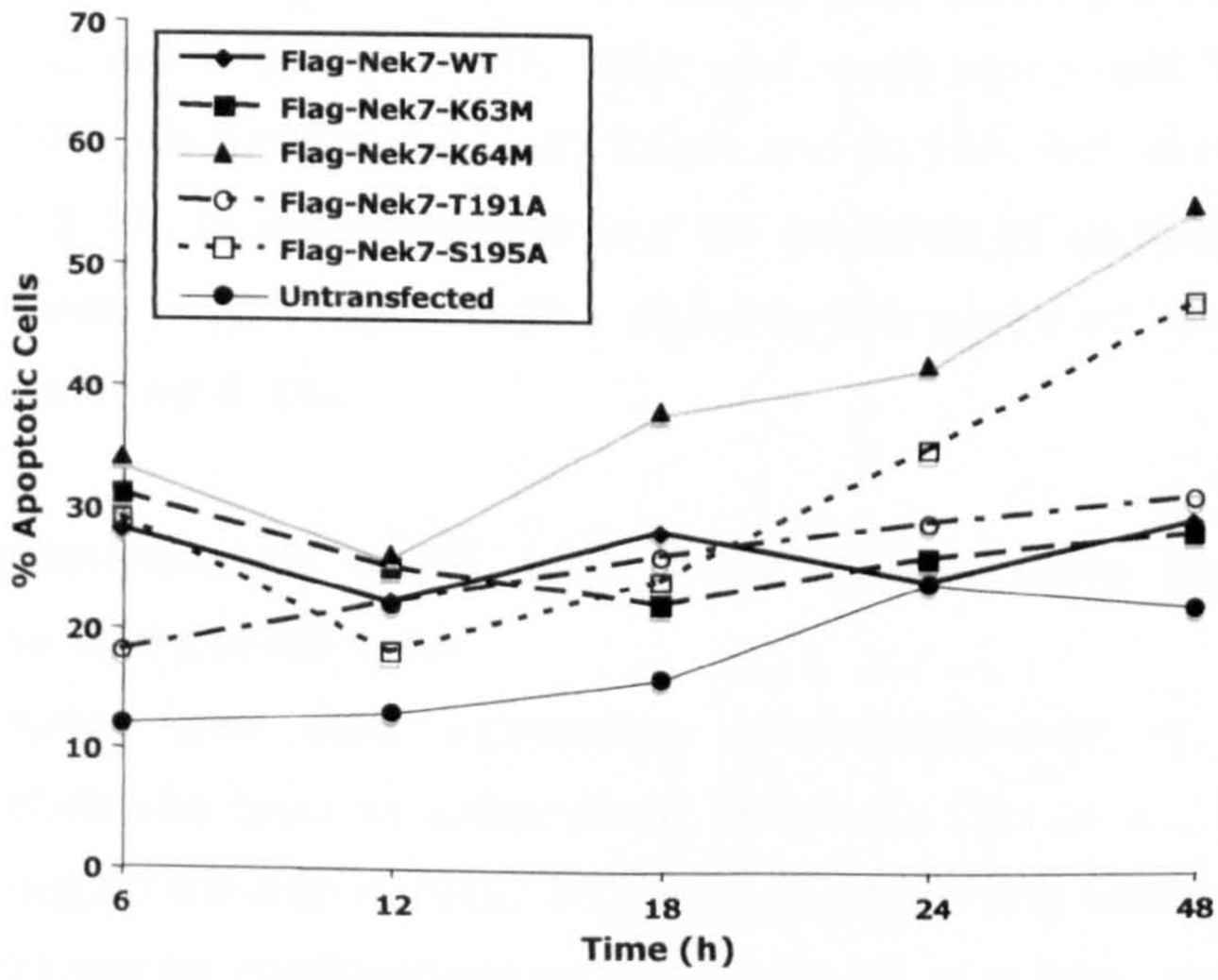

Figure 5.4 Expression of catalytically-inactive Nek6 and Nek7 causes increasing levels of apoptosis with time

HeLa cells were transiently transfected with Flag-Nek6 (**A**) or Flag-Nek7 (**B**) constructs, for the times indicated, before being methanol fixed and processed for immnuofluorescence microscopy with anti-FLAG antibodies to detect transfected cells and anti-cleaved caspase 3 antibodies to detect apoptotic cells. The percentages of transfected cells which were positive for cleaved caspase 3 stain at each time point is shown and data represent means (± S.D.) of counts of at least 50 cells in three separate experiments.

Immunofluorescence microscopy of HeLa cells transiently transfected with kinase-inactive Nek6 and Nek7 revealed that many of the cells which stained positively for cleaved caspase 3, also appeared to possess features of mitotic cells such as alignment of DNA on the metaphase plate or segregating masses of DNA (Figures 5.5 & 5.6). To determine whether cells did indeed accumulate in mitosis prior to the occurrence of apoptosis, microscopic analysis of cells transiently transfected for increasing times with the different Flag-Nek6 and -Nek7 constructs was carried out. Cells were processed for immunofluorescence microscopy with anti-Flag antibodies to detect transfected cells; anti-α-tubulin antibodies and DNA stained with Hoechst 33258 were used to determine the cell cycle stage. The number of cells in any stage of mitosis from prophase to cytokinesis was analyzed at the various time points (Figure 5.7). This analysis revealed that whilst the percentage of mitotic cells was relatively constant and comparable between untransfected cell populations and those transfected with WT Nek6 and Nek7 or Nek6 K74 and Nek7 K63 mutants, cell populations transfected with the kinase deficient mutants (K75M, T202A and S206A for Nek6 and K64M, T191A and S195A for Nek7) accumulated at mitosis, with levels of mitotic cells peaking 24 h after transfection and then decreasing again by 48 h. The peak was most prominent for the least active mutants, Nek6 K75M and S206A and Nek7 K64M and S195A, with a mitotic index of 10-15% compared to 2-4% in untransfected and WT transfected cell populations. For the Nek6 T202A and Nek6 T191A hypomorphic mutants there was an intermediate increase in mitotic index to around 6-8%.

5.2.4 Catalytically-inactive Nek6 and Nek7 cause cells to accumulate at metaphase and cytokinesis

It has been reported that cells expressing catalytically-inactive versions of Nek6 accumulate at metaphase prior to undergoing apoptosis (Yin *et al.*, 2003), whilst cells expressing a HA-tagged version of Nek7 mutated at G43 in the kinase subdomain of the protein showed increased multinucleation indicative of a mitotic defect, although the cause of this phenotype was not discussed (Yissachar *et al.*, 2006).

Having determined that kinase-deficient Nek6 and Nek7, when expressed in HeLa cells, cause accumulation of cells in mitosis, we sought to further characterize this mitotic defect, initially by assessing whether cells were accumulating at a particular stage of mitosis. To this end, cells were transiently transfected for 24 h with Flag-tagged WT and kinase deficient versions of the Nek6 and Nek7 kinases before being fixed and processed for immunofluorescence microscopy. 24 h is the point at which the mitotic index appeared to peak before the significant apoptosis seen after 48 h. Cells were processed for immunofluorescence microscopy with anti-Flag antibodies to detect

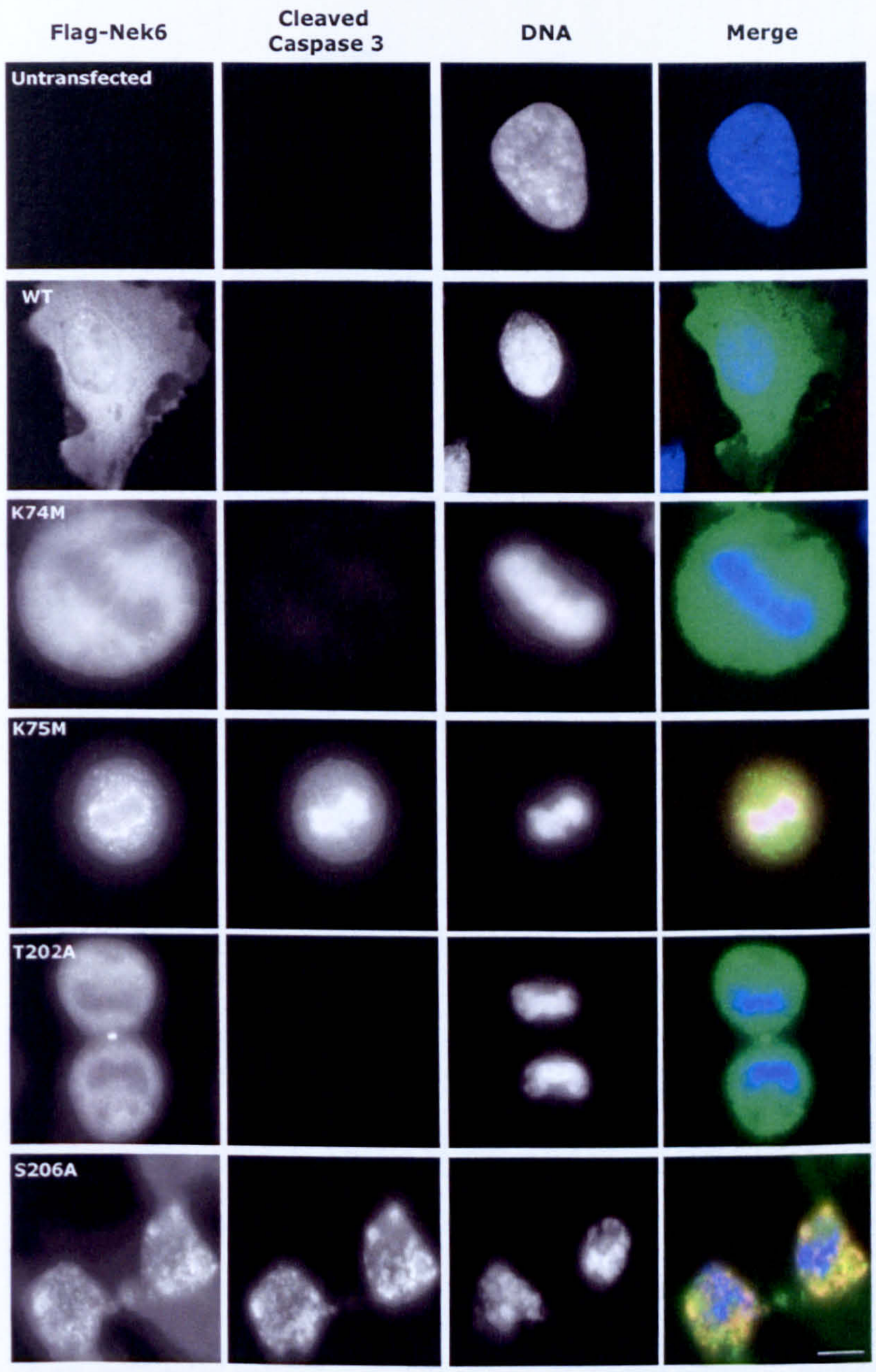

Figure 5.5 Kinase-inactive Nek6 triggers apoptosis in mitotic cells

HeLa cells growing on acid-etched coverslips were transiently transfected with Flag-Nek6 constructs for 24 h before being fixed and permeabilised with methanol. Cells were processed for immunofluorescence microscopy with anti-Flag antibodies to detect transfected cells (green on merge) and anti-cleaved caspase 3 antibodies to detect apoptosis (red on merge). DNA was stained with Hoechst 33258 (blue on merge). Scale bar, 10 μm.

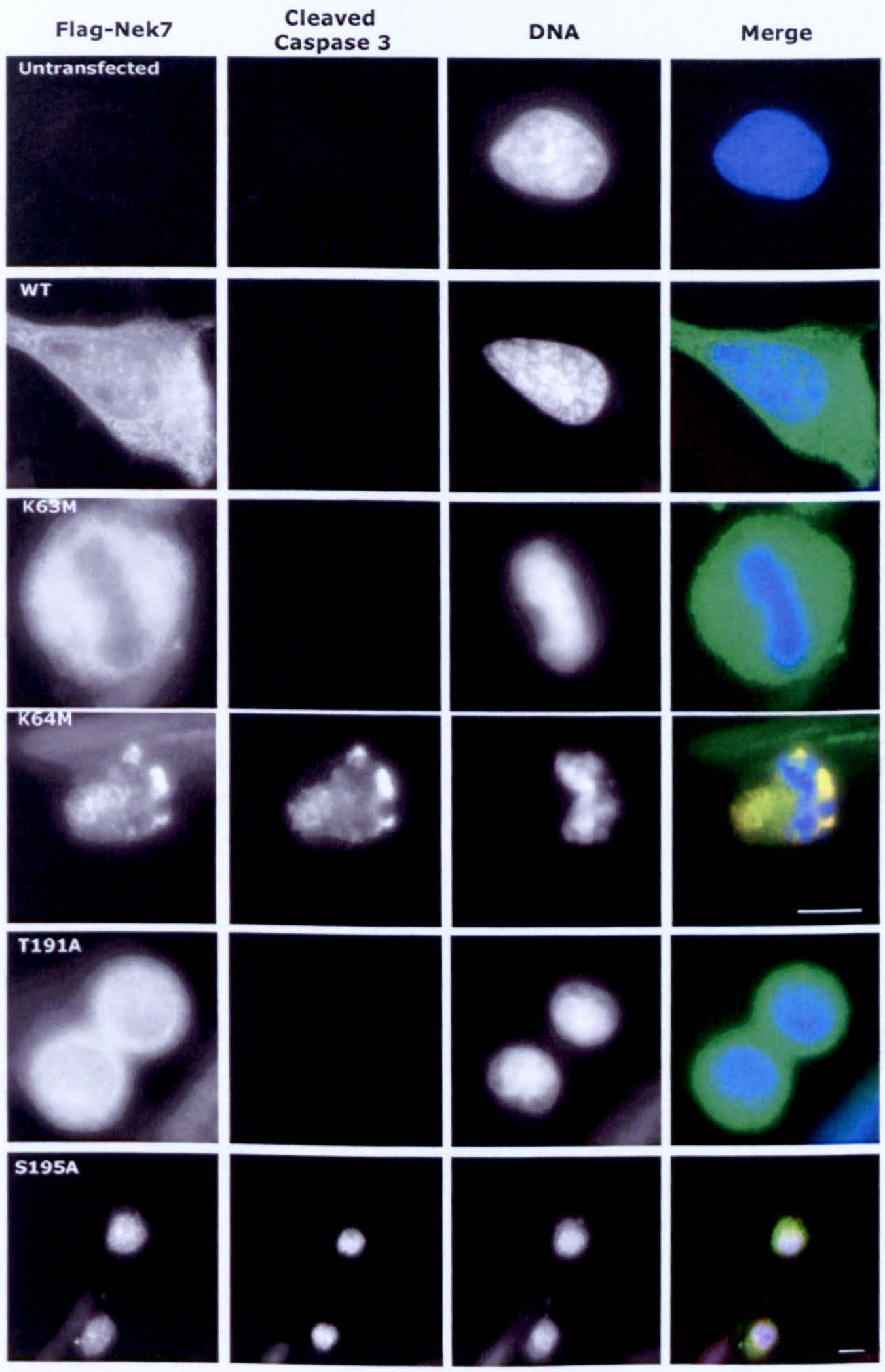

Figure 5.6 Kinase-inactive Nek7 triggers apoptosis in mitotic cells

HeLa cells growing on acid-etched coverslips were transiently transfected with Flag-Nek7 constructs for 24 h before being fixed and permeabilised with methanol. Cells were processed for immunofluorescence microscopy with anti-Flag antibodies to detect transfected cells (green on merge) and anti-cleaved caspase 3 antibodies to detect apoptosis (red on merge). DNA was stained with Hoechst 33258 (blue on merge). Scale bars, 10 μm.

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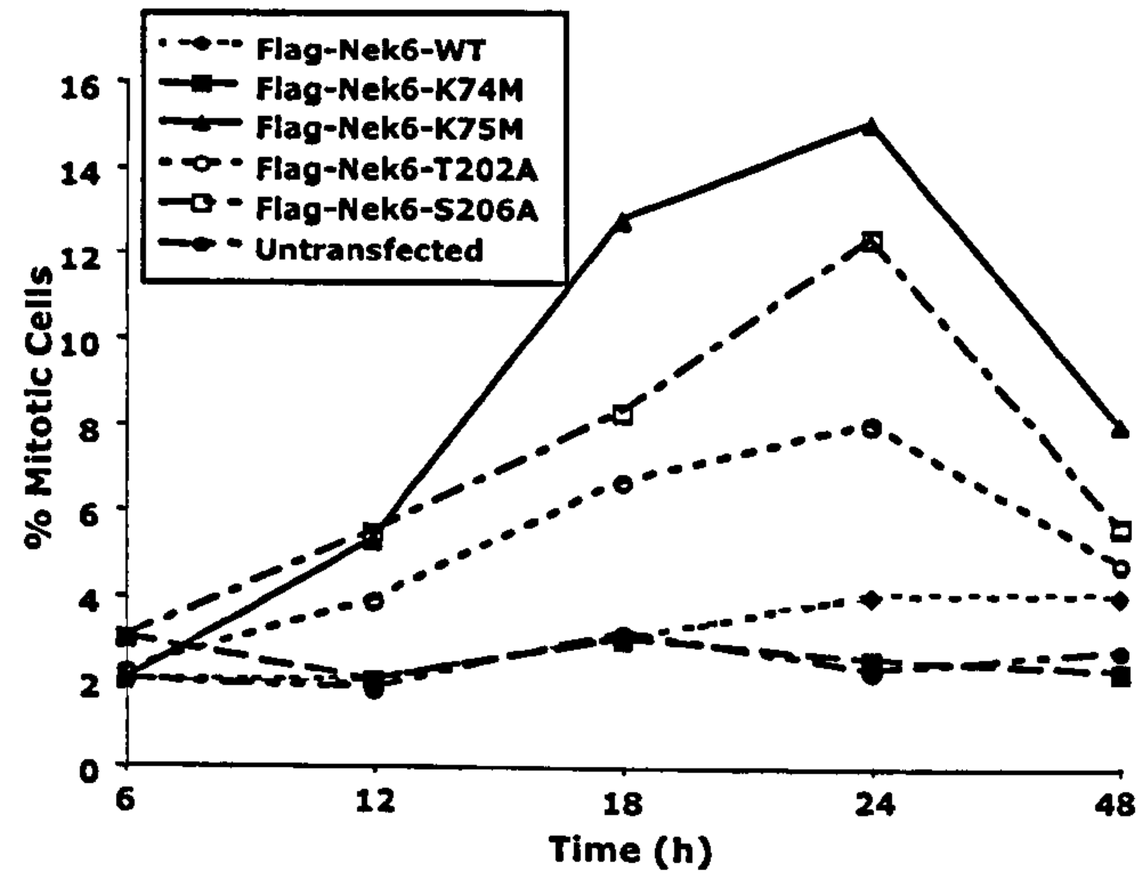

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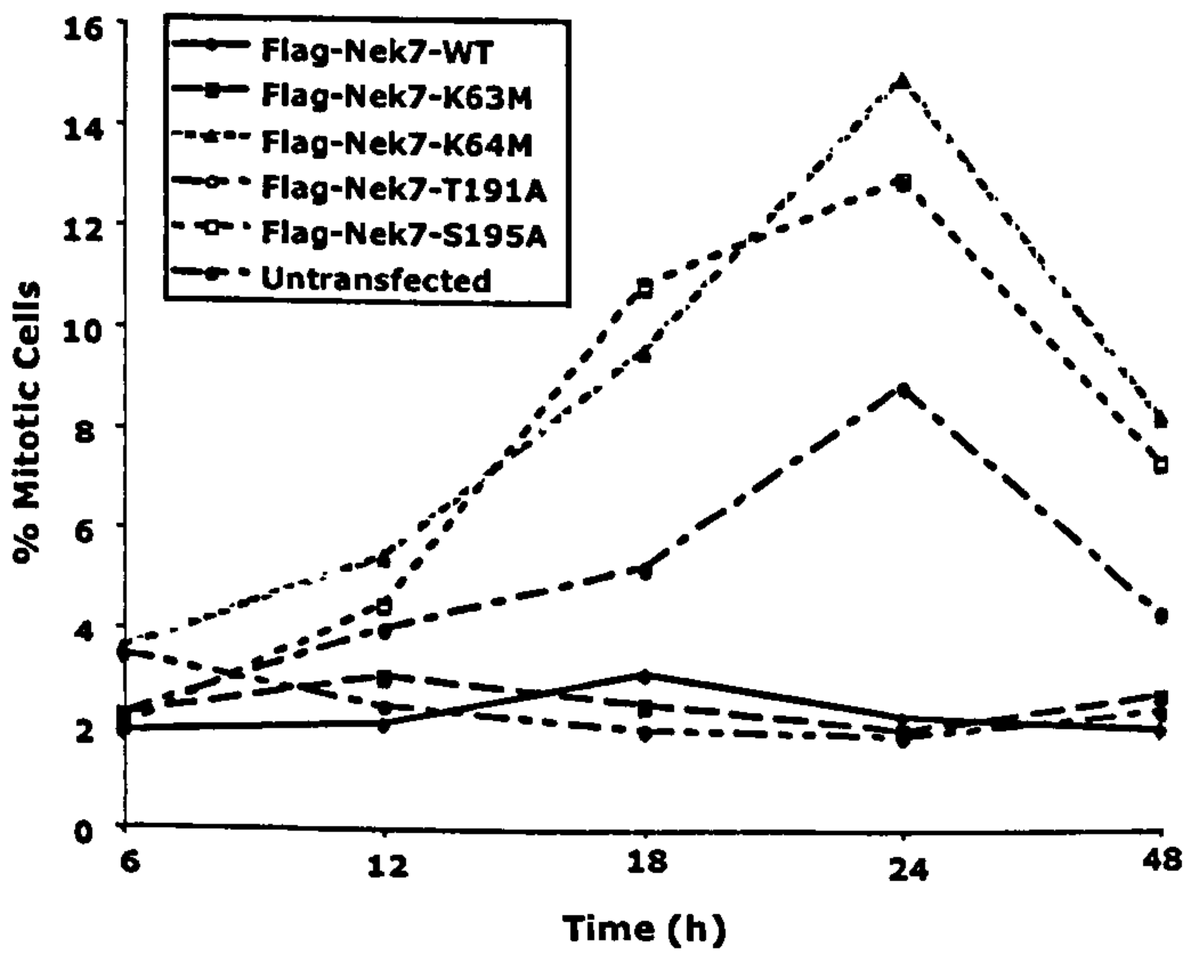

Figure 5.7 Kinase-inactive Nek6 and Nek7 mutants induce mitotic arrest
HeLa cells were transiently transfected with Flag-Nek6 (**A**) or Flag-Nek7 (**B**) constructs and fixed and permeabilised with methanol at various time points, as indicated, before being processed for immunofluorescence microscopy with anti-Flag antibodies to detect transfected cells. Hoechst 33258 was used to stain DNA and determine cell cycle stage. The percentage of transfected cells in mitosis was determined. Data are based on the mean of 3 separate experiments (± S.D.), with counts of at least 50 transfected cells in each case.

transfected cells, as well as anti-α-tubulin antibodies and DNA stain to determine cell cycle stage. Transfected mitotic cells were scored as being in prometaphase, with visible spindles and condensed DNA but which is not yet aligned on the metaphase plate; in metaphase when the mitotic spindle was formed and chromosomes were aligned along the metaphase plate; or in stages from anaphase onwards, when sister chromatid cohesin was lost until the completion of cytokinesis. Thus, this last group contained any daughter cells retaining obvious intercellular connections even if DNA was decondensed and the nuclear envelope had reformed (Figure 5.8B).

This analysis revealed that cells expressing all versions of the kinases showed levels of cells in prometaphase comparable to those seen in control, untransfected cells. Cells expressing wild-type Nek6 and Nek7, as well as those expressing versions of Nek6 and Nek7 which retained almost complete kinase activity (K74M and K75M, respectively) or hypomorphic mutants retaining intermediate levels of kinase activity (T202A and T191A, respectively), showed a moderate (3-4 fold) increase in the number of metaphase cells, whilst those cells expressing catalytically-inactive versions of Nek6 and Nek7 (K75M and K64M) showed a significantly higher (7-9 fold) increase in metaphase populations. Furthermore, cells expressing hypormorphic mutants of Nek6 and Nek7 (S206A and S195A, respectively) with around 20-30% activity, showed a moderate increase in metaphase cell populations comparable to that seen in cells expressing WT Nek6 and Nek7 but a significant (5-10 fold) increase in cells in late mitosis not seen in cells expressing almost completely inactive Nek6 and Nek7. The Nek6 and Nek7 T202A and T191A mutants, which retain 30-40% activity, showed an intermediate phenotype with moderate increases late mitotic populations (Figure 5.8A).

In summary, mutants of Nek6 and Nek7 with little or no kinase activity (<20%) blocked cells at metaphase, whilst those mutants which retained intermediate levels of activity (30-40%) allowed cells to progress beyond metaphase, instead blocking cells during the exit from mitosis.

5.2.5 RNAi depletion of Nek6 and Nek7

To confirm the involvement of Nek6 and Nek7 in regulation of mitotic progression, we wished to analyze the effect of the absence of the two kinases on cell cycle progression. To this end, for each kinase we obtained three oligoribonucleotides designed to specifically silence Nek6 and Nek7. The ability of each of these sets of RNAi oligos to effectively deplete Nek6 or Nek7, both singly and in a pool, was assessed in HeLa cells. RNAi depletion with each oligo and two pools of the three Nek6 and Nek7 specific RNAi

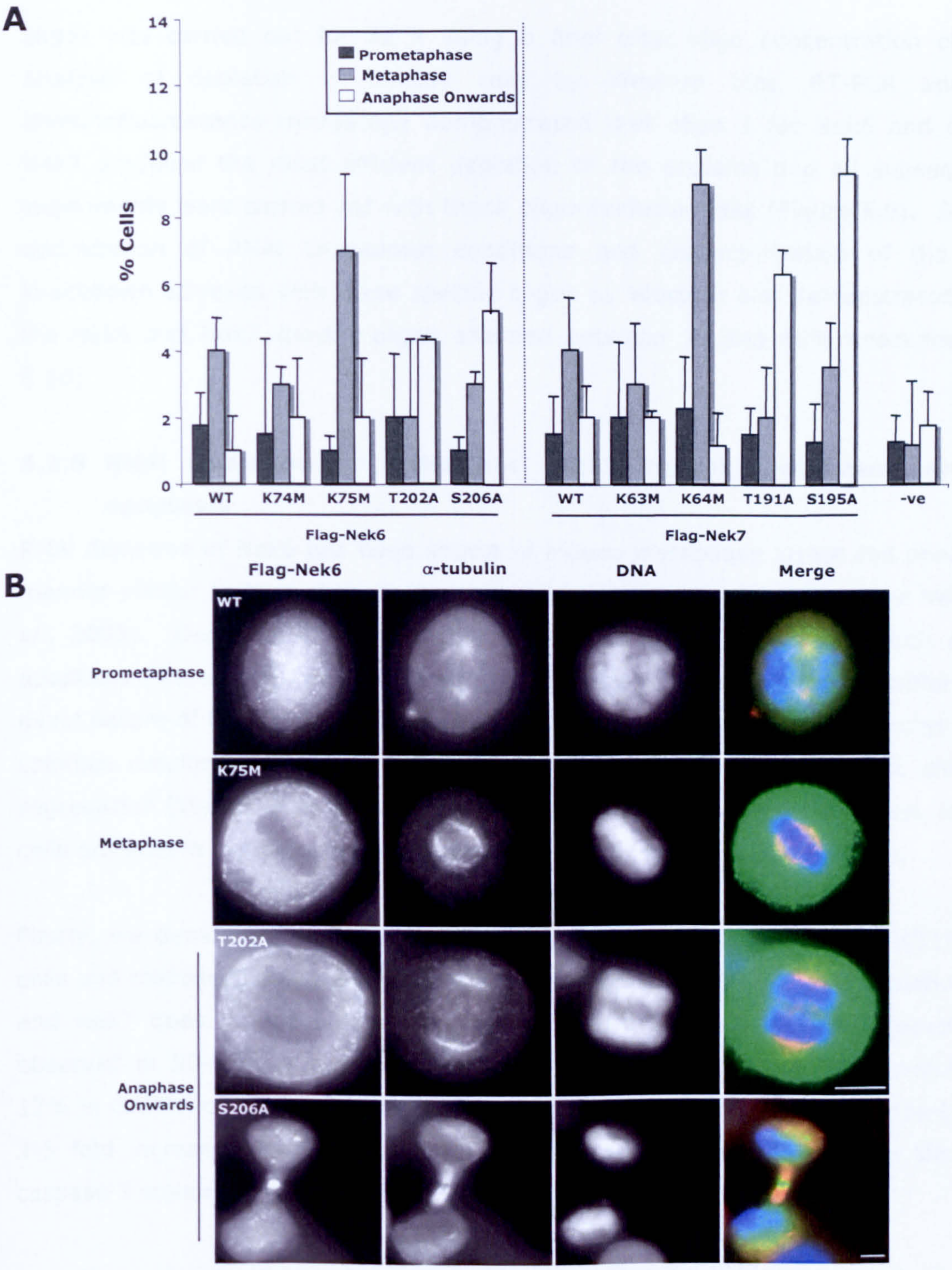

Figure 5.8 Kinase-inactive Nek6 and Nek7 mutants induce metaphase arrest, whilst hypormorphic mutants induce late mitotic arrest

(**A**) HeLa cells were transiently transfected with Flag-Nek6 and -Nek7 constructs, as indicated, for 24 h before being fixed and permeabilised and processed for immunofluorescence microscopy with anti-Flag antibodies, and anti-α-tubulin antibodies to detect the mitotic spindle and thus determine mitotic stage. DNA was stained with Hoechst 33258 and also used to determine mitotic stage. The histogram represents the observed frequency of transfected cells in the different stages of mitosis; anaphase onwards represents any cell stage from the onset of anaphase until cytokinesis. Data represent mean (± S.D.) of counts of at least 50 cells in three separate experiments. (**B**) Representative images of the different stages of mitosis. Scale bars, 10 μm.

oligos was carried out for 72 h using a final total oligo concentration of 100 nM. Analysis of depletion in treated cells by Western blot, RT-PCR and indirect immunofluorescence microscopy demonstrated that oligo 2 for Nek6 and oligo 2 for Nek7 produced the most efficient depletion of the proteins and all subsequent RNAi experiments were carried out with these oligoribonucleotides (Figure 5.9). Subsequent optimization of RNAi knockdown conditions and characterization of the levels of knockdown achieved with these specific oligos by Western blot demonstrated that both the Nek6 and Nek7 specific oligos afforded between 70 and 80% knockdown (Figure 5.10).

5.2.6 RNAi depletion of Nek6 and Nek7 causes metaphase arrest and apoptosis

RNAi depletion of Nek6 has been shown to induce metaphase arrest and apoptosis in a manner similar to that seen upon overexpression of catalytically-inactive Nek6 (Yin *et al.*, 2003). Depletion of Nek7 has likewise been shown to induce mitotic arrest and apoptosis (Yissachar *et al.*, 2006; Kim *et al.*, 2007). However, reports differ as to the exact nature of the arrest with it being reported to be due to the formation of multipolar spindles resulting in abnormal chromosome alignment and asymmetric chromosome segregation (Yissachar *et al.*, 2006) and a failure of centrosome separation, resulting in cells arrested in prometaphase with monopolar spindles (Kim *et al.*, 2007).

Firstly, we demonstrated via both flow cytometric analysis of annexin V/FITC stained cells and microscopic analysis of cleaved caspase 3 staining that RNAi depletion of Nek6 and Nek7 does indeed induce apoptosis. In the Annexin V assay, apoptosis was observed in 50-60% of the Nek6 and Nek7 depleted cell populations compared to 6-12% in GAPDH or mock-depleted populations (Figure 5.11A). This is comparable to the 3-5 fold increase in apoptosis upon depletion of Nek6 and Nek7 seen with cleaved caspase 3 staining (Figure 5.11B & C).

In order to assess whether RNAi depletion of Nek6 and Nek7 also induced mitotic arrest prior to apoptosis, microscopic observation of the DNA and cytoskeleton was performed following depletion of Nek6 and Nek7 in HeLa cells. RNAi depletion of both Nek6 and Nek7 resulted in a 2-3 fold increase in the mitotic index of these cell populations compared to GAPDH or mock-depleted populations (Figure 5.12A). Furthermore, a detailed analysis of the stage at which mitotic arrest had occurred showed that for both Nek6 and Nek7 depleted cell populations, there was a specific increase in the numbers of cells in metaphase (Figure 5.12B). Indeed, these cells appeared to have normal

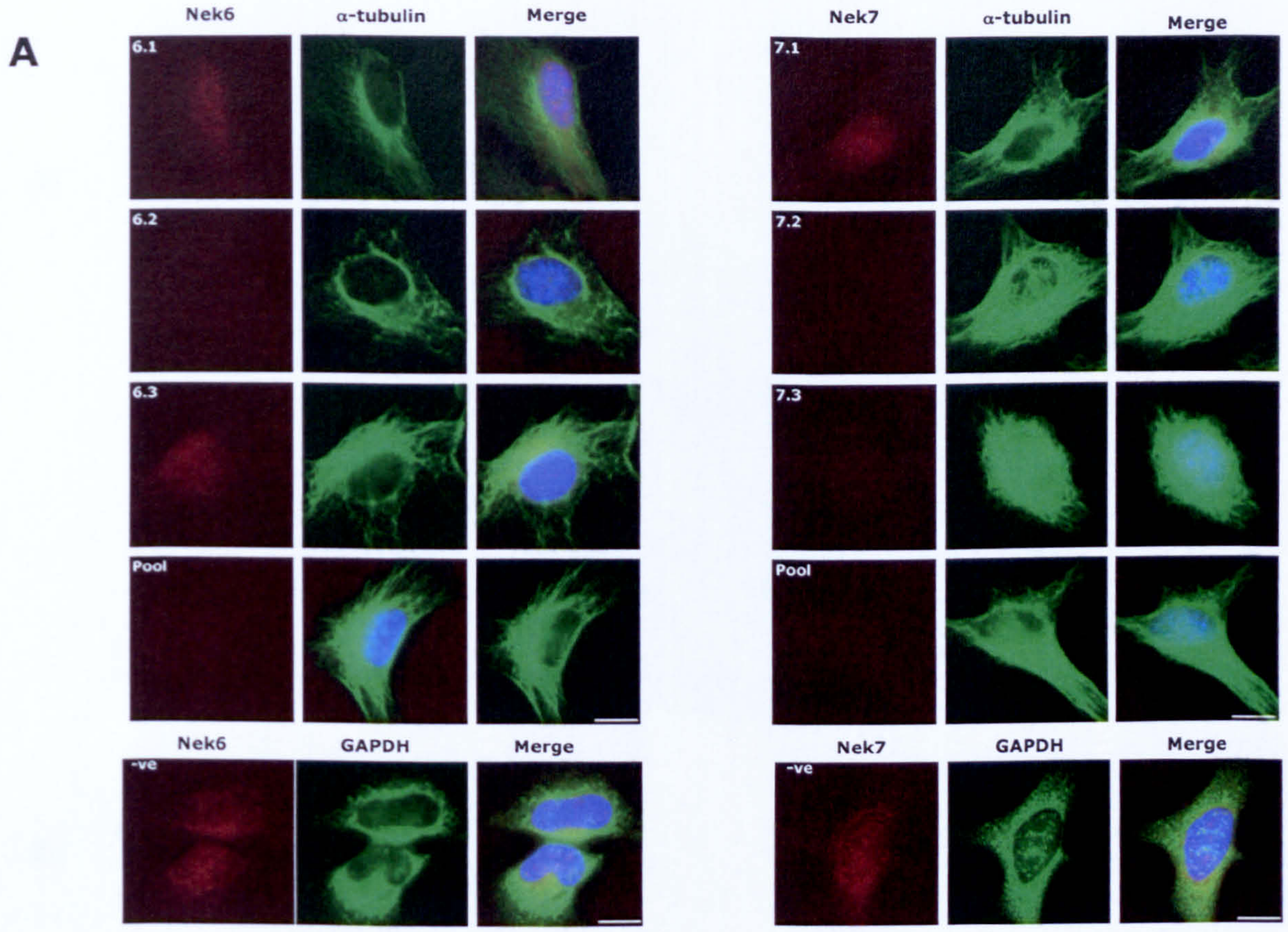

Figure 5.9 RNAi knockdown of Nek6 and Nek7 in HeLa cells

(**A**) HeLa cells were transfected with three individual Nek6 (6.1-6.3) or Nek7 (7.1-7.3) or pools of all 3 (6.P and 7.P) siRNA oligoribonucleotides for 72 h before being methanol-fixed and stained with anti-Nek6 or anti-Nek7 antibodies (red), as appropriate, and anti-α-tubulin antibodies (green). DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm. (**B**) HeLa cells were transfected with three individual Nek6 (6.1-6.3) or Nek7 (7.1-7.3) or pools of all 3 (6.P and 7.P) siRNA oligoribonucleotides for 72 h before cells were harvested and lysed and analysed by SDS-PAGE and Western blot with anti-Nek6, anti-Nek7, anti-GAPDH and anti-α-tubulin antibodies. +ve, GAPDH-depleted; -ve, mock-depleted. (**C**) HeLa cells were transfected with three individual Nek6 (6.1-6.3) or Nek7 (7.1-7.3) or pools of all 3 (6.P and 7.P) siRNA oligoribonucleotides for 72 h before cells were harvested and lysed and RNA recovered. RT-PCR was carried out with Nek6, Nek7, GAPDH and Actin-specific PCR primers. +ve, GAPDH-depleted; -ve, mock-depleted.

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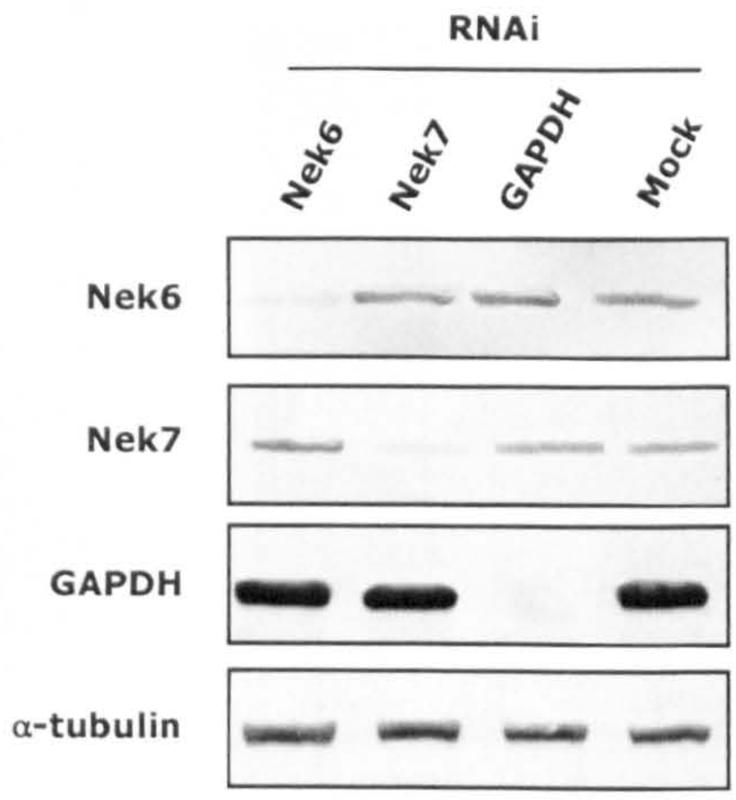

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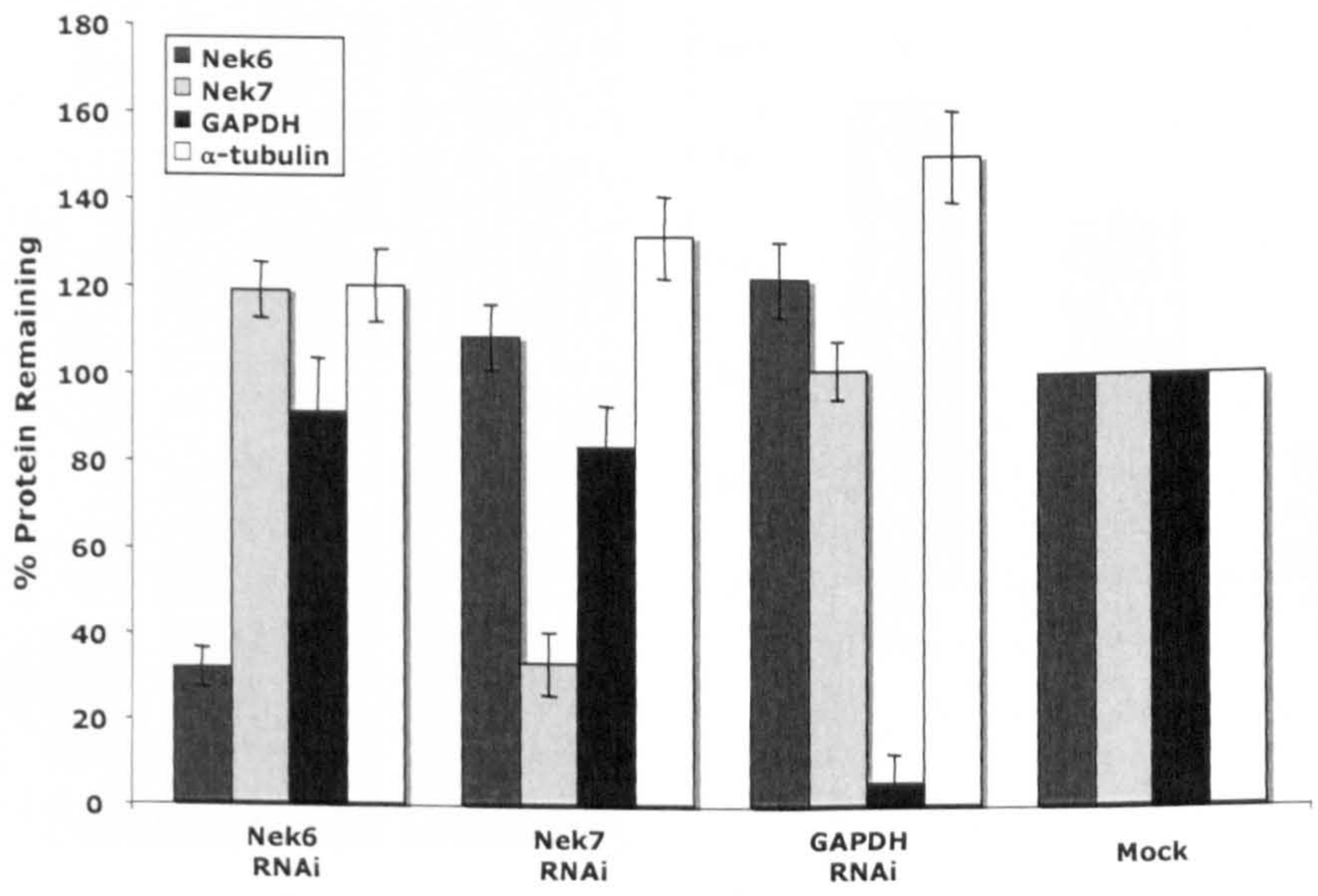


Figure 5.10 Quantification of RNAi knockdown of Nek6 and Nek7 in HeLa cells
(**A**) HeLa cells were transfected with Nek6, Nek7 or GAPDH-specific RNAi oligos for 72 h before being harvested, lysed and subjected to SDS-PAGE and Western blotting with anti-Nek6, anti-Nek7, anti-GAPDH or anti-α-tubulin antibodies. (**B**) Knockdown efficiency was quantified using ImageJ software. Protein remaining after knockdown is shown as a percentage of protein levels in mock-depleted extracts minus background intensities.

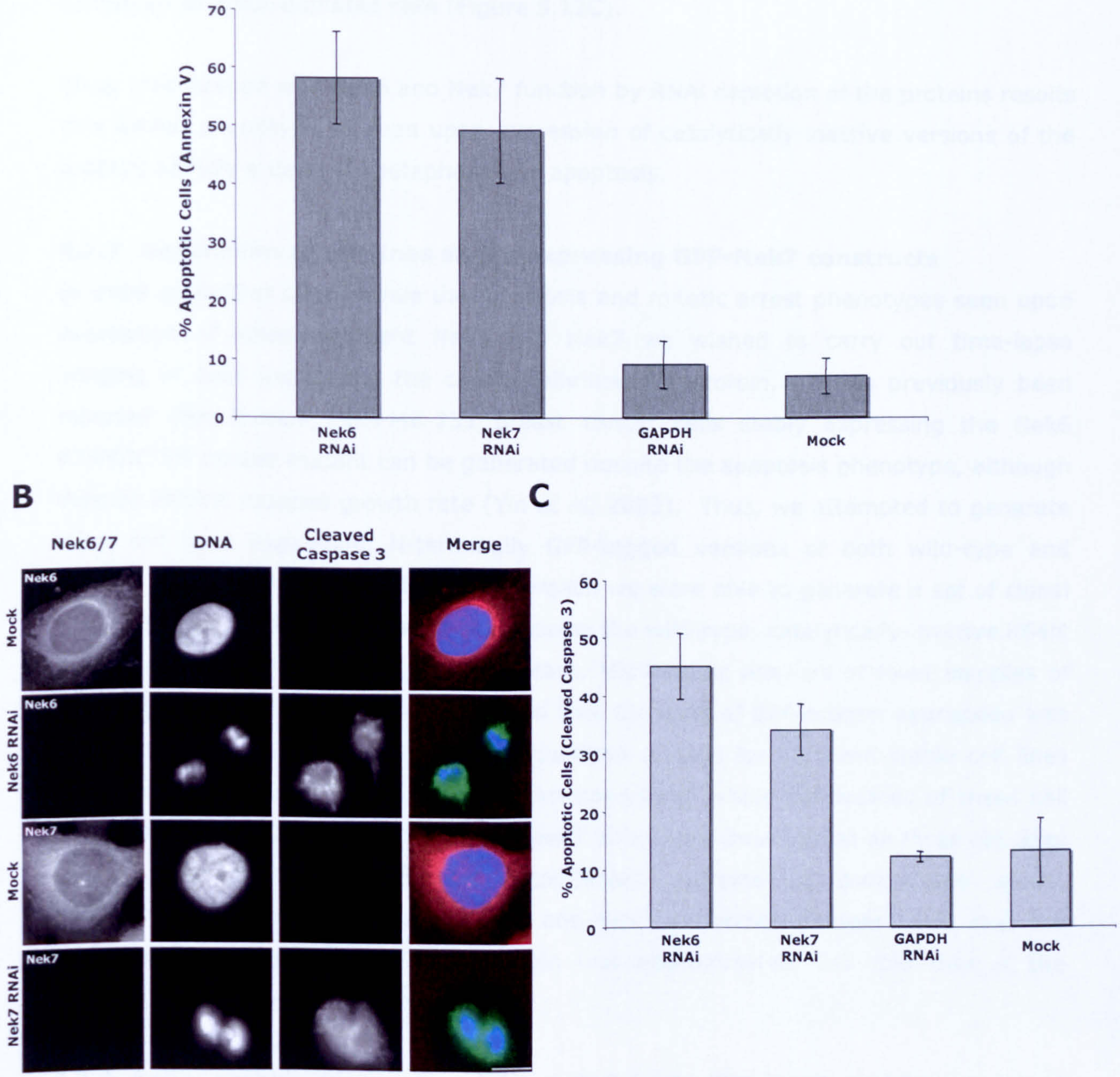


Figure 5.11 RNAi knockdown of Nek6 and Nek7 causes apoptosis in HeLa cells
(**A**) HeLa cells were transfected with Nek6, Nek7 or GAPDH specific RNAi oligos for 72 h before being assayed for their ability to take up annexin V/FITC stain via flow cytometry analysis. Mock transfected cells were treated identically but with no oligos. (**B**) HeLa cells were transfected with siRNA oligos, as indicated, for 72 h before being fixed and permeabilised with methanol and processed for immunofluorescence microscopy with anti-Nek6 or anti-Nek7 antibodies (red on merge) and anti-cleaved caspase 3 antibodies (green on merge). DNA was stained with Hoechst 33258 (blue on merge). Scale bar, 10 μm. (**C**) Numbers of apoptotic cells in each transfected cell population were counted. Data are based on means (± S.D.) of 3 separate experiments with counts of 300 cells in each case.

chromosome alignment on the metaphase plate, but less robust mitotic spindles compared to mock-depleted cells (Figure 5.12C).

Thus, interference with Nek6 and Nek7 function by RNAi depletion of the proteins results in a similar phenotype as seen upon expression of catalytically-inactive versions of the protein, namely a delay in metaphase and apoptosis.

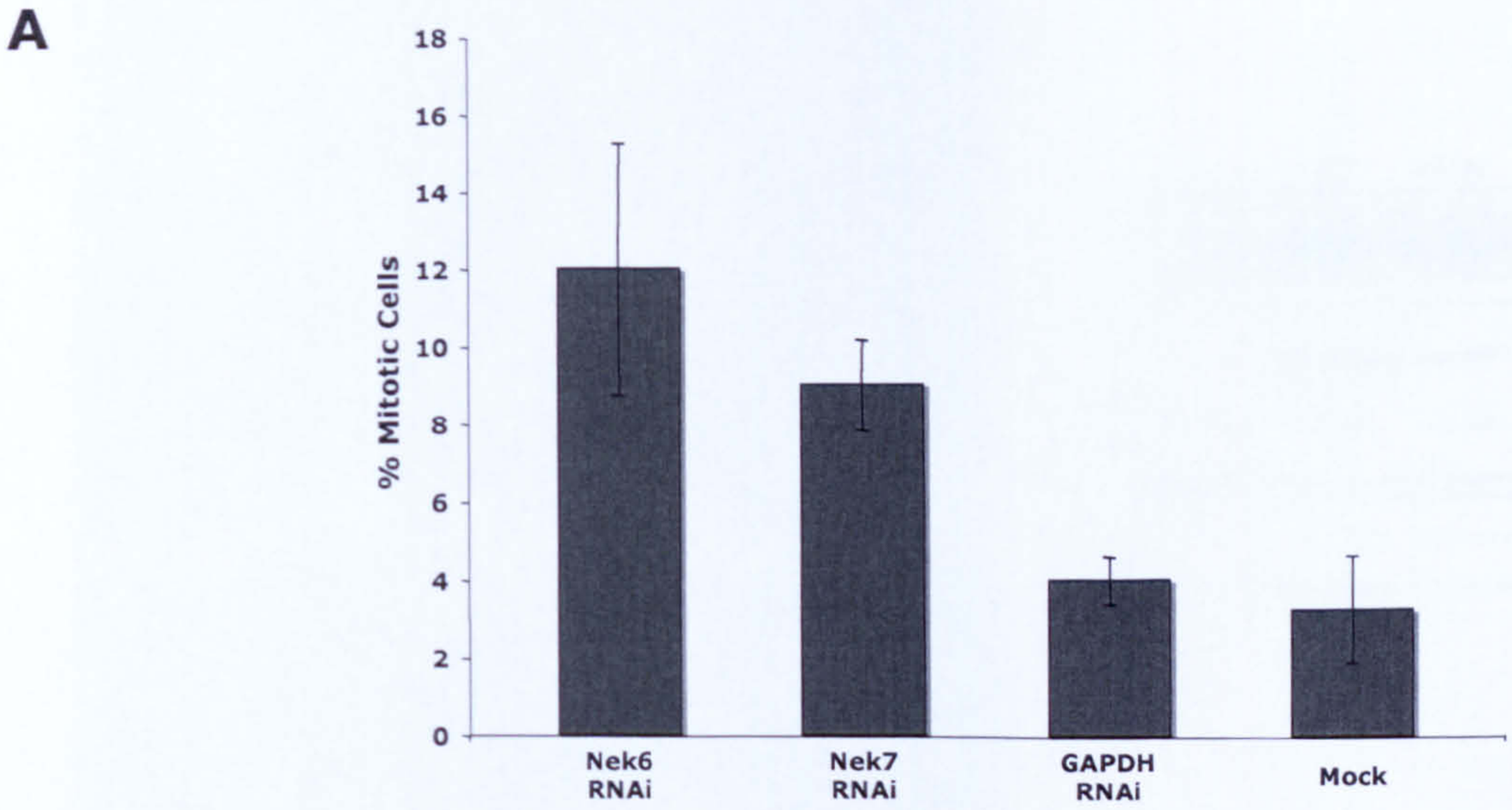
5.2.7 Generation of cell lines stably expressing GFP-Nek7 constructs

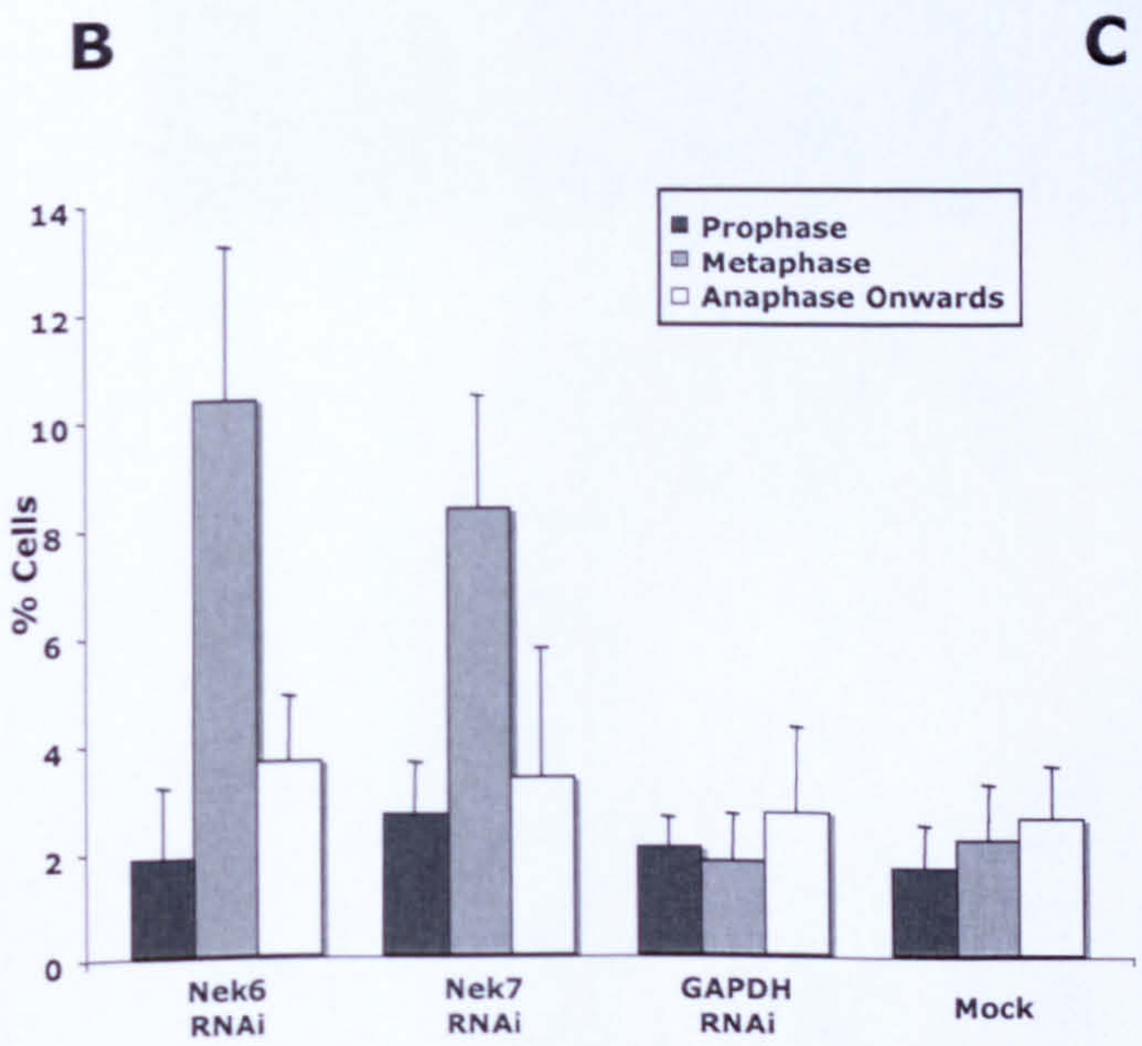
In order to further characterize the apoptosis and mitotic arrest phenotypes seen upon expression of kinase-deficient Nek6 and Nek7 we wished to carry out time-lapse imaging of cells expressing the catalytically-inactive protein. It has previously been reported that human MDA-MB-231 breast cancer cells stably expressing the Nek6 K74M/K75M double mutant can be generated despite the apoptosis phenotype, although they do exhibit reduced growth rate (Yin *et al*, 2003). Thus, we attempted to generate HeLa cell lines expressing N-terminally GFP-tagged versions of both wild-type and catalytically-inactive Nek7. Using this approach we were able to generate a set of clonal HeLa cell lines for GFP-Nek7, stably expressing the wild-type, catalytically-inactive K64M mutant and the hypomorphic S195A mutant. Microscopic analysis of fixed samples of expanded clonal populations demonstrated that the level of GFP-protein expression was relatively consistent across the whole population of cells for all three stable cell lines (Figure 5.13A). Furthermore, Western blot analysis of whole cell lysates of these cell populations, with both anti-GFP and anti-Nek7 antibodies showed that all three cell lines were expressing their respective recombinant proteins at comparable levels. Additionally, Western blot analysis with anti-Nek7 antibodies demonstrated that the expression level of the GFP-Nek7 protein was approximately 2-3 fold that of the endogenous Nek7 protein (Figure 5.13B).

5.2.8 Cells stably expressing kinase-deficient GFP-Nek7 exhibit reduced growth rates and increased mitotic index

To determine whether expression of Nek7 proteins affected proliferation, the growth rates of the three different Nek7 stable cell lines was compared to that of the parental HeLa cells by counting cell number over a period of 10 days. This approach indicated that stable expression of kinase-deficient Nek7 did indeed impair cell growth rate, whereas cells expressing wild-type Nek7 showed growth rates comparable to that of the parental HeLa cells (Figure 5.13C).

Microscopic analysis of the DNA and microtubule network of fixed cells showed that, as for transient expression of Flag-tagged versions of the Nek6 and Nek7 kinases, cells





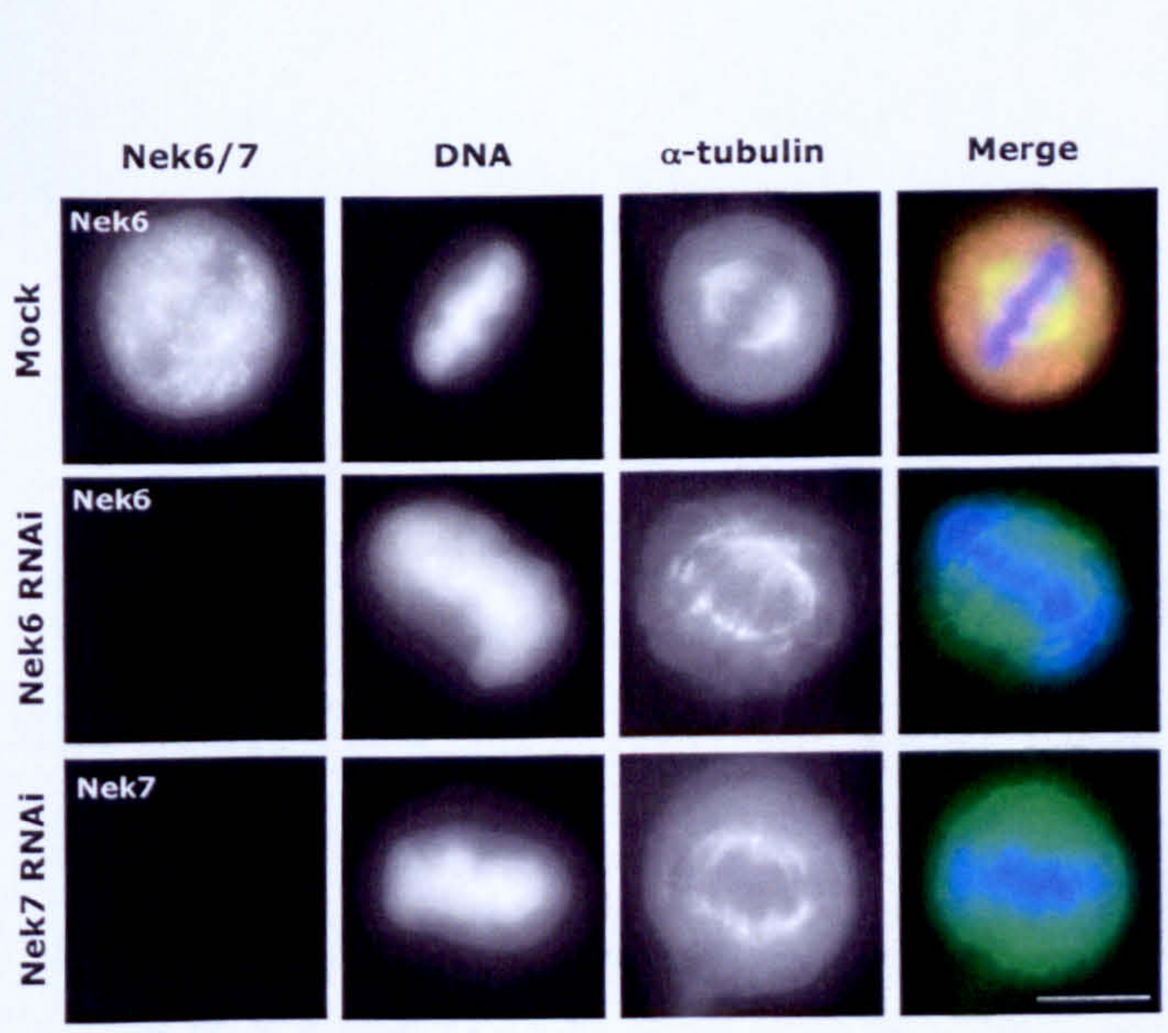


Figure 5.12 RNAi knockdown of Nek6 and Nek7 induces mitotic arrest

(**A**) HeLa cells were either mock-transfected or transfected with Nek6, Nek7 or GAPDH-specific RNAi oligos for 72 h before cells were fixed and permeabilised using methanol and processed for immunofluorescence microscopy with anti-Nek6, anti-Nek7 and anti-α-tubulin antibodies. DNA was stained with Hoechst 33258. The percentage of mitotic cells in each cell population was counted. Data are based on means (± S.D.) of 3 separate experiments with counts of at least 100 cells in each case. (**B**) The observed frequency of transfected cells in different stages of mitosis, as indicated, was measured. Data represent mean (±S.D) of 3 separate experiments with counts of at least 100 cells in each case. (**C**) Cells were stained with anti-Nek6 or anti-Nek7 antibodies (red on merge) and anti-α-tubulin antibodies (green). DNA was stained with Hoechst 33258 (blue on merge). Scale bar, 10 μm.

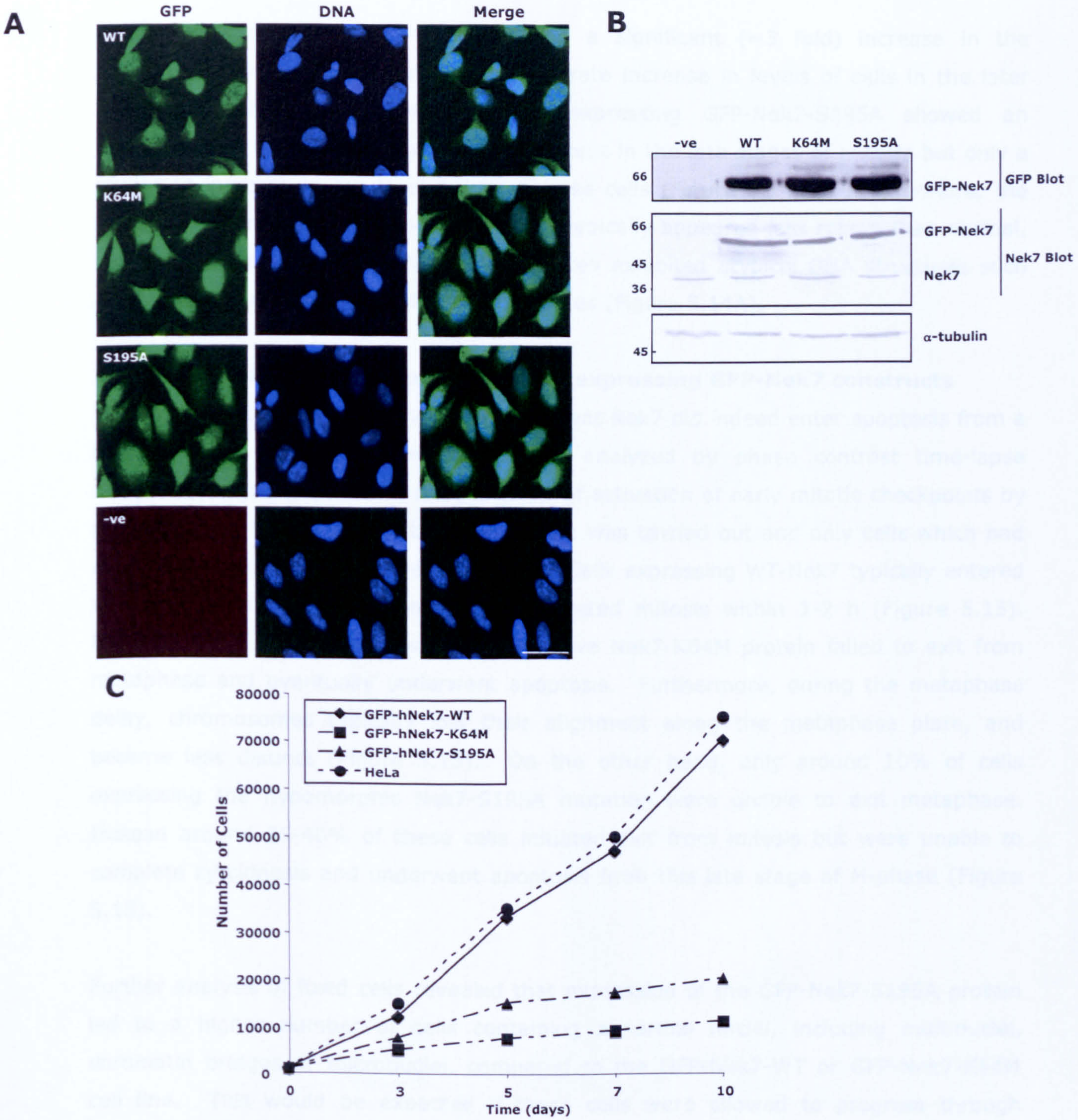


Figure 5.13 Generation of Nek7 stable cell lines with reduced growth rate

HeLa cell lines stably expressing GFP-hNek7-WT, GFP-hNek7-K64M and GFP-hNek7-S195A were established as described in Materials and Methods. (**A**) Either parental HeLa cells (-ve) or cell lines stably expressing GFP-Nek7 constructs were methanol-fixed and processed for immunofluorescence microscopy with Hoechst 33258 (blue). GFP was imaged directly. Scale bar, 30 μm. (**B**) Either parental HeLa cells or populations of clones stably expressing GFP-Nek7 constructs were harvested and lysed before being analysed by SDS-PAGE and Western blotting with anti-GFP, anti-Nek7 and anti-α-tubulin antibodies. Molecular weights (kDa) are as indicated on the left. (**C**) The growth rate of HeLa cells stably expressing GFP-Nek7 constructs was measured by cell number counting. Data represent means of three separate experiments and each experiment was performed using 3 individual clones of the different cell lines.

stably expressing GFP-Nek7-K64M showed a significant (~3 fold) increase in the metaphase cell population but only a moderate increase in levels of cells in the later stages of mitosis. Conversely, those expressing GFP-Nek7-S195A showed an approximate 4 fold increase in the levels of cells in the late stages of mitosis but only a moderate increase in the levels of metaphase cells (Figure 5.14B). Furthermore, the metaphase spindle of GFP-Nek7-K64M cells typically appeared less robust than normal, whilst those expressing GFP-Nek7-S195A often exhibited atypical DNA structures such as chromatin bridges and lagging chromosomes (Figure 5.14A).

5.2.9 Time-lapse imaging of cells stably expressing GFP-Nek7 constructs

To confirm that cells expressing kinase-deficient Nek7 did indeed enter apoptosis from a mitotic arrest, the stable cell lines were analyzed by phase contrast time-lapse microscopy. In order to limit the likelihood of activation of early mitotic checkpoints by photodamage, no imaging of the GFP protein was carried out and only cells which had already reached metaphase were selected. Cells expressing WT-Nek7 typically entered anaphase within 20-30 min and had completed mitosis within 1-2 h (Figure 5.15). However, 40% of cells expressing the inactive Nek7-K64M protein failed to exit from metaphase and eventually underwent apoptosis. Furthermore, during the metaphase delay, chromosomes typically lost their alignment along the metaphase plate, and became less distinct (Figure 5.15). On the other hand, only around 10% of cells expressing the hypomorphic Nek7-S195A mutation were unable to exit metaphase. Instead around 35-40% of these cells initiated exit from mitosis but were unable to complete cytokinesis and underwent apoptosis from this late stage of M-phase (Figure 5.15).

Further analysis of fixed cells revealed that expression of the GFP-Nek7-S195A protein led to a higher number of cells containing abnormal nuclei, including multinuclei, chromatin bridges or micronuclei, compared to the GFP-Nek7-WT or GFP-Nek7-K64M cell line. This would be expected if these cells were allowed to progress through metaphase but failed to complete cytokinesis (Figure 5.16).

5.2.10 Apoptosis is a direct result of mitotic arrest

The data presented in the preceding sections all add weight to the hypothesis that interference with Nek6 and Nek7 causes cells to arrest in mitosis and undergo apoptosis.

To confirm that apoptosis is indeed a direct result of mitotic arrest, cells expressing Nek6-WT or -K75M were either untreated or treated with hydroxyurea in order to arrest

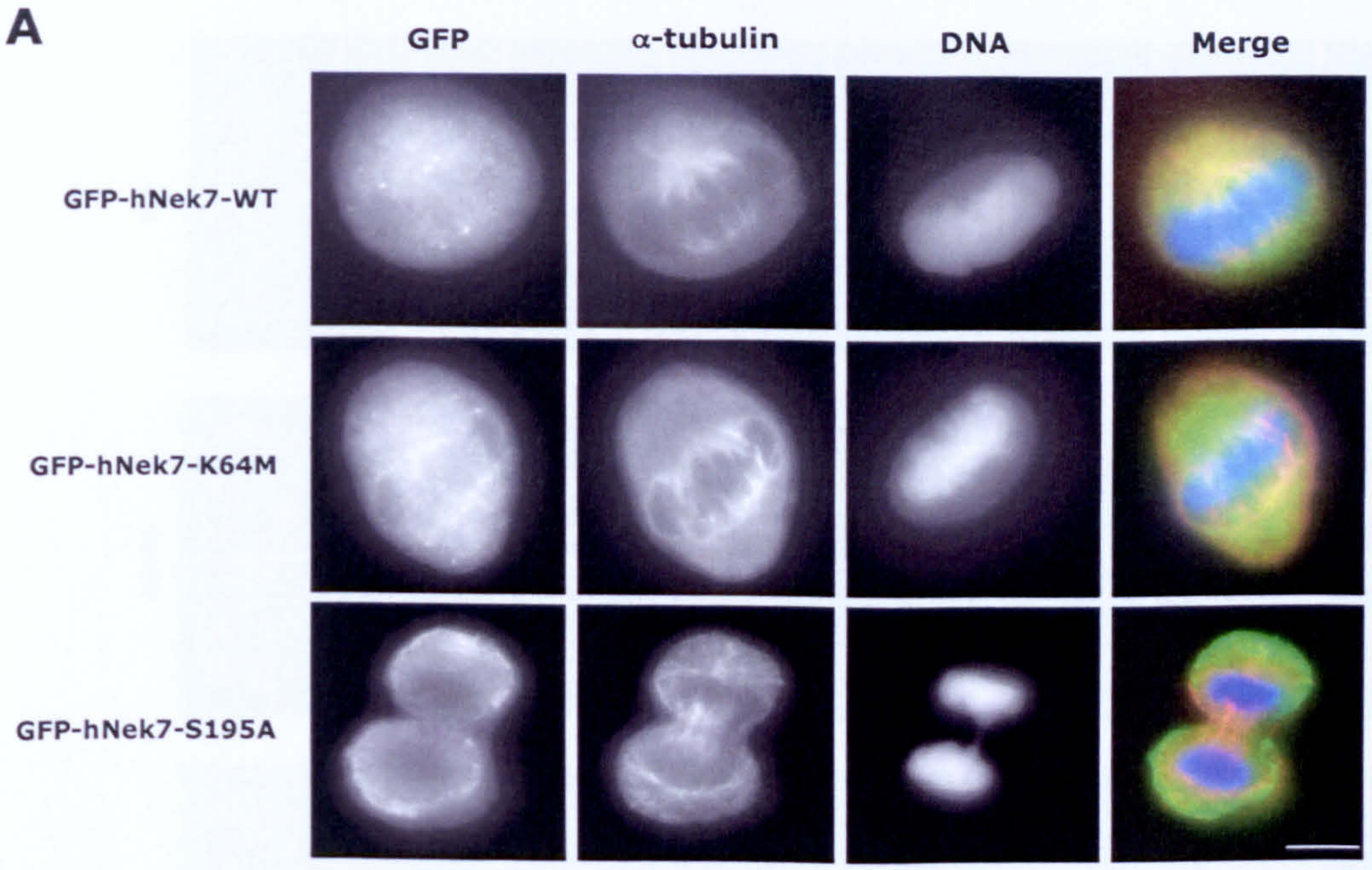


Figure 5.14 Cells stably expressing kinase-inactive Nek7 mutants have an increased mitotic index

(**A**) Cells stably expressing GFP-Nek7 constructs, as indicated, were fixed and permeabilised with ice-cold methanol and processed for immunofluorescence microscopy with anti-α-tubulin antibodies (red) and anti-GFP antibodies (green). DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm. (**B**) The percentage of cells at the various stages of mitosis, as indicated, was analyzed. Anaphase onwards designates any cells from anaphase onset until completion of cytokinesis. Data are based on means (± S.D.) of counts of at least 150 cells from 3 separate experiments.

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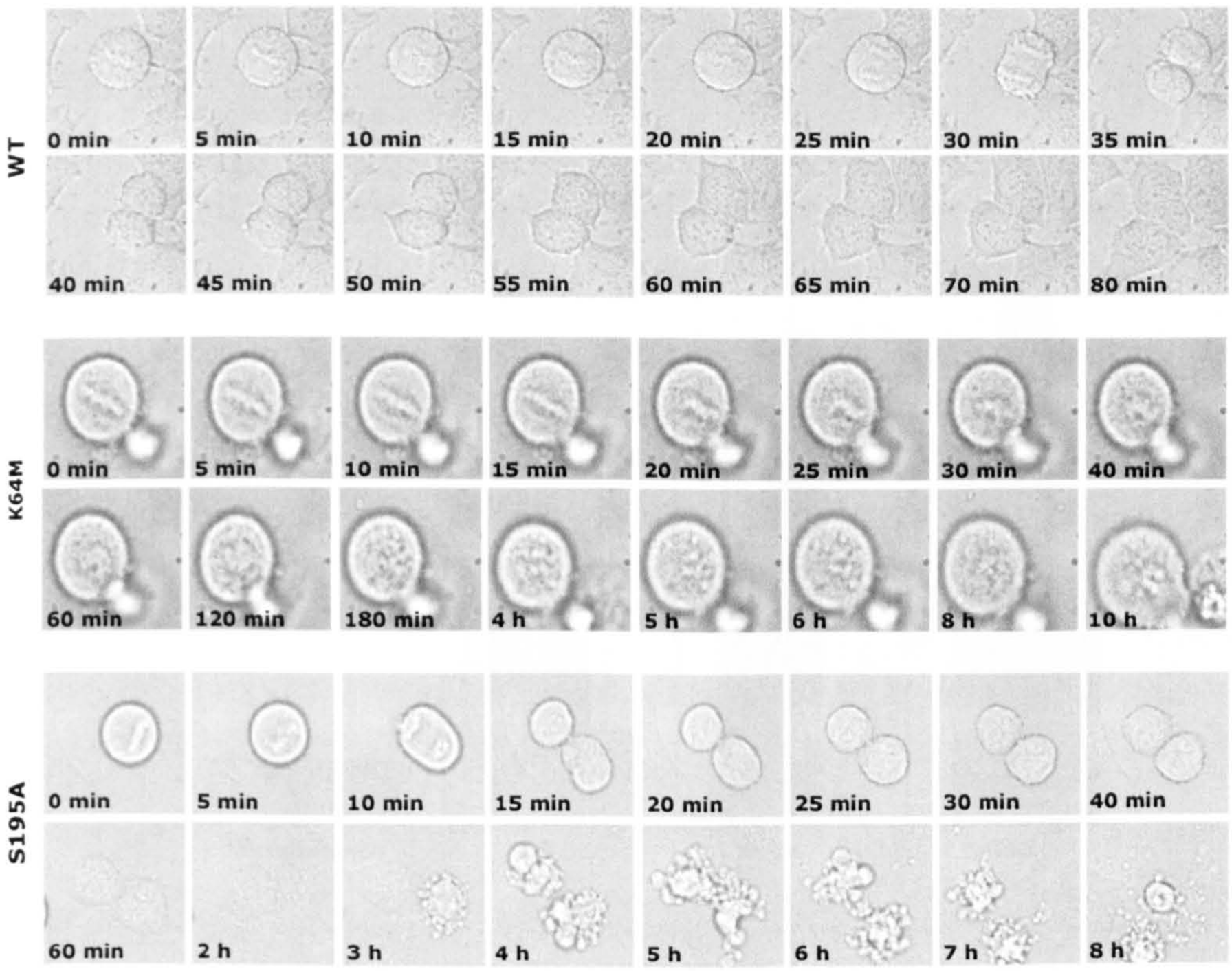

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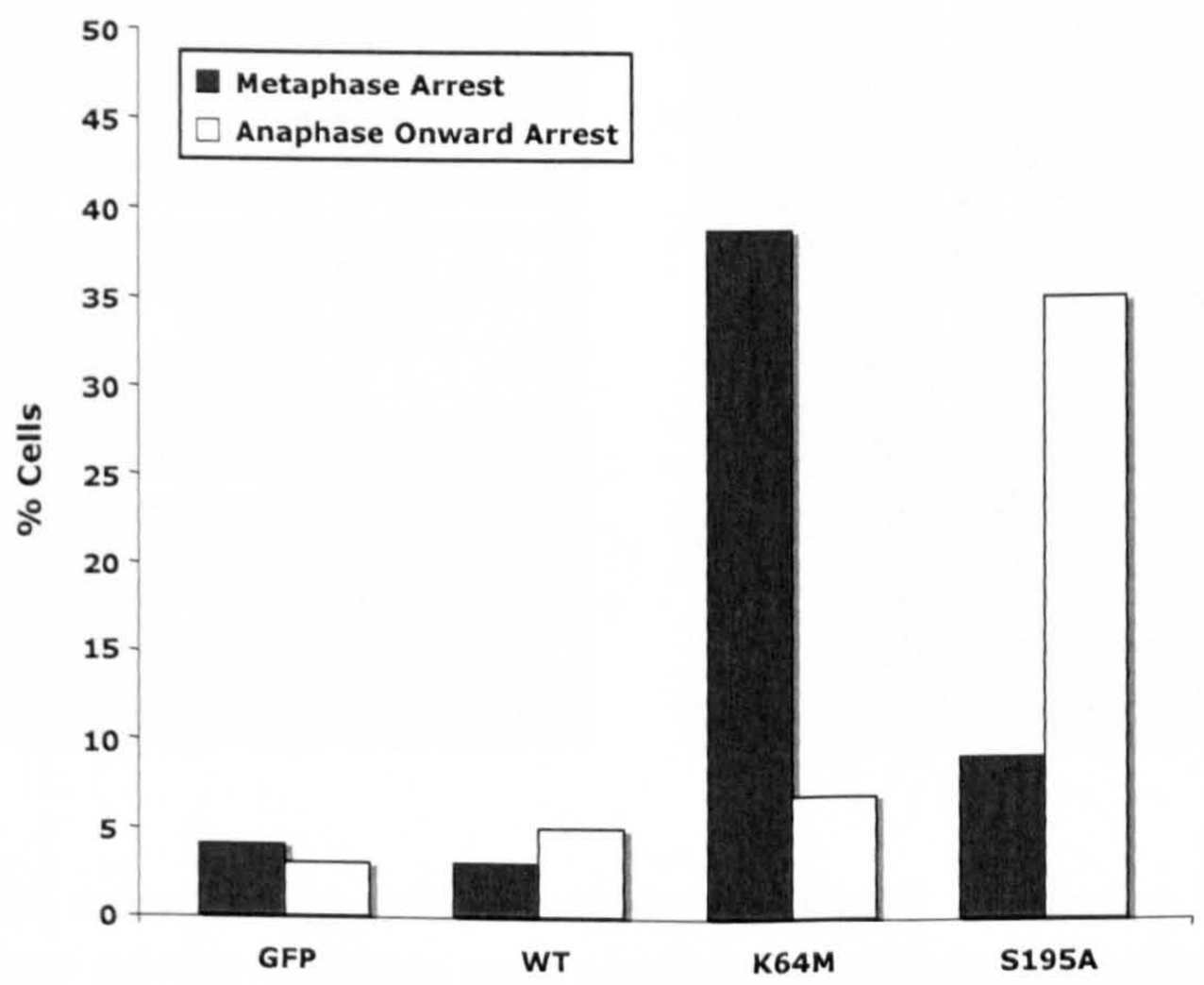

Figure 5.15 Time-lapse imaging of cells expressing GFP-Nek7 constructs

(**A**) HeLa cells stably expressing GFP-Nek7 constructs, as indicated, were subjected to time lapse recording of their mitotic progression to determine the ability of the cells to complete mitosis. Images are presented from metaphase until completion of cytokinesis or progression to cell death. (**B**) The outcome of each mitosis was scored according to whether cells, arrested in metaphase and underwent apoptosis or arrested between anaphase onset and completion of cytokinesis and underwent apoptosis. Between 40-50 cells were scored for each cell line.

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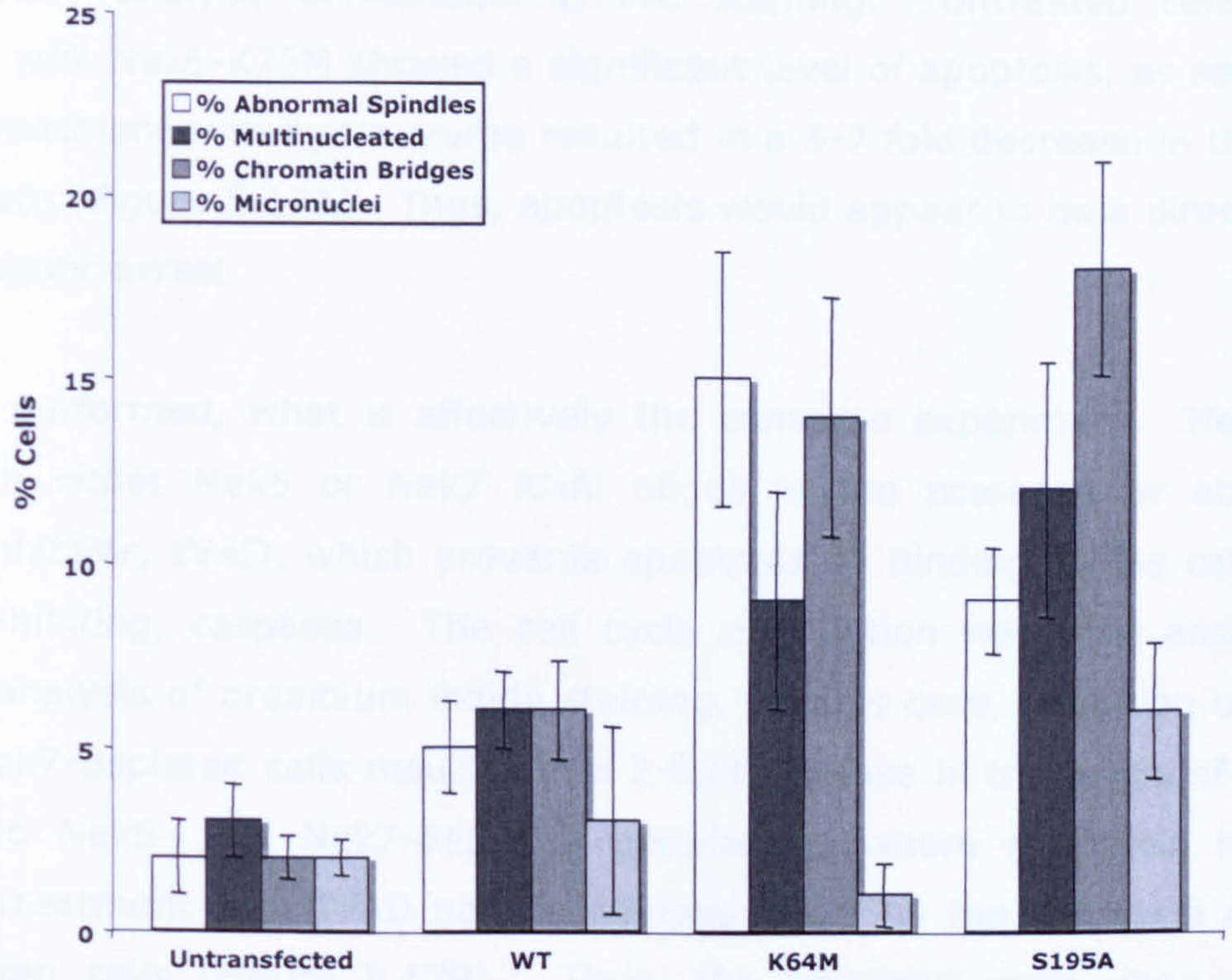

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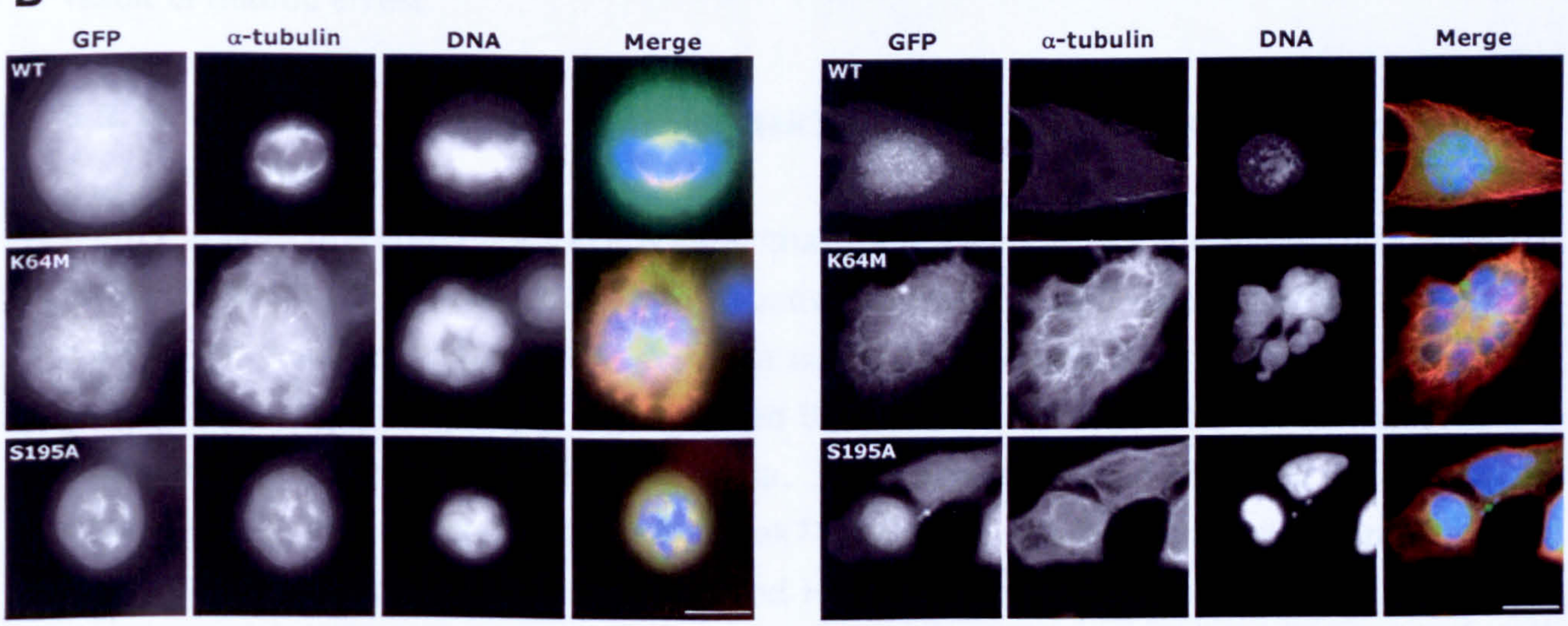


Figure 5.16 Cells expressing kinase-inactive Nek7 mutants exhibit increased numbers of nuclear abnormalities and abnormal mitotic spindles

(**A**) Cells stably expressing GFP-Nek7 constructs, as indicated, were fixed and permeabilised with methanol and processed for immunofluorescence microscopy with anti-α-tubulin antibodies (red) and anti-GFP-antibodies (green). DNA was stained with Hoechst 33258 (blue). The percentage of cells with abnormal mitotic spindles (multi- or mono-polar) or abnormal nuclei, multinuclei, chromatin bridges or micronuclei, as indicated, was analyzed. Data represent means (± S.D.) of counts of at least 150 cells from three separate experiments. (**B**) Representative images of the phenotypes scored are shown. Left panel, abnormal mitotic spindles; right panel, abnormal nuclei. Scale bars, 10 μm.

cells in S phase and thus prevent mitotic entry before being assayed for apoptosis via flow cytometry analysis of Annexin V/FITC staining. Untreated cells which were transfected with Nek6-K75M showed a significant level of apoptosis, as seen previously. However, treatment with hydroxyurea resulted in a 5-7 fold decrease in the numbers of apoptotic cells (Figure 5.17A). Thus, apoptosis would appear to be a direct result of the observed mitotic arrest.

Finally, we performed, what is effectively the converse experiment. HeLa cells were treated with either Nek6 or Nek7 RNAi oligos in the presence or absence of the apoptosis inhibitor, zVAD, which prevents apoptosis by binding to the catalytic site of, and thus inhibiting, caspases. The cell cycle distribution was then assayed via flow cytometric analysis of propidium iodide staining. In this case, inhibition of apoptosis in Nek6- or Nek7-depleted cells resulted in a 2-fold increase in the levels of 4n cells seen compared to Nek6- and Nek7-depleted populations where apoptosis had not been inhibited. Treatment with zVAD had no obvious effect on the cell cycle distribution of mock-depleted cells (Figure 5.17B). Thus, the apoptosis seen upon expression of catalytically-inactive Nek6 or Nek7 or RNAi depletion of these two proteins is a direct result of mitotic arrest.

5.2.11 Expression of kinase-inactive Nek7 interferes with Nek6 kinase activity and vice versa

From the data presented above, it is clear that expression of catalytically-inactive Nek6 or Nek7 results in a strong dominant-negative phenotype and we speculate that this may be the result of inhibition of a common upstream activator, such as Nek9. In order to assess the likelihood of this, we examined the effect of overexpression of kinase-dead Nek6 on Nek7 kinase activity and vice versa. Assuming expression of kinase dead Nek6 interferes with the upstream activator of Nek7 then Nek7 kinase activity will be inhibited in this situation and vice versa. To this end HEK 293 cells were transiently transfected with wild-type and kinase-inactive Nek6 and Nek7 and lysates were subjected to immunoprecipitation with the opposite antibody. Thus, cells transfected with Flag-Nek6 constructs underwent immunoprecipitation with anti-Nek7 antibodies and vice versa. Immune complexes were then used to *in vitro* kinase assays with β-casein as substrate (Figure 5.18). Whilst expression of active Nek7 had no effect on Nek6 kinase activity, expression of kinase-inactive Nek7 reduced Nek6 kinase activity by around 50%. Similarly, expression of kinase-inactive Nek7 resulted in immunoprecipitated Nek6 exhibiting a 50-60% decrease in kinase activity. Thus, it seems likely that the similar phenotypes seen upon interference with either Nek6 or Nek7 function are the result of

the two kinases acting in a strong dominant-negative manner to inhibit an upstream activator, leading to inhibition of both Nek6 and Nek7 simultaneously.

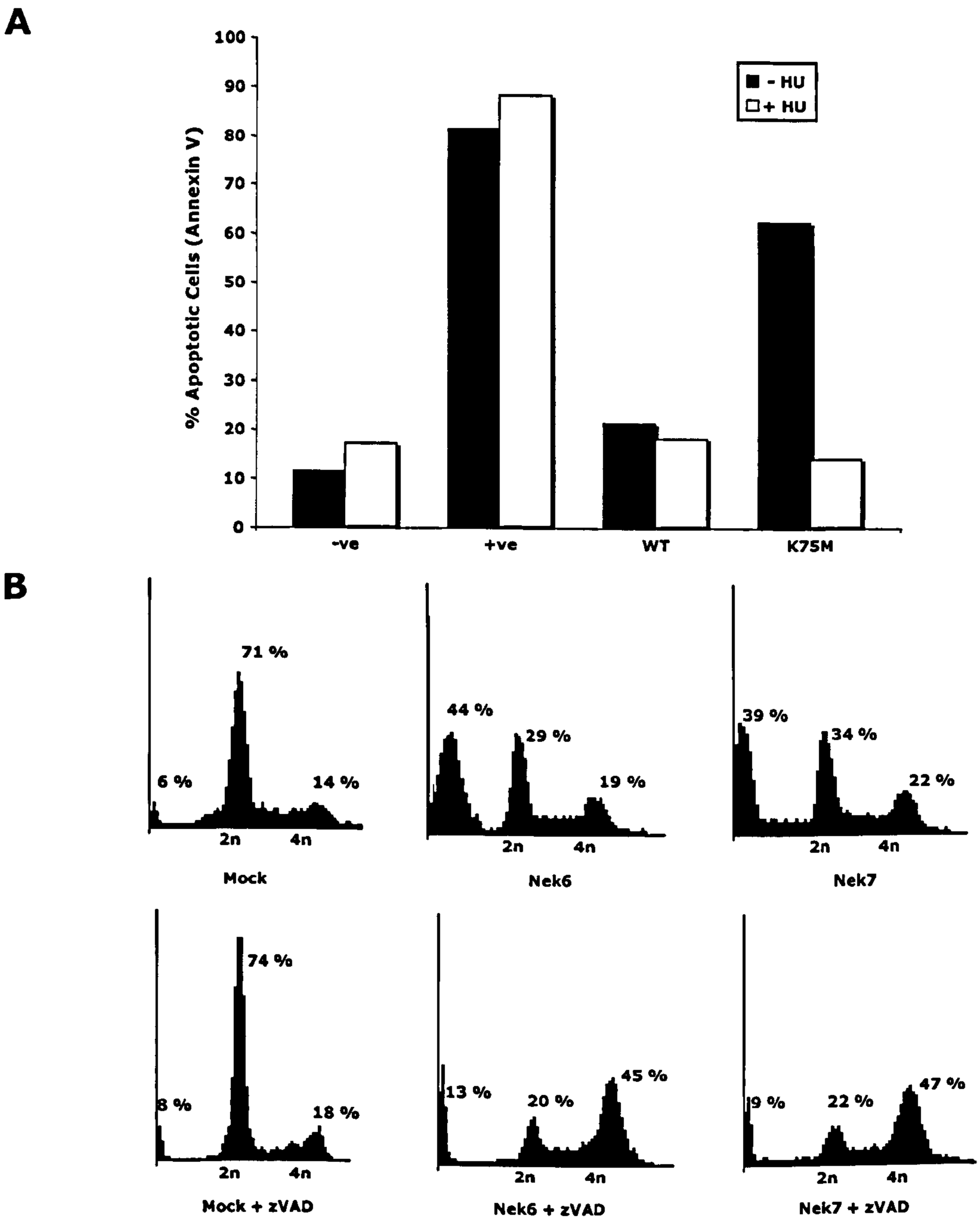
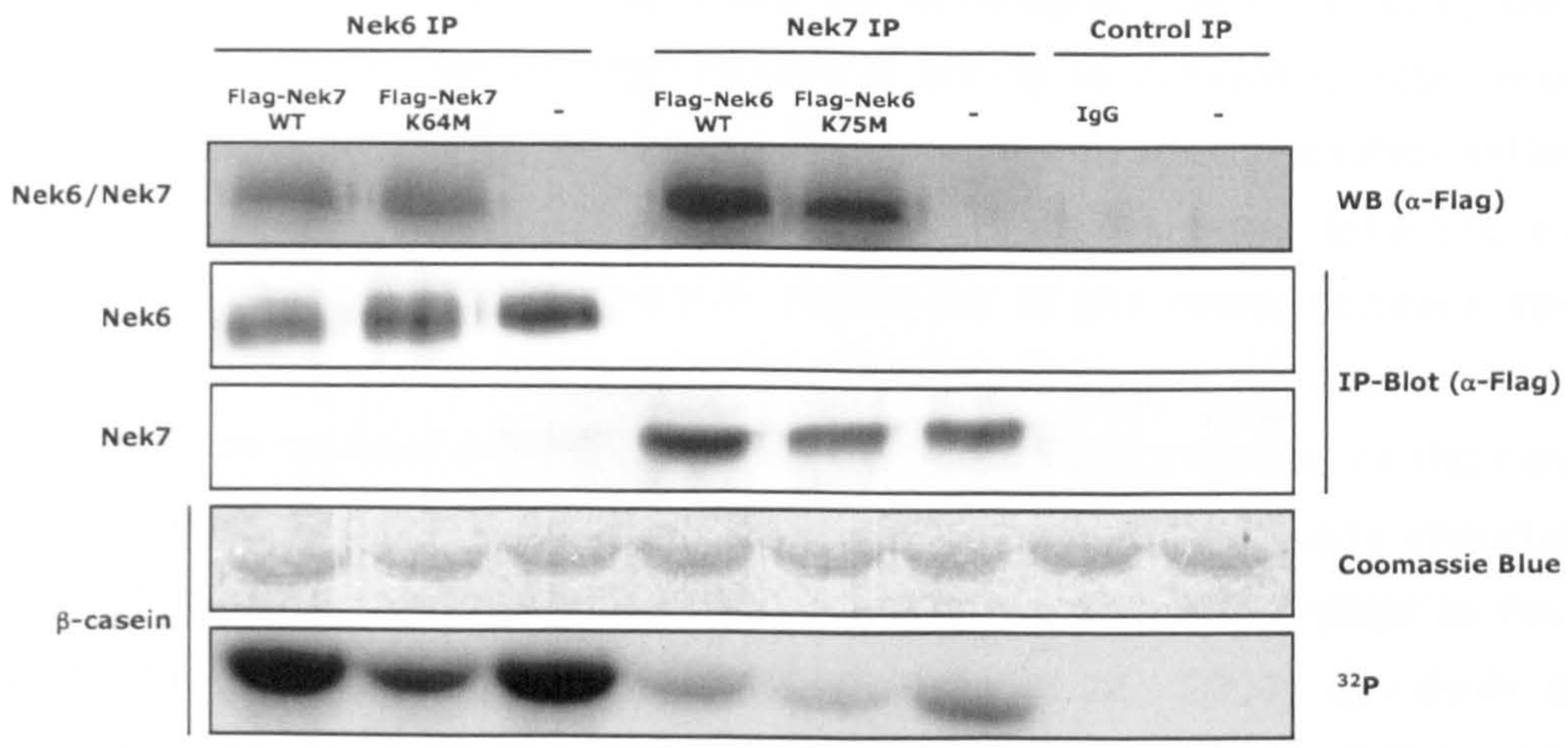


Figure 5.17 Apoptosis induced by kinase-inactive Nek6 is dependent upon mitotic arrest

(**A**) HeLa cells were transiently transfected with Flag-Nek6 constructs, as indicated, for 48 h in the presence or absence of hydroxyurea (+ HU and - HU, respectively) before being assayed for their ability to take up FITC-conjugated Annexin V via flow cytometry analysis. Data represent means (± S.D.) of three separate experiments. Negative control cells were treated identically but with no DNA (-ve), positive control cells were treated with 1 μM staurosporine for 6h (+ve). Cells were treated with HU in order to arrest cells in S phase and thus prevent the cells from entering mitosis. S-phase arrest prevents cells from undergoing apoptosis, indicating that apoptosis is a direct result of the mitotic arrest seen. (**B**) HeLa cells were treated with Nek6 or Nek7 siRNA oligos or mock-transfected for 72 h in the presence or absence of the apoptosis inhibitor, zVAD, as indicated. Cells were then harvested and fixed before being stained with propodium iodide and cell cycle distribution analyzed by flow cytometry.

A



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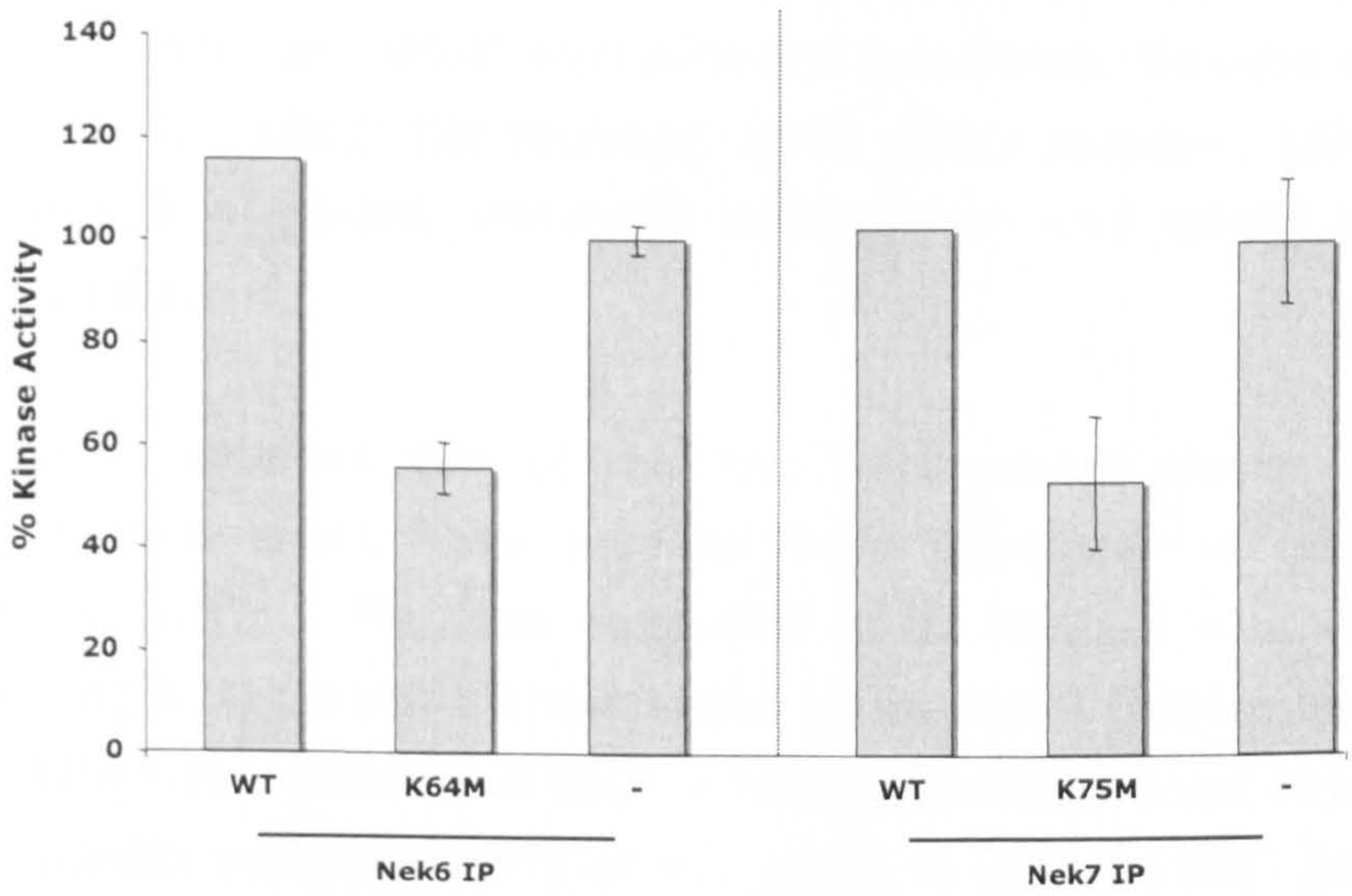


Figure 5.18 Expression of kinase-inactive Nek7 interferes with Nek6 kinase activity and vice versa

(**A**) HEK 293 cells were transiently transfected with Flag-Nek6 and Flag-Nek7 constructs, as indicated. After 24 h cells were harvested and lysed and samples subjected to SDS-PAGE and Western blotting with anti-Flag antibodies to determine transfection efficiency. Remaining lysates were then subjected to immunoprecipitation with anti-Nek6 or anti-Nek7 antibodies, as indicated. Immunoprecipitates were subjected to SDS-PAGE and Western blotting analysis with anti-Flag antibodies and used in kinase assays with β-casein as a substrate to determine the effect of expression of catalytically-inactive Nek6 on Nek7 activity and vice versa. Samples were analyzed by SDS-PAGE, Coomassie Blue staining and autoradiography (^{32}P). (**B**) Activity of the immunoprecipitated proteins is expressed as a percentage of wild-type activity in untransfected (-) cells, normalised to the amount of precipitated protein. Data is shown as mean (± S.D.) of three separate experiments.

5.3 Discussion

M-phase is the critical stage in the cell cycle when duplicated DNA is segregated into two nuclei, which are then separated into two clonal daughter cells during cytokinesis. Accurate and timely progression through mitosis is critical to prevent genetic instability and complex regulatory networks, predominantly involving protein phosphorylation and dephosphorylation, exist to ensure that this occurs. Protein kinases take the form of executioners, which are directly involved in regulation of the many complex steps of mitosis, and surveillance molecules, which act in checkpoint systems, such as the SAC, to ensure accurate completion of one stage is achieved before initiation of the next can occur. Unsurprisingly, these systems are intimately interlinked, and many important M-phase regulators perform multiple roles. Aurora-B for example is critical in the SAC signaling pathway (Biggins and Murray, 2001; Tanaka *et al.*, 2002; Lampson *et al.*, 2004), as well as being involved in cytokinesis (Carmena and Earnshaw, 2003). Indeed, the key mitotic regulators are typically multifunctional; Plk1, for example, is implicated in diverse mitotic functions including centrosome maturation, bipolar spindle assembly, sister chromatid cohesion, APC/C activation and cytokinesis (Sumara *et al.*, 2004; van Vugt *et al.*, 2004;), whilst the founding NIMA family member, NIMA, has roles in promoting entry into mitosis, chromatin condensation and spindle organization (De Souza *et al.*, 2000).

Nek6 and Nek7 represent two of the four NIMA-related kinase encoded by the mammalian genome which have thus far been implicated in mitotic progression (O'Regan *et al.*, 2007). They are postulated to be involved in a mitotic regulatory cascade involving a third mitotic NIMA-related kinase, Nek9 (Belham *et al.*, 2003). The final mitotic NIMA-related kinase is Nek2, which shares the highest level of homology in its catalytic domain with NIMA (Fry *et al.*, 1995; Lu *et al.*, 1993) and is involved in maintenance and modulation of centrosome architecture (Fry *et al.*, 1998).

Nek9 is believed to be responsible for the phosphorylation of critical residues within the activation loop of Nek6 and Nek7 leading to their activation in mitosis (Belham *et al.*, 2003). Based on the phenotypes that arise from inhibiting Nek9, it is assumed that this acts to regulate mitotic spindle formation and function. However, little is known about the possible downstream events of such a cascade and it is possible that Nek9 has other targets that are involved in these processes. Indeed, the functional data on Nek6 and Nek7 is limited and somewhat contradictory, with separate reports describing interference with Nek6 as having a severe effect on cell cycle progression, resulting in mitotic arrest and apoptosis (Yin *et al.*, 2003), or as having no effect on cell cycle progression (Minoguchi *et al.*, 2003). Furthermore, although published data on Nek7

generally agree that interference with its function has an effect on cell cycle progression, reports differ as to the severity and exact nature of the defect (Kim *et al.*, 2007; Yin *et al.*, 2006).

Thus, having shown that both Nek6 and Nek7 are cell-cycle regulated and at least Nek6 is localized to some degree to mitotic structures, in this chapter we aimed to carry out a detailed functional characterization of Nek6 and Nek7 in an attempt to clarify the proposed mitotic roles of these two kinases. We found that interference with Nek6 or Nek7 function, either by expression of kinase-deficient proteins or RNAi depletion, resulted in marked growth inhibition, as well as a significant accumulation of cells in mitosis and apoptosis. Furthermore, the delay in mitosis ultimately results in progression to apoptosis. Thus, interfering with both Nek6 and Nek7 function clearly has an effect on cell cycle progression.

Importantly, the generation of mutant versions of Nek6 and Nek7 with varying degrees of kinase activity has led to the conclusion that Nek6 and Nek7 may act at two separate stages of mitosis. Expression of completely inactive Nek6 and Nek7 mutants predominantly led to a metaphase delay, whilst expression of hypomorphic mutants that retained some activity tended to cause an accumulation of cells in late mitosis.

The demonstration that interference with Nek7 function results in a delay at both metaphase and cytokinesis, dependent upon the level of Nek7 kinase activity, is consistent with reports by Kim *et al.* (2007) who demonstrated that co-depletion of both Nek7 and Mad2 (in order to bypass the SAC) resulted in a decrease in the number of cells which were positive for the mitotic marker, phospho-histone H3 but an accumulation of 4n cells indicative of failed cytokinesis. This led to the conclusion that the metaphase delay seen was as a result of the activation of the SAC but that further, interference with Nek7 function may also result in a cytokinesis failure. The data presented here, demonstrating a delay in cytokinesis in cells expressing hypomorphic Nek6 mutants is, to my knowledge, the first time Nek6 has also been implicated in the regulation of mitotic exit. It has been shown that expression of the completely inactive KK/MM Nek6 mutant as well as RNAi depletion of endogenous Nek6 induces metaphase arrest and apoptosis (Yin *et al.*, 2003). However, although the effect of bypassing the SAC has not been examined, here, expression of a hypomorphic Nek6 or Nek7 mutant appeared to mimic the effect of bypassing the SAC.

To ensure accurate distribution of sister chromatids, the SAC is activated in mammalian cells until all chromosomes are attached to spindle microtubules. Once this is

accomplished, the SAC is inactivated and activation of the APC/C then mediates the separation of sister chromatids, allowing anaphase to proceed (Mussacchio and Hardwick, 2002; Hoyt, 2001). Thus, a delay at metaphase is indicative of prolonged activation of the SAC. This may mean either that Nek6 and Nek7 kinase activity is required for recruitment or activation of SAC inhibitors or that there may be insufficient kinetochore attachment or lack of tension across the mitotic spindle. The latter possibility is consistent with reports that Nek7 is involved in centrosome maturation and spindle nucleation (Kim *et al.*, 2007; Yissachar *et al.*, 2006) and also with the idea of a mitotic signaling cascade involving Nek9, which appears to function in spindle nucleation through both centrosome- and Ran-GTP-dependent, chromosome-mediated pathways (Roig *et al.*, 2005).

The delay in mitotic exit seen upon expression of hypomorphic Nek6 and Nek7 mutants suggests that Nek6 and Nek7 not only play a role in metaphase progression but are important for cytokinesis. Indeed many mitotic kinases including Plk1, Aurora A, and indeed NIMA, have been shown to act at several stages throughout mitosis. Alternatively, the delay in cytokinesis could be the result of a defective SAC resulting in aberrant progression through metaphase, and chromosome missegregation. Indeed, cells stably expressing GFP-Nek7-S195A exhibited a high degree of nuclear abnormalities, including chromatin bridges, which may be indicative of this sort of mitotic defect. Thus, an alternative interpretation of the data is that the residual levels of kinase activity remaining in the hypormorphic Nek6 and Nek7 mutants, whilst sufficient for cells to overcome the SAC, still resulted in missegregation of DNA and this was the cause of the cytokinesis failure. If this interpretation is correct, it would suggest that Nek6 and Nek7, consistent with their interaction with Nek9, are primarily involved in regulation of mitotic spindle organization or the SAC. On the other hand, along with an increase in the number of cells with chromatin bridges, expression of hypomorphic Nek6 or Nek7 mutants also led to an increase in the number of multinucleated cells. This is perhaps more indicative of failed cytokinesis as opposed to chromosome missegregation, and, as discussed above, co-depletion of Nek7 and Mad2 has been shown to result in an increase in the number of 4n cells (Kim *et al.*, 2007). It has also been reported that RNAi depletion of Nek7 results in the formation of cells with multipolar spindles, a phenotype which can be attributable to failed cytokinesis (Yissachar *et al.*, 2007). Furthermore, as discussed in chapter 4, both endogenous and recombinant Nek6 localize to both the spindle midzone and the midbody in the later stages of mitosis further supporting a direct role in cytokinesis progression.

Elucidation of a cytokinetic role for proteins which also function at earlier stages of mitosis is difficult as expression of catalytically-inactive versions of a protein or RNAi depletion of the endogenous protein typically highlights the earlier mitotic role consequently masking the later cytokinetic contribution (Eggert *et al.*, 2006). Recently, small molecule inhibitors designed specifically to target mitotic protein kinases have found a crucial application in dissecting the mitotic roles of protein kinases. Such drugs are powerful cell biology tools for a number of reasons, not least because they can be used in dose-response experiment to uncover a range of phenotypes and because, due to their rapid mode of action, studies can be conducted with exact temporal control (Taylor and Peters, 2008). Such inhibitors have already been used to good effect to uncover the mitotic roles of Aurora and Polo-like kinases and the development of specific Nek6 or Nek7 inhibitors would allow analysis of the roles the Nek6 and Nek7 play in late mitotic events. Another such approach would be the development of analogue-sensitive kinases whose specific inhibitors could be added at the appropriate time following metaphase (Blethrow *et al.*, 2004).

An interesting aspect of Nek6 and Nek7 biology centers on whether two such similar kinases have redundant or distinct functions. Earlier chapters have shown that whilst both Nek6 and Nek7 are regulated in a similar manner, their localization patterns may differ. It is clear from this section of work that interference with both Nek6 and Nek7 function by expression of kinase-deficient versions of the proteins results in a similar phenotype for both. However, expression of kinase-inactive Nek6 interfered with the activation of Nek7 and vice-versa. A possible explanation for this is that the two kinases act in a strong dominant manner to inhibit a common upstream activator, perhaps Nek9. Thus, the consequences of expressing kinase-deficient versions of Nek6 and Nek7 may result from interference with phosphorylation of other substrates of Nek9 and would thus lead to the same phenotypes whether Nek6 or Nek7 mutants were used. However, RNAi depletion of either Nek6 or Nek7 led to the same apoptosis phenotype, as was seen upon expression of recombinant kinase, which would suggest a more direct role for Nek6 and Nek7 in these processes. Indeed, RNAi depletion of Nek6 and Nek7 only resulted in metaphase arrest and not the cytokinesis defect seen upon expression of hypomorphic Nek6 and Nek7 mutants, which may suggest that the cytokinesis defect is a specific consequence of inhibiting another Nek9 substrate, whilst Nek6 and Nek7 play a more direct role in metaphase progression. Nevertheless the fact that RNAi depletion of both Nek6 and Nek7 resulted in the same apoptosis phenotype suggests that loss of Nek6 is not complemented by the presence of Nek7 and vice versa. This would seem to suggest a lack of redundancy between Nek6 and Nek7.

Chapter 6

Nek6 and Nek7 are required for Mitotic Spindle Integrity

6.1 Introduction

Functional studies on Nek9 have shown that it plays a major role in the organization of the mitotic spindle (Roig *et al.*, 2002; Roig *et al.*, 2005) and that this role may, at least in part, be mediated through its interaction with Nek6 and Nek7 (Belham *et al.*, 2003). In the previous chapter, we described the effect of interference with Nek6 and Nek7 function by expression of kinase-deficient mutants and RNAi depletion of proteins. Such analyses demonstrated that, consistent with reports by Yin *et al.* (2003) and Kim *et al.* (2007), Nek6 and Nek7 have functions in regulation of the metaphase to anaphase transition and possibly also cytokinesis.

The segregation of duplicated DNA to daughter cells at mitosis is a complex biomechanical process achieved in large part through the action of the bipolar microtubule-based scaffold known as the mitotic spindle (McIntosh *et al.*, 2002; Walczak and Heald, 2008). This structure is nucleated from opposite poles of the cell at the onset of mitosis by separated and duplicated centrosomes. These then act to tether microtubules at their minus ends whilst the plus ends radiate out towards the centre of the cell, eventually capturing sister chromatids through their kinetochores, resulting in their alignment on the metaphase plate. Once all sister chromatid pairs are correctly bioriented on the spindle at the metaphase plate, sister chromatids are separated to opposite poles of the cell as a result of the depolymerisation of spindle microtubules, the action of microtubule-based motor proteins, the further separation of spindle poles and the rapid loss of sister chromatid cohesion. As cells enter cytokinesis, microtubules become important in the midbody, acting to keep segregated genomes apart and playing an integral role in signaling cytokinesis. In this context, it is important that these microtubules remain stable and the proteins of the midbody matrix are thought to include microtubule stabilizing elements that impart this stability to overlapping plus ends (Mullins and McIntosh, 1982; O'Connell and Khojakov, 2007).

The nucleation, organization and stabilization of the mitotic spindle is regulated largely by post-translational modifications of these microtubule-, as well as centrosome-associated proteins (Rapley *et al.*, 2005; Sharp *et al.*, 2000; Karsenti and Vernos 2001; Scholey *et al.*, 2003). Thus, for example, many mitotic kinases, including Aurora A and Plk1, are known to localize to the centrosome and be required for correct mitotic spindle assembly. Similarly, the CPC is required to stabilize chromatin-nucleated microtubules (Sampath *et al.*, 2004), Aurora B kinase activity also controls the centromeric localization and catalytic activity of the microtubule depolymerase, MCAK (Andrews *et al.*, 2004; Lan *et al.*, 2004). Meanwhile, Nek9 activity has been shown to be important for both centrosomal and chromatin-mediated spindle assembly, the former may be due

to a direct interaction with the γ-TuRC and the latter may reflect its ability to phosphorylate TPX2 (Roig *et al.*, 2005). Indeed post-translational modification of microtubule-based motor proteins is emerging as a key regulator of spindle microtubule stability (Gadde and Heald, 2004). The role of the centrosome as a docking site for mitotic kinases means that beyond the canonical role of the MTOC in microtubule assembly, it plays key roles in the regulation of signaling and cell cycle control (Cuschieri *et al.*, 2007).

Having shown that interference with the function of the mitotically regulated Nek6 and Nek7 protein kinases results in metaphase and cytokinesis delays, we next wished to examine the functional basis for these defects. Nek9 was identified as a protein kinase capable of interacting with Nek6 and phosphorylating both Nek6 and Nek7 (Belham *et al.*, 2003). It is believed to have a key role in mitotic spindle nucleation both through its interaction with the γ-TuRC as well as through the RCC1-Ran-GTP chromatin-mediated spindle nucleation pathway. Active Nek9 concentrates at spindle poles in mitosis. Furthermore, the *Xenopus* Nek9 orthologue coprecipitates with components of the γ-TuRC and localizes to the poles of spindles formed *in vitro*. Immunodepletion of the protein from egg extracts results in formation of aberrant spindles as well as slowing aster assembly induced by Ran-GTP (Roig *et al.*, 2005). Thus, it seems likely that the mitotic delay observed upon interference with Nek6 or Nek7 function may, at least in part, be as a result of interference with the downstream effectors of Nek9 function. Although it should be noted that microinjection of anti-Nek9 antibodies into the Ptk2 rat kangaroo kidney epithelial cell line, typically resulted in a variety of mitotic defects, including a significant arrest in prometaphase with condensed DNA and separated centrosomes but an interphase array of microtubules. This defect was also the predominant phenotype upon microinjection of anti-Nek9 antibodies into CF-PAC1 cells, a human cell line that contains levels of Nek9 comparable to HeLa and HEK 293 cells (Roig et al., 2002). This is in obvious contrast to the metaphase and cytokinesis delays seen upon interference of Nek6 and Nek7 function. Also of note is the report that RNAi depletion of Nek9 does not result in a significant phenotype (Roig *et al.*, 2005; Pelka *et al.*, 2007). However, this may be a result of incomplete knockdown resulting in sufficient remaining enzyme to sustain physiological Nek9 function (Roig *et al.*, 2005), although depletion did appear to induce significant apoptosis (Pelka *et al.*, 2007).

This chapter describes detailed analyses of the phenotypes displayed upon interference with Nek6 and Nek7 function, in particular in relation to the metaphase delay.

6.2 Results

6.2.1 Expression of catalytically-inactive Nek6 and Nek7 disturbs mitotic spindle integrity

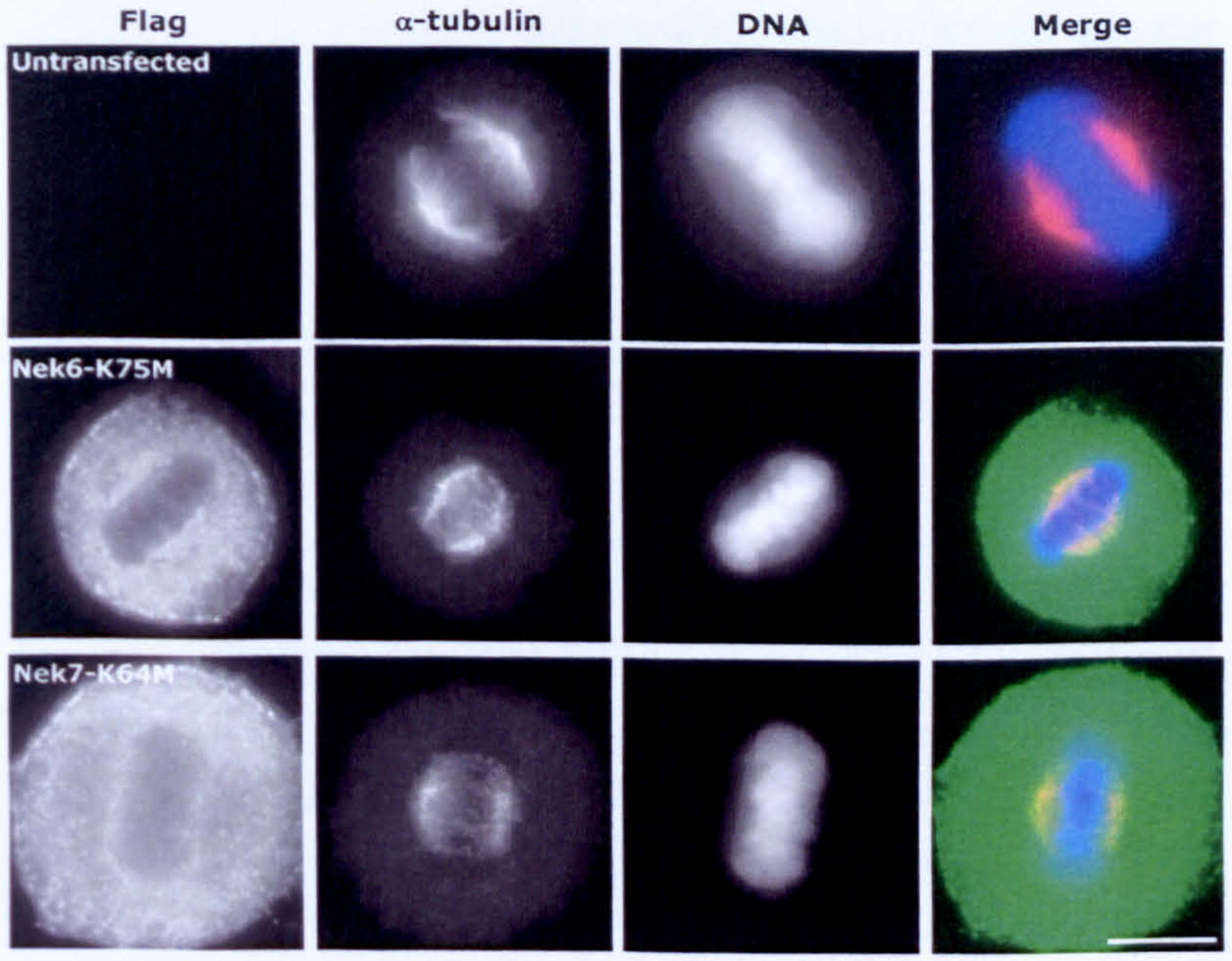
Expression of Nek6 and Nek7 mutants with no catalytic activity results in a delay in progression through metaphase, typically resulting in apoptosis (this study and Yin *et al.*, 2003). To investigate the cause of this arrest, quantitative immunofluorescence microscopy was used to examine the mitotic spindle in cells expressing WT and kinase-dead Nek6 and Nek7 constructs. Observation of the mitotic spindle structures in these cells demonstrated that whilst control cells exhibited robust spindles with a strong α-tubulin signal, cells expressing catalytically-inactive Nek6-K75M or Nek7-K64M exhibited smaller, apparently more fragile spindles with less distinct spindle microtubules and less well focused spindle poles (Figure 6.1A). Quantitative analysis of α-tubulin staining in immunofluorescence images all taken under the same conditions and processed in the same fashion confirmed that there was a clear reduction in overall spindle microtubule intensity (Figure 6.1B). This analysis showed that whilst Nek6- or Nek7-WT transfected cells had an overall spindle intensity comparable to the control cells, cells transfected with the inactive Nek6-K75M or Nek7-K64M mutants had an overall spindle intensity of only around 35-45% compared to the untreated cells. Those cells transfected with the Nek6-K74M and Nek7-K63M mutants that retain almost full activity showed only a slight reduction in spindle intensity at around 75-85%. Thus, this effect is likely to be a function of the loss of kinase activity of the Nek6 and Nek7 proteins.

We conclude that interference with Nek6 and Nek7 function by expression of kinase-deficient mutants results in the formation of smaller, less robust mitotic spindles.

6.2.2 RNAi depletion of Nek6 and Nek7 disturbs mitotic spindle integrity

Metaphase spindle microtubule intensity was next examined in cells depleted of Nek6 and Nek7 using quantitative immunofluorescence microscopy. Examination of the α-tubulin stain revealed that, as for expression of catalytically-inactive Nek6 and Nek7, cells depleted of Nek6 and Nek7 exhibit metaphase spindles which appeared fragile, with less distinct microtubules and less focused spindle poles compared to mock-depleted cells (Figure 6.2A). Quantitative analysis of the intensity of the α-tubulin stain demonstrated that the overall metaphase spindle microtubule intensity was reduced by approximately 50-60% in cells depleted of Nek6 or Nek7 compared to mock-depleted or GAPDH-depleted cells (Figure 6.2B).

A



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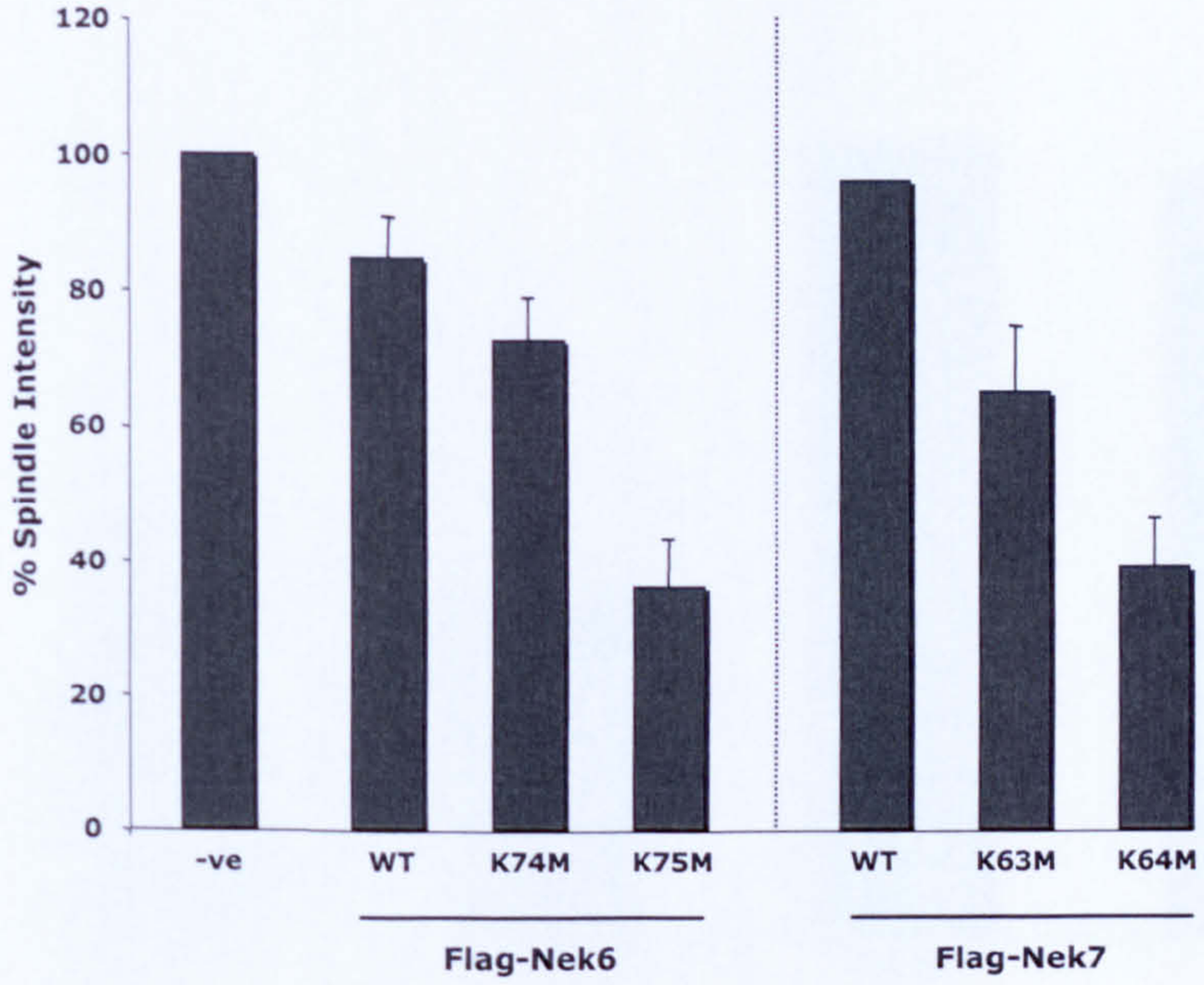
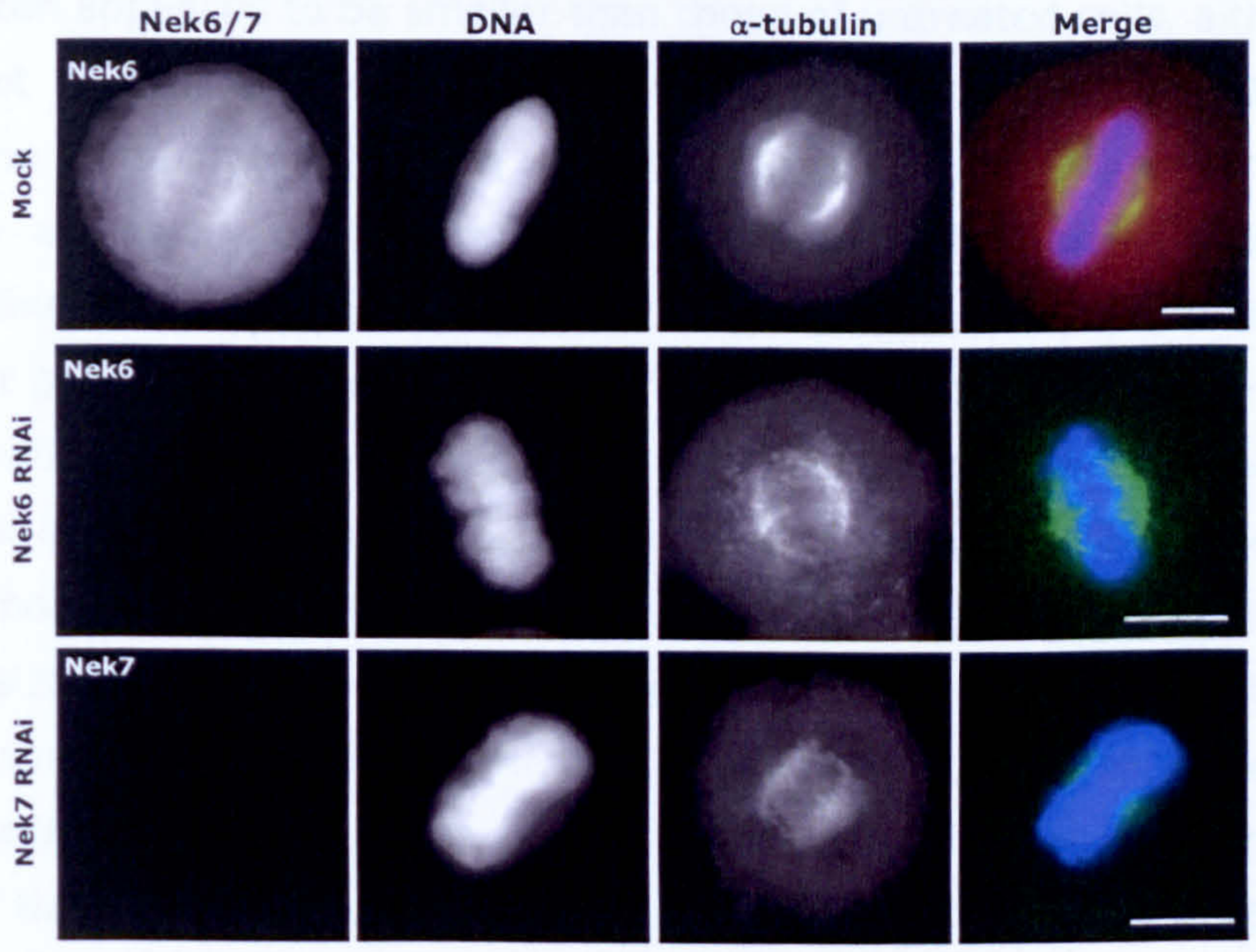


Figure 6.1 Expression of catalytically-inactive Nek6 and Nek7 disturbs mitotic spindle integrity

(**A**) HeLa cells were transiently transfected with Flag-Nek6 and Flag-Nek7 constructs, as indicated, for 24 h before being fixed and permeabilised with methanol and processed for immunofluorescence microscopy with anti-Flag antibodies (green) to detect transfected cells and anti-α-tubulin antibodies to detect spindle microtubules (red). DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm. (**B**) The intensity of the metaphase spindle in transfected cells is expressed relative to the intensity of the spindle in untransfected cells (-ve). Data represent means (± S.D.) of measurements from 50 cells for each construct.

A



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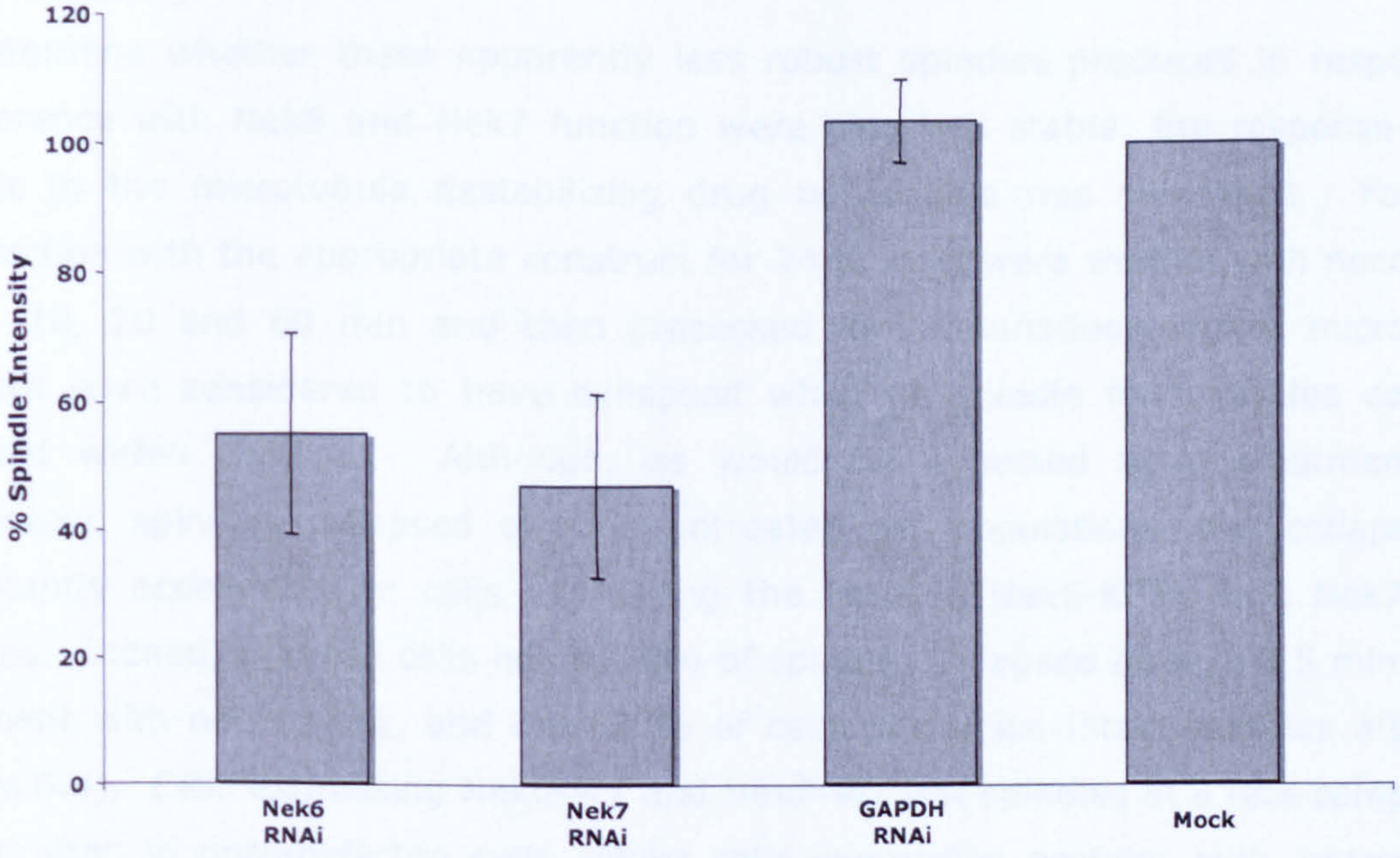


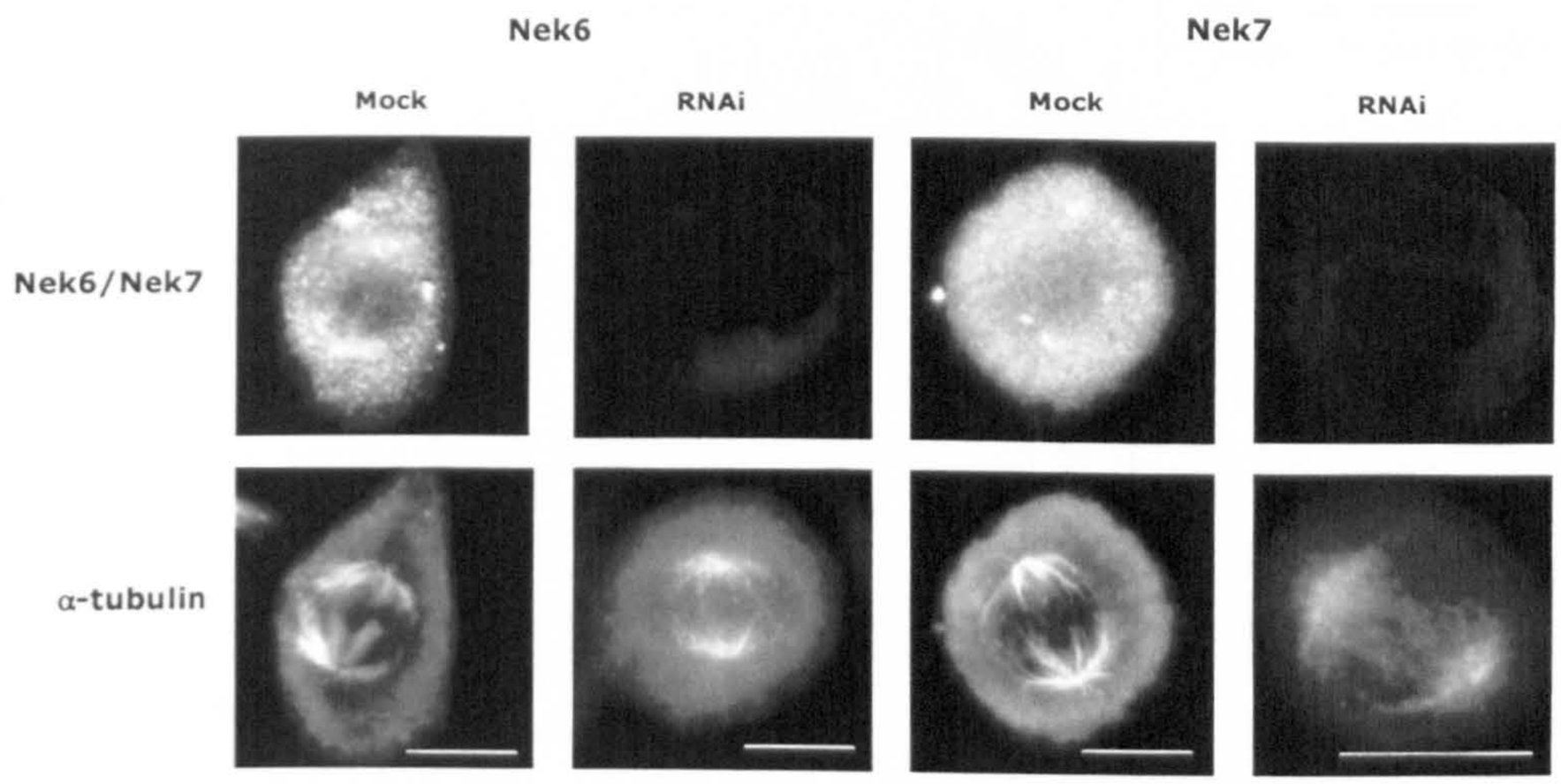
Figure 6.2 RNAi depletion of Nek6 and Nek7 disturbs mitotic spindle integrity
(**A**) HeLa cells were transfected with Nek6 or Nek7 siRNA oligos or were mock-transfected for 72 h before being fixed and permeabilised with methanol and processed for immunofluorescence microscopy with anti-Nek6 or -Nek7 antibodies (red) and anti-α-tubulin antibodies to detect spindle microtubules (green). DNA was stained with Hoechst 33258 (blue). Scale bar, 10 μm. (**B**) The intensity of the metaphase spindle in depleted cells is expressed relative to the intensity of the spindle in mock-depleted cells. Data represent means (± S.D.) of measurements from 50 cells .

Close examination of the metaphase spindles of RNAi depleted cells suggested that the spindles often appeared to be smaller than those of untreated cells, although overall cell size did not appear significantly different (Figures 6.1A, 6.2A & 6.3A). In order to quantify the comparative spindle lengths, further quantitative immunofluorescence microscopy analysis was undertaken in cells depleted of Nek6 and Nek7. In this analysis a line was drawn across the middle of the cell, from membrane to membrane, such that it passed through the centre of each spindle pole and thus the centre of the mitotic spindle. Pixel intensity was measured at regular intervals along this line such that a pixel intensity profile could be produced charting the pixel intensity across the cell, with characteristic peaks at the spindle poles. Analysis of this data showed that, whilst cell size did not change significantly, the distance between the two spindle poles was significantly shorter in Nek6 and Nek7 RNAi depleted cells as compared to GAPDH-depleted cells. Moreover, these peaks in intensity were significantly lower in cells depleted of Nek6 and Nek7 compared to GAPDH-depleted cells (Figure 6.3B).

6.2.3 Interference with Nek6 and Nek7 function leads to reduced spindle stability

To determine whether these apparently less robust spindles produced in response to interference with Nek6 and Nek7 function were also less stable, the response of the spindle to the microtubule destabilizing drug nocodazole was measured. Following transfection with the appropriate construct for 24 h, cells were treated with nocodazole for 5, 10, 20 and 60 min and then processed for immunofluorescence microscopy. Spindles were considered to have collapsed when no spindle microtubules could be detected within the cell. Although, as would be expected upon treatment with nocodazole, spindles collapsed even in untreated cell populations, this collapse was significantly accelerated in cells expressing the inactive Nek6-K75M and Nek7-K64M proteins. Indeed, in these cells nearly 50% of spindles collapsed after just 5 minutes of treatment with nocodazole, and only 20% of cells possessed intact spindles after 1 h (Figure 6.4). Cells expressing Nek6-WT and Nek7-WT lost spindles at a rate comparable to that seen in untransfected cells, whilst cells expressing proteins with intermediate levels of activity (Nek6-K74M and Nek7-K63M) lost spindles at an intermediate rate. Thus, the metaphase delay associated with expression of catalytically-inactive Nek6 and Nek7 appears to result from interference with mitotic spindle stability.

A



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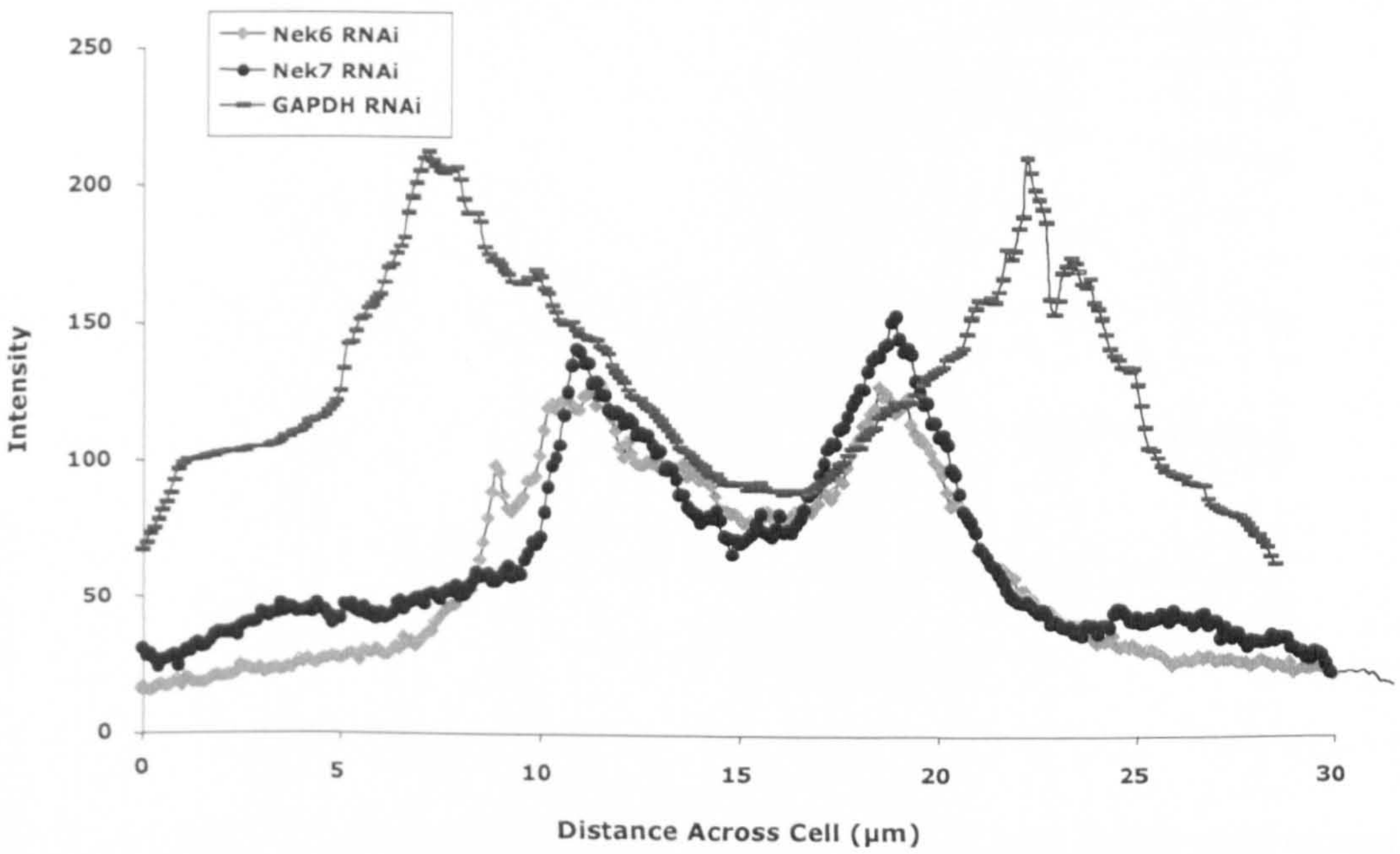

Figure 6.3 Nek6 and Nek7 depleted cells exhibit shorter mitotic spindles
(**A**) HeLa cells were either mock-transfected or transfected with Nek6 or Nek7 siRNA oligos for 72 h before being fixed and permeabilised with ice-cold methanol and processed for immunofluorescence microscopy with anti-Nek6 or -Nek7 antibodies and anti-α-tubulin antibodies. Scale bars, 10 μm. (**B**) The intensity and length of the metaphase spindle in Nek6, Nek7 and GAPDH-depleted cells was measured using ImageJ software. Data represent means of measurements of at least 50 cells for each condition.

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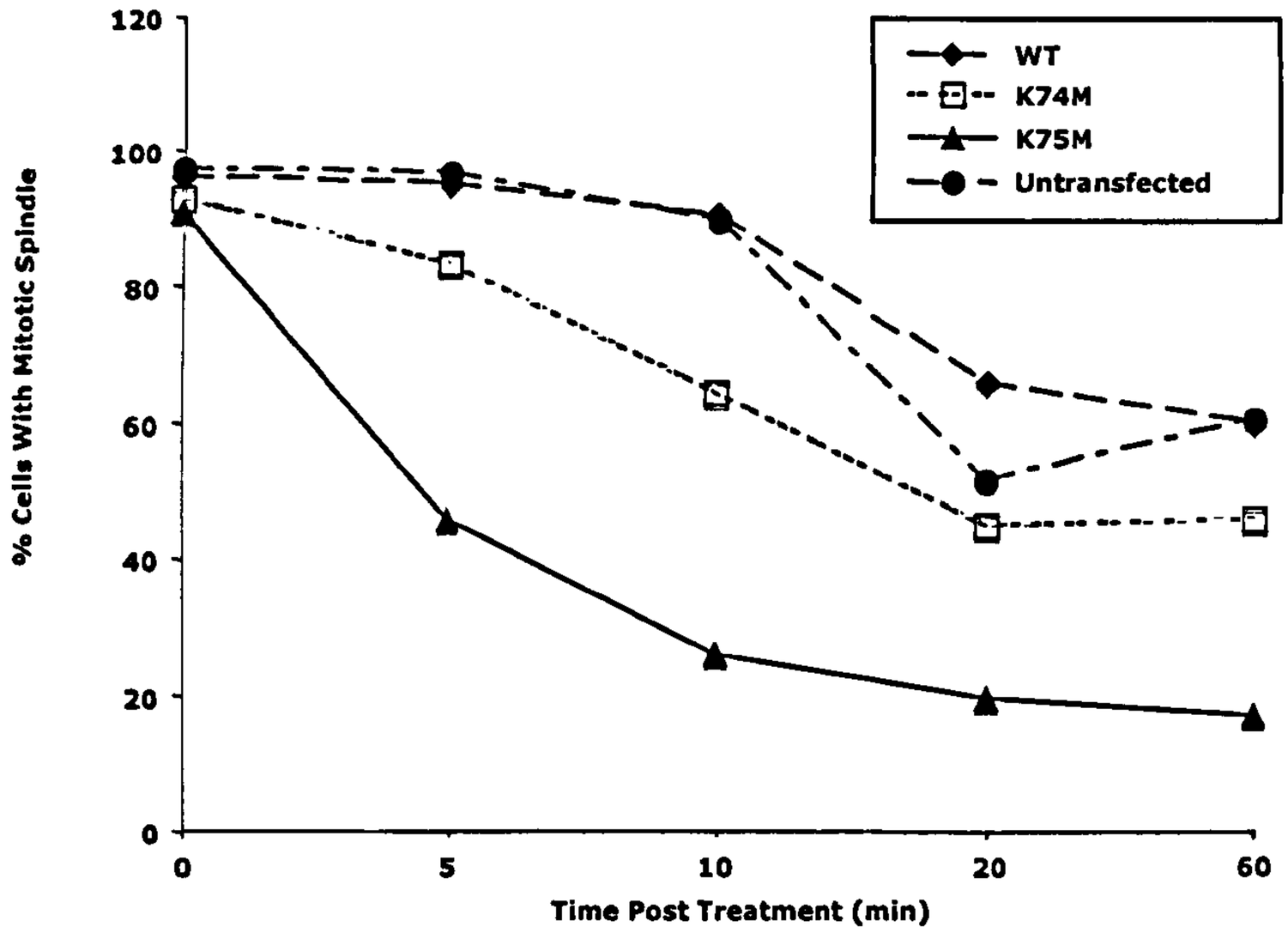

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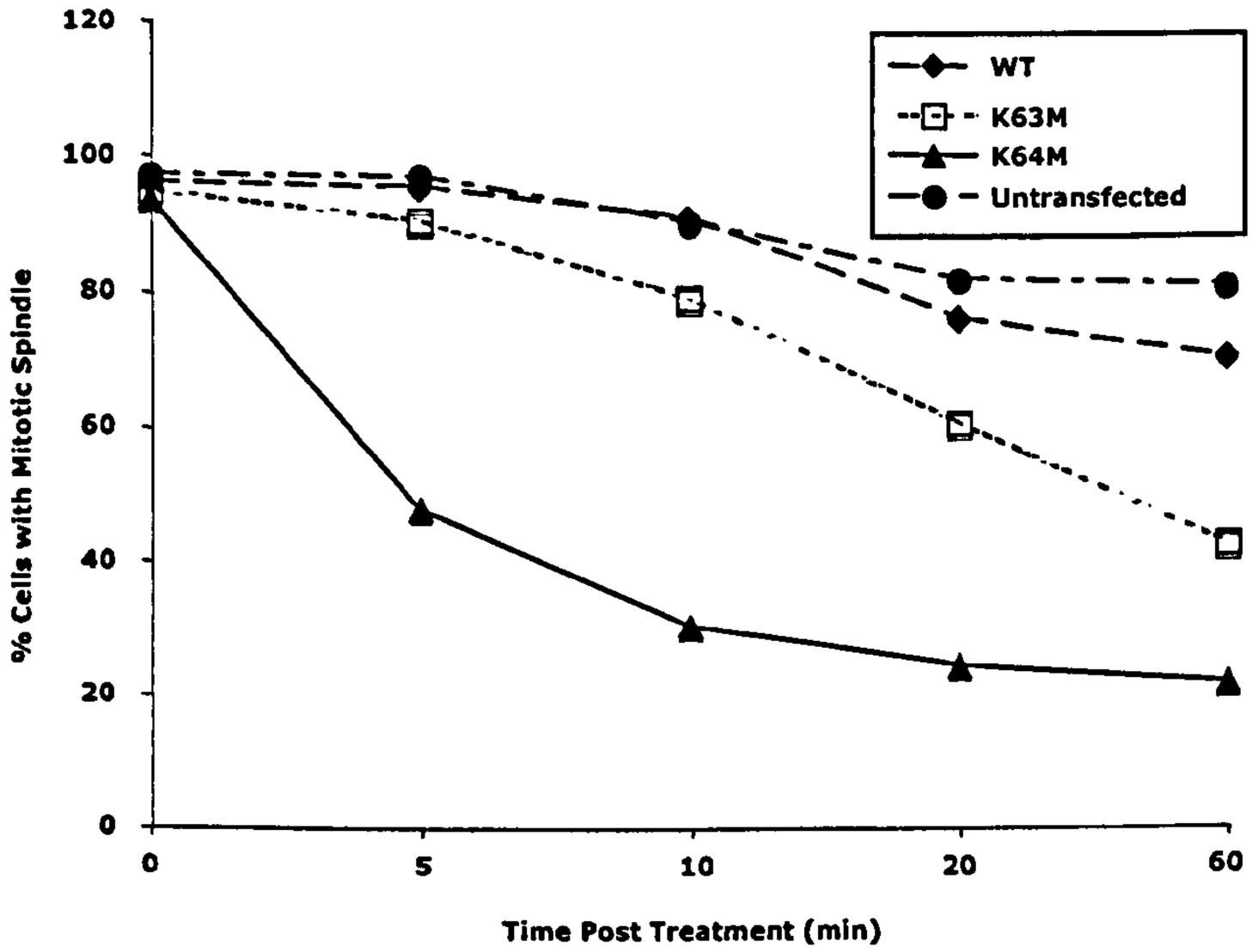


Figure 6.4 Expression of catalytically-inactive Nek6 and Nek7 decreases mitotic spindle stability

HeLa cells were transiently transfected with Flag-Nek6 (**A**) or Flag-Nek7 (**B**) constructs for 24 h. Cells were then treated with nocodazole before being methanol fixed at the time points indicated. Fixed cells were then processed for immunofluorescence microscopy with anti-FLAG antibodies to detect transfected cells and anti-α-tubulin antibodies to detect the microtubule network. Transfected metaphase cells were scored for the integrity of their mitotic spindles. Cells were scored as no longer having a mitotic spindle when no spindle microtubules could be seen radiating across the cell. Data represent means of counts of at least 30 cells for each condition in three separate experiments.

6.2.4 Interference with Nek6 and Nek7 function affects γ-tubulin recruitment to the spindle poles

Formation of the mitotic spindle occurs predominantly as a result of the nucleating activity of the γ-TuRC at the centrosome (Doxsey, 2001; Francis and Davis 2000; Hagan and Petersen 2000). It has been reported that recruitment of γ-tubulin to the centrosome is impaired in cells in which Nek7 was depleted (Kim *et al.*, 2007), although there are no reports to date concerning the effect of interference with Nek6 function on γ-tubulin recruitment. We thus wished to determine whether the fragile spindle phenotypes produced as a result of interfering with Nek6 and Nek7 function could arise in part from an effect on γ-tubulin recruitment. To this end, quantitative immunofluorescence imaging was used to analyze centrosomal γ-tubulin levels in untransfected cells compared to cells expressing the Nek6 and Nek7 wild-type and mutant proteins. Examination of the γ-tubulin stain revealed that, in untransfected and Nek6- and Nek7-WT transfected cells, γ-tubulin was predominantly centrosomal, with little cytoplasmic staining. In contrast, in cells expressing catalytically-inactive versions of Nek6 and Nek7, the cytoplasmic signal became much stronger and the centrosomal signal more diffuse (Figure 6.5A). Quantitative analysis revealed that whilst the γ-tubulin intensity of Nek6- or Nek7-WT expressing cells was comparable to that of untransfected cells, in cells expressing the inactive Nek6-K75M or Nek7-K64M mutants the intensity was reduced to 35-45% of that seen in control cells (Figure 6.5B). Again, those cells expressing mutants of Nek6 and Nek7 with intermediate levels of activity displayed intermediate levels of reduced γ-tubulin intensity.

This quantitative immunofluorescence imaging of centrosomal γ-tubulin was repeated in cells in which endogenous Nek6 or Nek7 had been depleted by RNAi. As for expression of catalytically-inactive Nek6 and Nek7, γ-tubulin staining in RNAi depleted Nek6 and Nek7 cells gave a stronger cytoplasmic stain and a less intense, less strongly focused centrosomal signal compared to mock- or GAPDH-depleted cells (Figure 6.6A). Quantitative analysis of the centrosomal γ-tubulin signal revealed a 60-70% decrease in intensity in Nek6 and Nek7 depleted cells compared to mock- or GAPDH-depleted cells (Figure 6.6B).

Thus the fragility of mitotic spindles that arises from interference with Nek6 and Nek7 function may in part be mediated by a reduction in γ-tubulin recruitment and therefore microtubule nucleation from the spindle poles.

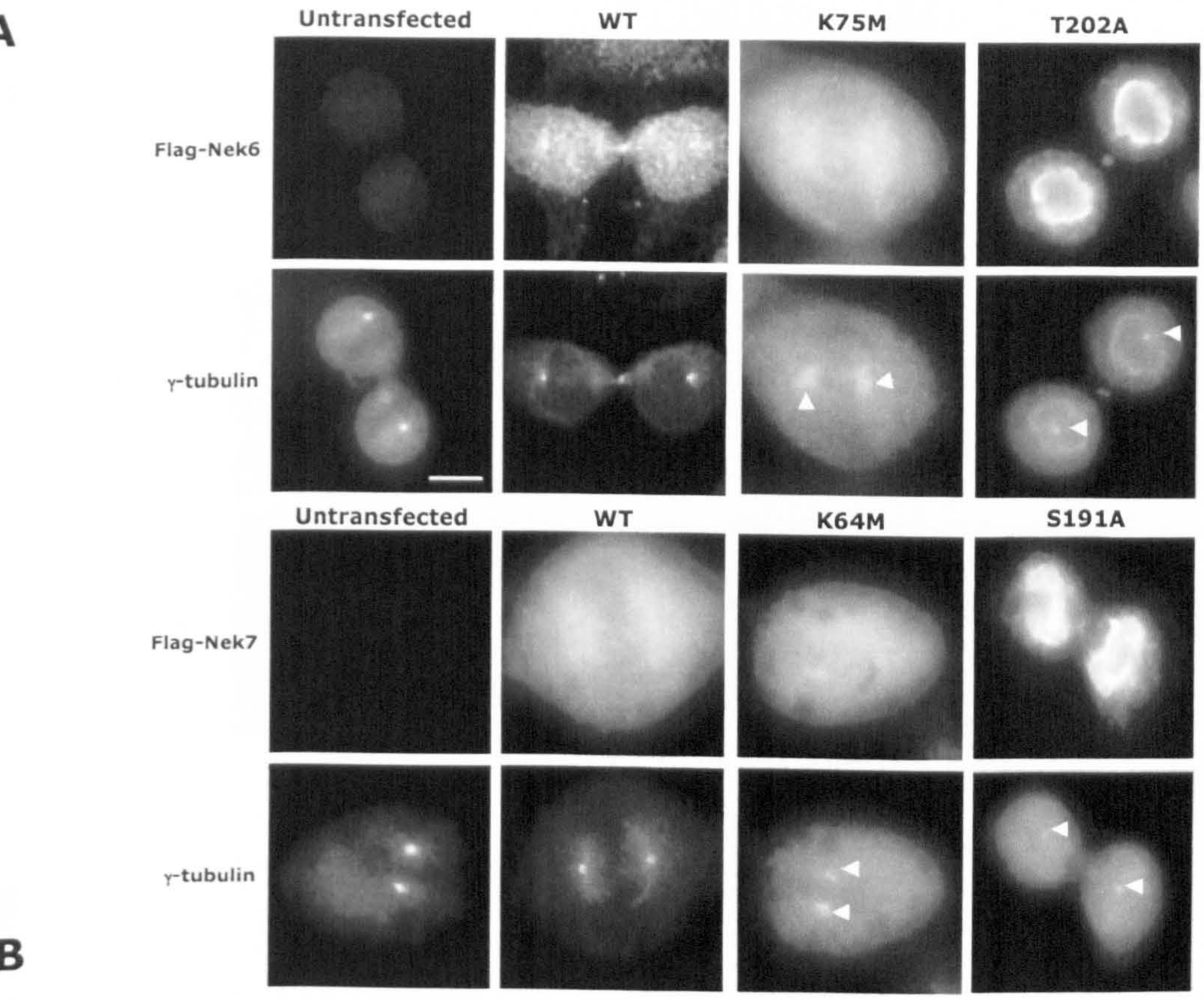


Figure 6.5 Expression of catalytically-inactive Nek6 and Nek7 interferes with centrosomal γ-tubulin recruitment

(**A**) HeLa cells were either untransfected or transiently transfected with Flag-Nek6 and Flag-Nek7 constructs, as indicated, for 24 h before being fixed and processed for immunofluorescence microscopy with anti-Flag antibodies and anti-γ-tubulin antibodies. Arrowheads, indicate centrosome position; scale bar, 10 μm. (**B**) The abundance of γ-tubulin at the centrosome was measured using quantitative fluorescence imaging. Relative abundance is expressed as a percentage of γ-tubulin intensity in untransfected (-ve) cells. Data represents means (±S.D.) of measurements of at least 50 cells in 3 separate experiments.

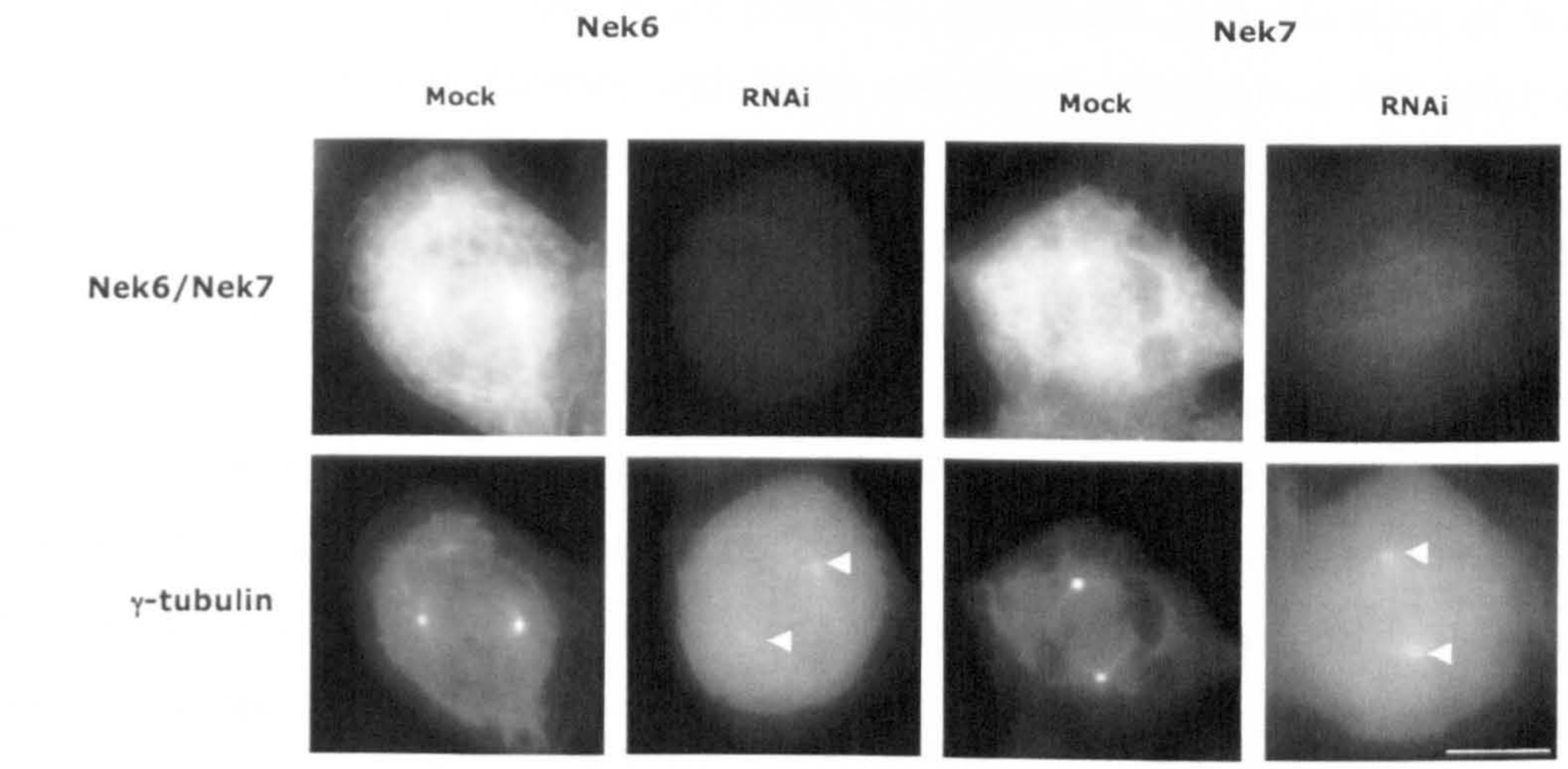


Figure 6.6 RNAi depletion of Nek6 and Nek7 interferes with centrosomal γ-tubulin recruitment

(**A**) HeLa cells were either mock-transfected or transfected with Nek6 or Nek7 RNAi oligos, as indicated, for 72 h before being fixed and processed for immunofluorescence microscopy with anti-Nek6 or -Nek7 antibodies and anti-γ-tubulin antibodies. Arrowheads indicate centrosome position; scale bar, 10μm. (**B**) The abundance of γ-tubulin at the centrosome was measured in cells depleted of Nek6, Nek7 or GAPDH using quantitative fluorescence imaging. Relative abundance is expressed as a percentage of γ -tubulin intensity in mock depleted cells (Mock). Data represent means (±S.D.) of measurements from 50 cells in 3 separate experiments.

6.2.5 Nek6 and Nek7 coprecipitate with γ-tubulin

It has been shown that active Nek9 localizes to the centrosome and that Nek9 coprecipitates not only with Nek6 but also with γ-tubulin and components of the γ-TuRC (Roig *et al.*, 2005). In view of the potential centrosomal localization of Nek7, along with the evidence that interference with Nek6 and Nek7 results in a reduction in centrosomal γ-tubulin, an obvious question is whether Nek6 and Nek7 also interact with γ-tubulin. To this end we assessed whether γ-tubulin coprecipitates with either Nek6 or Nek7. Extracts from either untransfected HEK 293 cells or cells transfected with Flag-Nek6-WT and –K75M or Flag-Nek7-WT and –K64M, synchronized in either G1/S or M phase of the cell cycle, were immunoprecipitated with anti-Flag antibodies and Western blotted with anti-γ-tubulin antibodies (Figure 6.7). Synchronized cell extracts were used to preclude the possibility of cell cycle stage specific binding of Nek6 or Nek7 to γ-tubulin masking an interaction. This analysis demonstrated that γ-tubulin coprecipitates specifically with both Nek6 and Nek7 and that this interaction does not appear to be cell cycle stage dependent as γ-tubulin was detectable in the immunoprecipitates in both G1/S and M lysates. Likewise, γ-tubulin coprecipitated with both active and catalytically-inactive Nek6 and Nek7. Interestingly, Nek6 and Nek7 do not appear to co-precipitate, although comparatively higher levels of recombinant proteins relative to endogenous levels of Nek6 and Nek7 may have masked such an interaction.

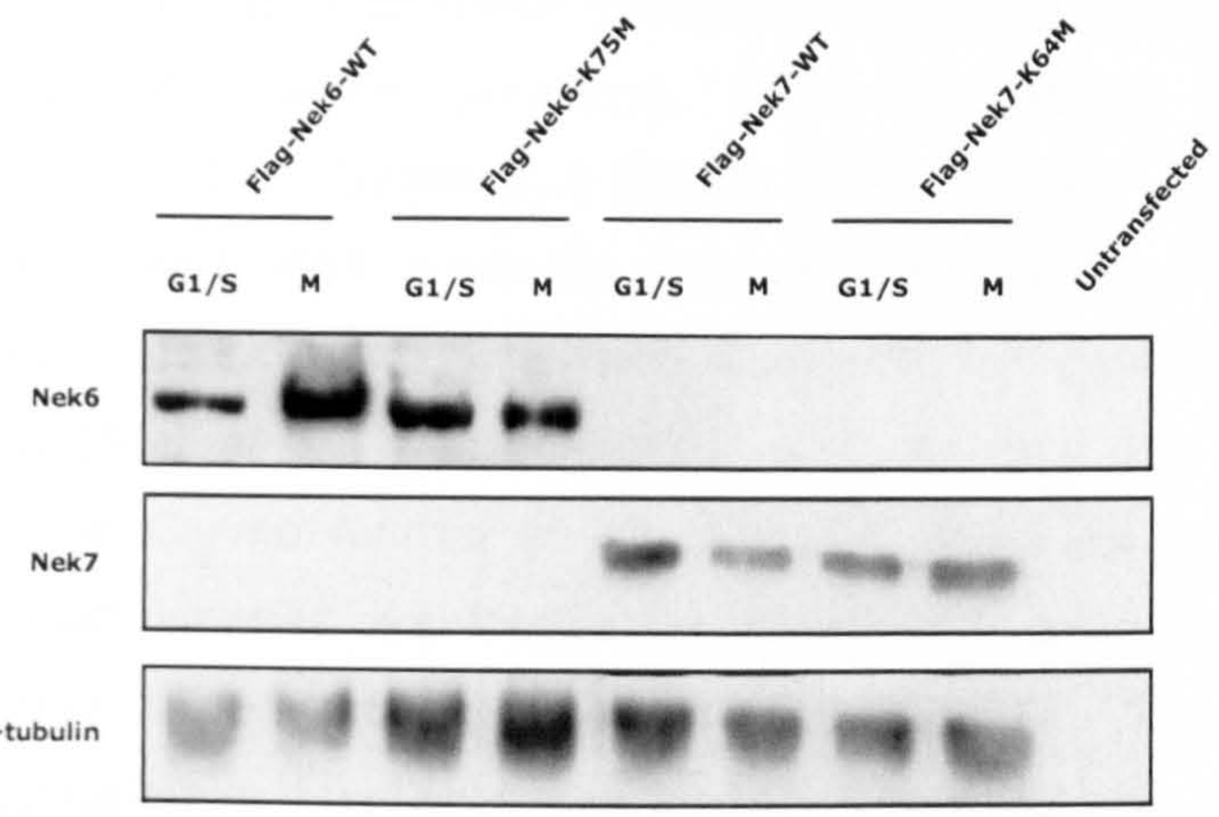


Figure 6.7 Nek6 and Nek7 interact with γ-tubulin

HEK 293 cells were transiently transfected with Flag-Nek6 and Flag-Nek7 constructs, as indicated. After 24 h cells were synchronised at G1/S and M-phase of the cell cycle before cells were lysed and recombinant proteins immunoprecipitated using anti-FLAG antibodies. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with anti-Nek6, anti-Nek7 and anti-γ-tubulin antibodies, as indicated.

6.3 Discussion

Progression through mitosis requires the formation of a robust mitotic spindle. This can be considered to be fully formed at the completion of metaphase when sister chromatids are bioriented on spindle microtubules. These microtubules are nucleated predominantly from the MTOC but also via a chromatin-induced pathway (O'Connell and Khodjakov, 2007). The striking structural rearrangement of microtubules which occurs upon entry into, progression through and exit from, mitosis is underpinned by a complex regulatory network of kinases and phosphatases. Indeed, regulation of the nucleation, organization and maintenance of the mitotic spindle is achieved in large part through phosphorylation and dephosphorylation of mitotic spindle and centrosome-associated proteins and many of these phosphorylations are carried out by centrosome and spindle-associated kinases, including, Cyclin-dependent, Aurora- and Polo-like kinases (Rapley *et al.*, 2005; Gadde and Heald, 2004; Scholey *et al.*, 2003). Hence, for example, Aurora A and Plk1 promote the recruitment and/or activation of PCM components (such as TPX2 and γ-tubulin) required for centrosome maturation and spindle nucleation (Giet *et al.*, 2002; Heald, 2000; Berdnik and Knoblich, 2002; Lane and Nigg, 1996; do Carmo-Avides *et al.*, 2001). Although less well characterized, mitotic NIMA-related kinases are beginning to emerge as important mitotic spindle regulators (O'Connell *et al.*, 2003; O'Regan *et al.*, 2007). The data presented here suggests that the delay in metaphase progression that results from interference with Nek6 and Nek7 function may be due to decreased stability of the mitotic spindle.

Microtubules are highly dynamic structures, not least as a result of their inherent instability. However, this instability must be suppressed in the microtubules of the mitotic spindle and this is thought to be achieved, at least in part by the phosphorylation and dephosphorylation of MAPs (Gassmann *et al.*, 2004) as well as possibly by direct post-translational modification of tubulin subunits (Verhet and Gaertlig, 2007). We found that expression of inactive Nek6 and Nek7 or RNAi depletion of Nek6 and Nek7 results in fragile mitotic spindles. This is similar to results with Borealin, a member of the CPC, whose depletion by RNAi results in cells which progressed through the early stages of mitosis normally, ultimately forming an apparently normal metaphase plate, however the spindle then began to lose its integrity resulting in aberrant mitotic exit (Gassmann *et al.*, 2004). This phenotype is believed to be related to the function of Borealin in the CPC in delivering Aurora B to the sites of MAPs, where it may regulate their activity by phosphorylation (Lan *et al.*, 2004).

RNAi depletion of Nek6 and Nek7 or expression of catalytically-inactive mutants also resulted in reduced levels of γ-tubulin at the centrosome. Thus, the reduced integrity of

the mitotic spindle may have arisen, at least in part, as a result of reduced microtubule nucleation capacity at the centrosomes. In view of the proposed role of Nek9 in nucleation of the mitotic spindle (Roig *et al.*, 2005), this would fit well with the proposed model of a mitotic kinase cascade involving phosphorylation and activation of Nek6 and Nek7 by Nek9 (Belham *et al.*, 2003).

However, although Nek7 may be localized to the centrosome, Nek6 appears to be predominantly localized to the mitotic spindle itself rather than the spindle poles. Nonetheless, co-immunoprecipitation experiments suggest that both Nek6 and Nek7 specifically interact with γ-tubulin at all stages of the cell cycle. It has been reported that γ-tubulin localizes not only to spindle poles but also to the mitotic spindle microtubules themselves (Lajoie-Mazenc *et al.*, 1994; Luders *et al.*, 2006) and that this localization may underlie another non-centrosomal pathway for spindle microtubule generation (Mahoney *et al.*, 2006). In this pathway, a protein complex called Augmin is believed to be required to recruit γ-tubulin to the mitotic spindle, where it may function to increase spindle microtubule density and facilitate chromosome capture (Goshima *et al.*, 2008; Mahoney *et al.*, 2006). Thus, the fragile spindle phenotype seen upon interference with Nek6 function may reflect a failure in this pathway and thus a role for Nek6 in promoting the interaction of γ-tubulin with spindle microtubules. The function of this pathway appears, in part, to be to promote the formation of K-fibers, that is to say the capture of kinetochores by spindle microtubules. Thus, a failure in this pathway may result in a longer period in prometaphase. Interestingly, it has been reported that interference with Nek7 and Nek9 function results in prometaphase delay in certain conditions (Roig *et al.*, 2003; Yissachar *et al.*, 2007). However, whether this is as a result of failed microtubule nucleation from centrosomes or the spindle, or a combination of both, remains to be determined. More detailed time-lapse imaging of HeLa cell lines stably expressing GFP-Nek7 variants compared to Nek6 variants may help to elucidate this function, as would photobleaching experiments using GFP-tubulin cell lines depleted of Nek6 and Nek7. It will also be important to determine whether Nek6 and Nek7 interact with other components of the γ-TuRC or Augmin complex.

We have shown that expression of hypomorphic mutants of Nek6 and Nek7 results in a delay in cytokinesis, which may reflect a role for these proteins in regulation of mitotic exit. As cells progress through anaphase and into telophase and cytokinesis, interpolar microtubules in the spindle midzone bundle into the central spindle (Glotzer, 2005) eventually becoming highly compacted into the midbody (Mullins and Biesele, 1977). Both recombinant and endogenous Nek6 localize to the spindle midzone and the midbody of late mitotic cells. In view of the proposed role of Nek6 in maintaining

mitotic spindle integrity in early mitosis, perhaps this represents a conserved role for Nek6 in regulating spindle microtubule stability at different stages of mitosis. Several mitotic kinases have been shown to redistribute to the central spindle during anaphase and then become localized to the midbody of cytokinetic cells including, Plk1 and Aurora B. Disruption of either of these proteins results in lack of proper midbody assembly and cytokinesis failure (Brennan *et al.*, 2007; Petroczki *et al.*, 2007; Santamaria *et al.*, 2007; Schumach *et al.*, 1998; Giet and Glover, 2001). Pertinently, the cytoskeletal protein, Tektin 2, which localizes to centrosomes and is proposed to be a component of the mitotic spindle matrix, has recently been shown to be required for correct central spindle organization during anaphase as well as proper localization of MKLP1, PRC1 and Aurora B to the midzone and formation of the midbody matrix (Durcan *et al.*, 2008).

The data presented in this chapter indicate that both Nek6 and Nek7 are required for regulation of mitotic spindle stability. This may result from roles in a variety of mechanisms including recruitment of γ-tubulin to the centrosome and thus centrosome maturation and centrosome-directed microtubule nucleation. Alternatively, it could indicate a role in recruitment of γ-tubulin to the mitotic spindle, regulating a spindle-mediated pathway of microtubule nucleation and amplification. Finally, it could suggest a role in regulation of proteins that directly control spindle microtubule stability. However, the relative contributions that Nek6 and Nek7 make to any of these pathways remains to be determined and it is possible that differential contributions to several different pathways may provide the basis for differences between these two highly similar proteins.

Chapter 7

Identification of Substrates of Nek6 and Nek7

7.1 Introduction

Functional characterization of the Nek6 and Nek7 serine/threonine protein kinases remains in its infancy. Examining the effects of interfering with their function can tell us a great deal about the physiological role of these kinases but key to determining how they execute these roles is identification of their downstream targets.

Post-translational modification of proteins is one of the principle regulatory mechanisms in eukaryotes. In particular protein phosphorylation has been demonstrated to be crucial to the proper regulation of nearly all cellular processes, including mitosis (Meng *et al.*, 2008). Protein kinases represent around 2% of the 30,000 proteins encoded by the human genome and approximately one-third of mammalian proteins contain covalently bound phosphate. Moreover, many proteins are phosphorylated by more than one protein kinase. Thus an average protein kinase can be considered to phosphorylate at least 20 *in vivo* substrates (Knebel *et al.*, 2001). Thus, the identification of the major physiological substrates of even a single kinase represents a significant challenge (Knebel *et al.*, 2001; Meng *et al.*, 2008). Perhaps most fundamentally, progress in tracing signaling pathways has been severely limited due to a lack of straightforward methods for kinase substrate identification (Cohen, 2002; Zhang *et al.*, 2002).

Nevertheless, a number of methodologies have been developed in the post-genomic era in order to facilitate the identification of kinase substrates. These include the screening of peptide libraries for identification of phosphorylation consensus sequences which can then be used to screen protein sequence databases (Obata *et al.*, 2000; Hutti *et al.*, 2004; Lizcano *et al.*, 2002), yeast two hybrid screens, use of phosphospecific antibodies to identify the phosphorylated substrates of the kinase of interest (Zhang *et al.*, 2002), and the use of synthetic ATP analogues only exploitable by the kinase of interest as a result of modification of its ATP pocket (Malay *et al.*, 2004; Shah and Shokat, 2003). However, all of these methodologies first require detailed characterization of the kinase of interest prior to substrate identification (Troiani *et al.*, 2005).

Another such methodology, Kinase Substrate Tracking and Elucidation (KESTREL) (Knebel *et al.*, 2001; Cohen and Knebel, 2006), takes a much simpler approach and relies upon the *in vitro* phosphorylation of proteins in fractionated cell extracts using recombinant kinases of high specific activity. This approach has a number of potential pitfalls including high background phosphorylation from kinases and their substrates present in a whole cell extract, *in vitro* phosphorylation of proteins that are not true *in vivo* substrates and difficulties associated with the identification of low abundance proteins. To overcome these pitfalls, a number of 'tricks' can be employed. Firstly,

background phosphorylations can be reduced by using short incubation times, adding high concentrations of the kinase of interest, employing ATP of very high specific radioactivity, and substituting Mg^{2+} ATP for Mn^{2+} ATP where possible. Secondly, to facilitate the identification of *bona fide* substrates, fractions can be phosphorylated in parallel with several closely related kinases identifiable by possession of similar peptide and/or protein *in vitro* substrate specificities. Those proteins phosphorylated by just one of the kinases in a given screen are more likely to be true physiological substrates. Thirdly, by fractionating the extract, kinases in the extract are likely to be separated from their *bona fide* substrates. Finally, identification of potential substrates has been significantly facilitated by the development of tryptic mass fingerprinting and tandem-MS microsequencing technologies (Cohen and Knebel, 2006). This method has been successfully employed in the identification of substrates of a number of protein kinases including protein kinase B and serum- and glucocorticoid-induced kinase (Cohen and Knebel, 2006) and, perhaps more pertinently to this work, Aurora A (Troiani *et al.*, 2005).

Very little is known about the possible substrates of the Nek6 and Nek7 kinases. It has been suggested that Nek6 strongly phosphorylates histone H1 in vitro and that this phosphorylation reflects a conservation of NIMA and Nek6 function in histone phosphorylation and chromatin condensation (Hashimoto *et al.*, 2003). However, we have been unable to detect a strong preference for histone H1 as a substrate, and indeed other reports have suggested Nek6 phosphorylates all common *in vitro* substrates (Histone H1, MBP and β-casein) equally (Minoguchi *et al.*, 2003; Belham *et al.*, 2003; Yin *et al.*, 2003). Moreover, whilst overexpression of NIMA results in Cdc2-independent premature chromatin condensation (O'Connell *et al.*, 1994) there is no evidence that interference with either Nek6 or Nek7 function has any effect on chromatin condensation (this work; Yin *et al.*, 2003; Kim *et al.*, 2007; Yissachar *et al.*, 2007).

It has been reported that Nek6 may interact with the peptidyl-prolyl isomerase Pin1 in the hepatic carcinoma cell line Hep3B (Chen *et al.*, 2006). Pin1 is essential for cell cycle progression in yeast and mammalian cells and its depletion induces a similar mitotic arrest/apoptosis phenotype to that seen upon interference with Nek6. Pin1 is overexpressed in a number of human cancers and its upregulation in hepatocellular carcinoma (HCC) cells was found to correlate with Nek6 upregulation in 70% of HCCs. However, the exact nature of this interaction remains unclear and it would seem likely that such an interaction reflects a progression to tumorigenesis rather than a normal cell cycle function(Chen *et al.*, 2006).

A screen of peptide libraries in order to identify phosphorylation consensus sequences and thus substrate specificity determinants for Nek6 suggests that Nek6 has a strong preference for a leucine residue 3 residues N-terminal to the site of phosphorylation and aromatic residues 4 amino acids N-terminal and 1 amino acid C-terminal to the phosphorylation site (Lizcano *et al.*, 2002). However, whilst this analysis suggested that the ribosomal S6 protein kinase (S6K) is not a physiological substrate of Nek6, as was originally thought (Belham *et al.*, 2001), it did not encompass the identification of any novel Nek6 or Nek7 substrates and no *bona fide* Nek6 or Nek7 substrates have been identified to date.

This chapter describes work carried out both at the University of Leicester by myself and through collaboration with Dr Axel Knebel (Kinasource Ltd, Dundee, UK) with a view to identification of Nek6 and Nek7 substrates.

7.2 Results

7.2.1 Identification of substrates of Nek6 and Nek7 using a KESTREL screen

Identification of physiological substrates of any kinase is key to understanding its function. To date no substrates of Nek6 and Nek7 have been described and so we decided to search for substrates of Nek6 and Nek7 using an unbiased kinase substrate tracking and elucidation (KESTREL) screen (Cohen and Knebel, 2006). Briefly, this method relies upon the *in vitro* phosphorylation of proteins in fractionated cell extracts using recombinant kinases at high specific activity as detailed in section 7.1. NIMA-related kinases are unusual serine/threonine kinases insomuch as they have the capacity to use Mn^{2+} as a cofactor rather than Mg^{2+} (Lu *et al.*, 1993; Fry *et al.*, 1995; Fry and Nigg, 1997; Noguchi *et al.*, 2002). Using Mn^{2+} in our KESTREL screen rather than Mg^{2+} would likely improve specificity. Thus, prior to the screen being carried out, we assessed the ability of Nek6 and Nek7 to use Mn^{2+} as a cofactor.

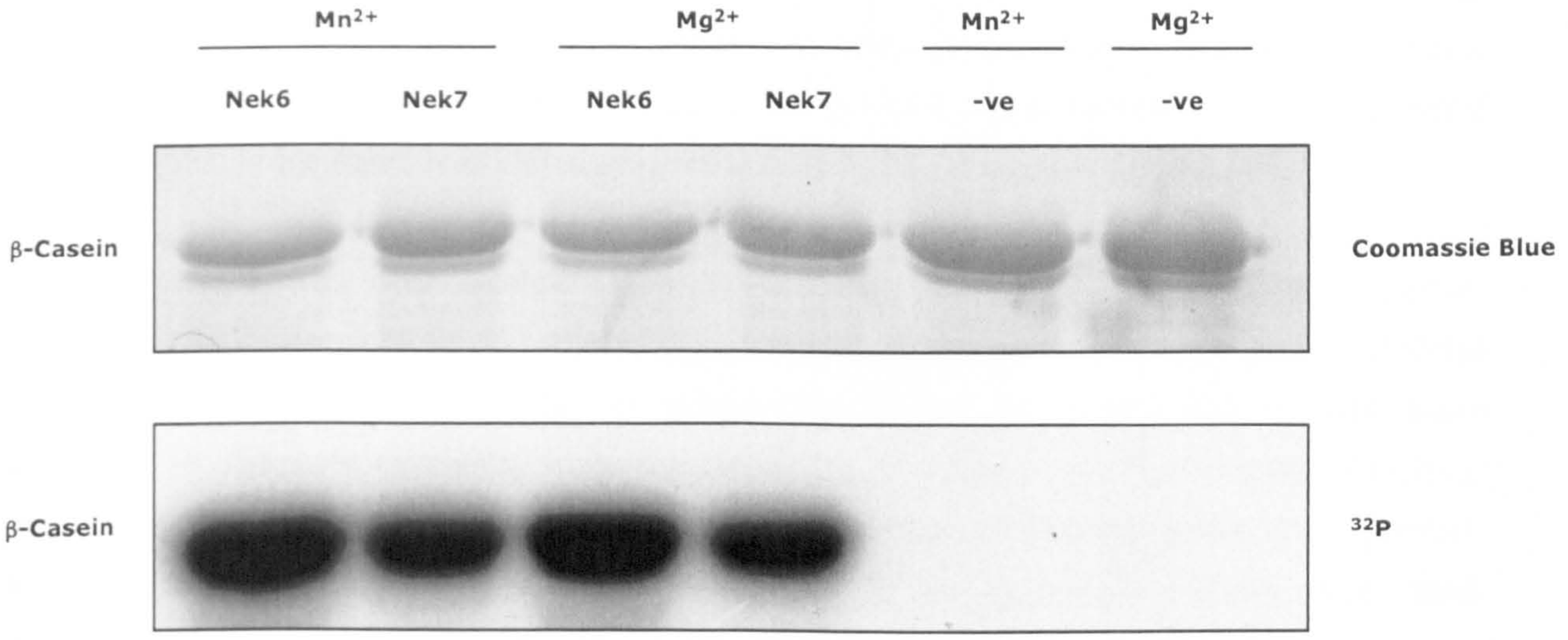
In vitro kinase assays were carried out using purified recombinant His_6Nek6 and His_6Nek7 (Upstate) with a β-casein substrate and kinase buffer containing either $MnCl_2$ or $MgCl_2$. Analysis of samples by SDS-PAGE, Coomassie Blue staining and autoradiography revealed that both kinases phosphorylated the β-casein substrate strongly in the presence of either Mn^{2+} or Mg^{2+} (Figure 7.1A). Quantification of the phosphorylation of the β-casein substrate under the different conditions via scintillation counting confirmed that both Nek6 and Nek7 phosphorylated β-casein to a similar extent whether Mn^{2+} or Mg^{2+} was present in the reaction (Figure 7.1B).

Thus, in common with most other NIMA-related kinases (Fry and Nigg, 1997) both Nek6 and Nek7 can successfully use Mn^{2+} as a cofactor in *in vitro* kinase assays and this, coupled with their high specific activity and the ready availability of kinases of similar specificities, makes them highly suitable for used in a KESTREL screen.

Hence, a KESTREL screen was performed, by Dr Axel Knebel (Kinasource Ltd, Dundee), to search for specific substrates of Nek6 and Nek7. Mn^{2+}-ATP was used instead of Mg^{2+}-ATP and fractions were phosphorylated in parallel with the four mitotic NIMA-related kinases (Nek2, Nek6, Nek7 and Nek9) on the basis that it is unlikely that genuine substrates will be phosphorylated by all of these kinases.

Cytosolic and nuclear extracts were prepared from HEK 293 cells and separated by heparin sepharose chromatography. These extracts were then incubated for 5 min with recombinant Nek2, Nek6, Nek7 and Nek9 and samples analysed by autoradiography.

A



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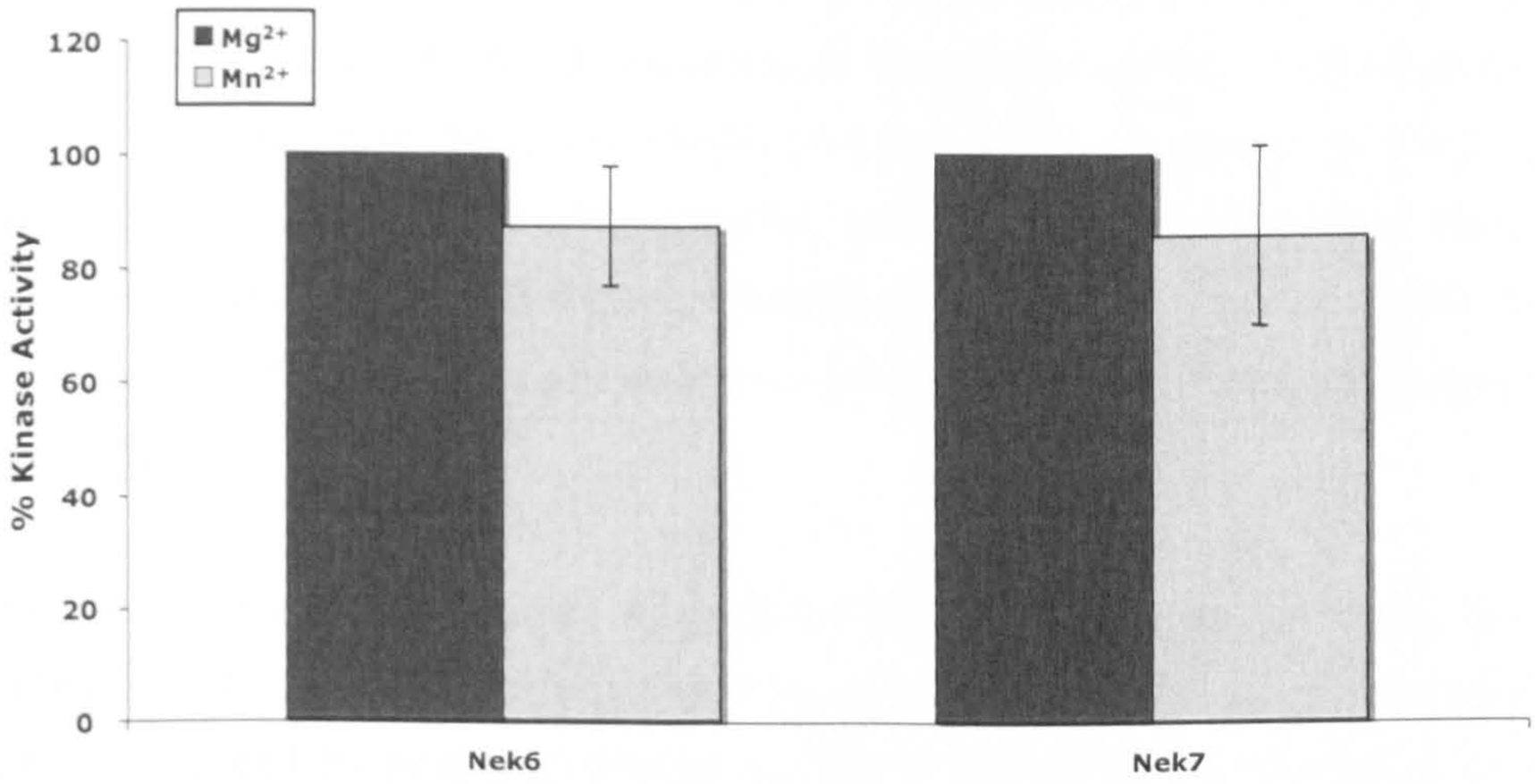


Figure 7.1 Nek6 and Nek7 show equal preference for Mn^{2+} and Mg^{2+} as a cofactor

(**A**) The ability of Nek6 and Nek7 to use Mn^{2+} as a cofactor was tested in an *in vitro* kinase assay using purified His_6Nek6/7 or no kinase (-ve) along with β-casein as a substrate. Proteins were incubated in kinase buffer containing Mn^{2+}-[γ^{32}P]-ATP or Mg^{2+}-[γ^{32}P]-ATP for 30 min at 30°C and analyzed by SDS-PAGE, Coomassie Blue staining and autoradiography (^{32}P). (**B**) Activity of the proteins with the Mn^{2+} cofactor is expressed as a percentage of activity of each with Mg^{2+} cofactor. Data represent means (±S.D.) of three separate experiments.

Analysis of the autoradiograph showed that whilst for Nek7 and Nek9, there were no specific substrates identified, a 28 kDa protein was present in a nuclear fraction which appeared to be specifically phosphorylated by Nek2 (described in Wu *et al.*, 2007). Meanwhile, a number of proteins of around 60-80 kDa were identified in the cytosolic extract which were specifically phosphorylated by Nek6 (Figure A.1A).

This procedure was then scaled up and 1000 mg HEK 293 cytosol was prepared, desalted, chromatographed on heparin sepharose and analysed for the presence of Nek6 substrates. As before, a cluster of substrates of around 60-80 kDa in size were apparent in cytosolic extracts from fractions 13-23 (Figure A.1B). Heparin fractions containing the highest levels of substrate phosphorylation (16-18) were then pooled, desalted and chromatographed on a Source 15 Q ion-exchange column, and Nek6 kinase assays performed to identify Nek6 substrate-containing fractions. Analysis of autoradiographs for the presence of Nek6 substrates, revealed the same cluster of bands was phosphorylated, peaking at fraction 16 onwards (Figure A.1C). Finally, fractions 16 to 18 of the Q-column were subjected to chromatography on a Superdex 200 gel filtration column and Nek6 kinase assays. Analysis by autoradiography for Nek6 substrates revealed the continued presence of the same cluster of substrates, peaking in fractions 3-7 and again in fractions 15-20 (Figure A.1D). Notably, in all three columns, the substrates co-eluted from the column, which may suggest that they exist in a complex. Moreover, these substrates were present in two distinct peaks from the gel filtration column indicating that there are at least 2 different sized complexes containing these proteins.

Following these sequential rounds of purification, the fractions from the Superdex 200 gel filtration column which contained the peak levels of substrate phosphorylation were separately subjected to phosphorylation by Nek6 before being analyzed by SDS-PAGE, colloidal Coomassie Blue staining and autoradiography (Figure A.2A). Ten distinct bands were efficiently phosphorylated across the two fraction peaks and eight of these were excised, and submitted for identification by MALDI-tof mass spectrometry (University of Dundee). This resulted in the unambiguous identification of four proteins: Cortactin A, Hsp70, β-tubulin and actin. All four proteins were present in the second gel filtration peak, however, Cortactin A was not obviously present in the first peak.

Finally, the initial rate of phosphorylation of proteins by Nek6 was calculated. The aliquots from the Superdex gel filtration column peaks were again subjected to phosphorylation by Nek6 at incubation times of 3, 10 and 30 min before being analysed by SDS-PAGE, colloidal Coomassie Blue staining and autoradiography (Figure A.2B).

This analysis showed that the Cortactin A present in Superdex peak 2 was the best substrate for Nek6, with strong phosphorylation after only 3 minutes and a calculated activity of 39.9 Units/mg. The initial rate against Hsp70 was 8.6 Units/mg. β-tubulin and actin appeared to be phosphorylated at similar rates to one another, although this rate was somewhat slower than that for Cortactin A and Hsp70.

In summary, the KESTREL screen, whilst being unable to identify any substrate of Nek7, has highlighted four potential Nek6 substrates: Cortactin A, Hsp70, β-tubulin and actin.

7.2.2 Identification of Nek6 substrates using co-immunoprecipitation

A second approach to substrate identification was then undertaken with Nek6 in an attempt to confirm the results obtained with the KESTREL screen as well as to search for Nek6 substrates specifically phosphorylated in mitosis. To this end, immunoprecipitates were prepared from HEK 293 cells which had been transfected with Flag-Nek6-WT and synchronized in G1/S or M phase of the cell cycle. These were then subjected to *in vitro* phosphorylation by incubation with ^{32}P-γ-ATP and samples analyzed by SDS-PAGE, Coomassie Blue staining and autoradiography (Figure 7.2). This analysis highlighted three bands which were strongly phosphorylated in mitotic, but not G1/S, samples. These bands were excised, digested with trypsin and subjected to identification by mass spectrometry fingerprinting at the University of Leicester Protein and Nucleic Acid Chemistry Laboratory. Of the proteins identified by this analysis, one was Nek6 itself, one was the NF-M neurofilament protein, and the final protein was Hsp70. Thus, using a different approach, one of the proposed Nek6 substrates identified by the KESTREL screen, Hsp70, has again been identified as a potential mitotic Nek6 substrate.

7.2.3 Validation of Nek6 substrates: interaction of Nek6 and Nek7 with microtubules

The identification of β-tubulin as a substrate of Nek6, together with the localization of Nek6 on mitotic spindle microtubules and the spindle defects seen upon interference with Nek6 and Nek7, led us to question whether Nek6, and perhaps also Nek7, may directly associate with microtubules.

To this end, we assessed the ability of Nek6 and Nek7 to interact with microtubules using an *in vitro* sedimentation assay. Nek6, Nek7 and the control proteins, Rab4 and γ-tubulin, were generated by *in vitro* translation in the presence of [^{35}S]-methionine (Figure 7.3A) before being incubated in the presence or absence of taxol-stabilized microtubules. Following centrifugation on a sucrose cushion, pellet and supernatant fractions were analyzed by SDS-PAGE and autoradiography (Figure 7.3B). Proteins

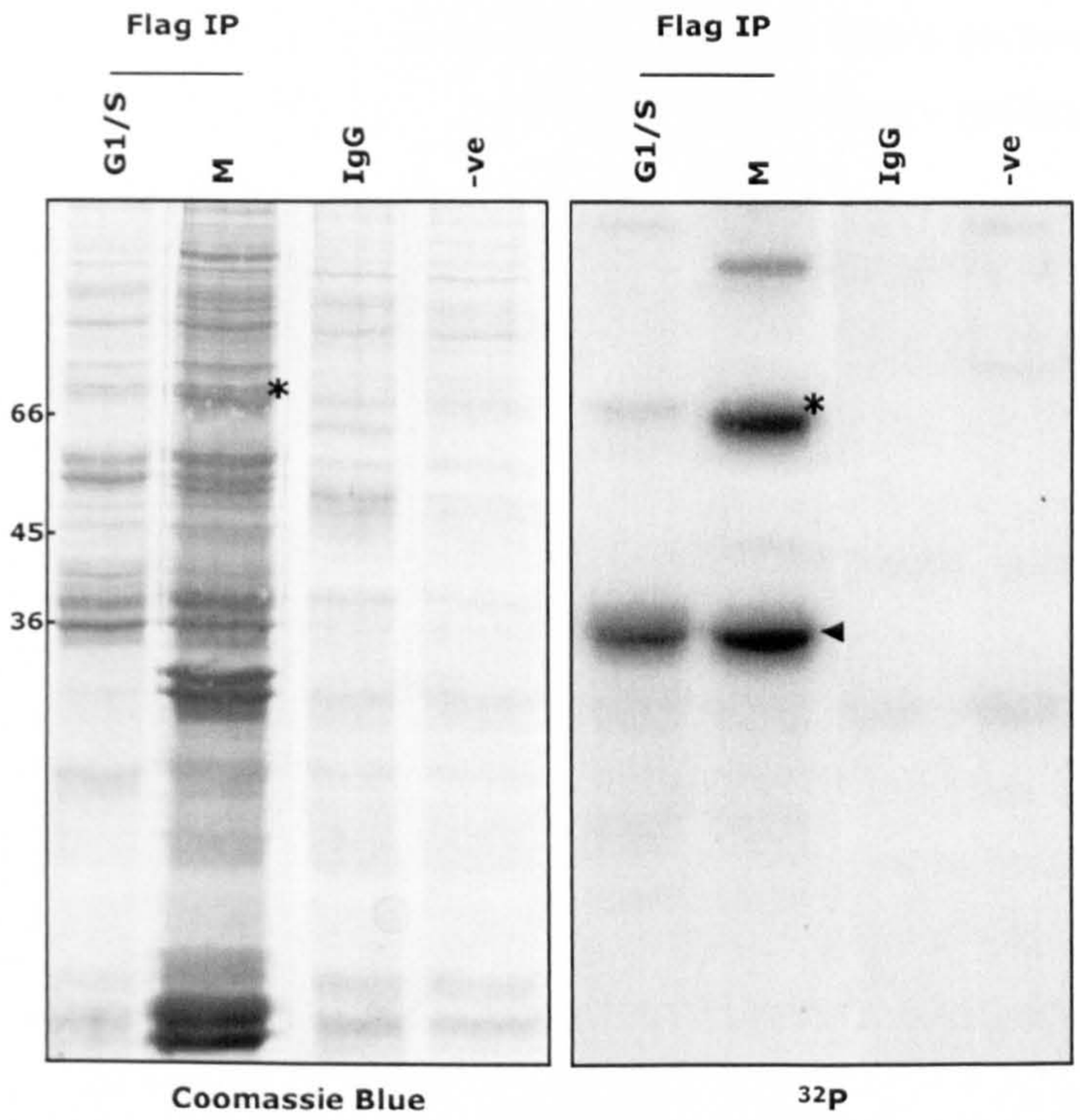


Figure 7.2 Identification of Nek6 substrates using coimmunoprecipitation
Immunoprecipitates were prepared from Flag-Nek6-transfected HEK 293 cells with anti-Flag antibodies, whole rabbit IgGs or no antibodies (-ve). For anti-Flag immunoprecipitates, cells were synchronised at either G1/S or M phase of the cell cycle, whereas for control immunoprecipitations asynchronous cell populations were used. Immunoprecipitates were incubated in complete kinase buffer containing Mn^{2+}-[γ^{32}P]-ATP for 30 min at 30°C prior to being analysed by SDS-PAGE, Coomassie Blue staining (left panel) and autoradiography (^{32}P; right panel). Bands specifically radiolabelled in mitotic immunoprecipitates were excised, digested with trypsin and subjected to identification by Mass Spectrometry Fingerprinting. The asterisk (*) indicates a band at 70 kDa, identified as Hsp70; arrowhead indicates Nek6 protein. Molecular weights (kDa) are indicated on the left.

capable of associating with microtubules should be present in the pellet fractions in the presence, but not the absence, of taxol-stabilized microtubules. Analysis of autoradiographs revealed that a significant proportion, 70-80% of both the Nek6 and Nek7 proteins sedimented with taxol-stabilized microtubules (Figure 7.3C).

The ability of Nek6 and Nek7 to directly phosphorylate microtubules *in vitro* was also assessed by incubation of taxol-stabilised microtubules with Nek6 or Nek7 in the presence of γ-^{32}P-ATP, followed by SDS-PAGE, Coomassie Blue staining and autoradiography (Figure 7.3D). This revealed that both Nek6 and Nek7 are capable of phosphorylating microtubules, at least *in vitro*, and much more efficiently than Nek2.

Thus, Nek6, and indeed Nek7, are capable of interacting with, and phosphorylating, microtubules. However, whether this is a true reflection of their *in vivo* function requires further investigation.

7.2.4 Validation of Nek6 substrates: interaction of Nek6 with Cortactin A and Hsp70

The two other major substrates of Nek6 identified in the KESTREL screen were a regulator of actin dynamics, Cortactin A, and a member of the heat shock protein family of chaperones, Hsp70. In order to validate these proteins as potential binding partners and substrates of Nek6, and possibly Nek7, we first assessed the ability of Nek6 and Nek7 to bind these proteins using a co-immunoprecipitation approach.

Both wild-type and catalytically-inactive Flag-Nek6 and Flag-Nek7 proteins were immunoprecipitated from lysates of transfected cells which had been synchronized in G1/S or M-phase of the cell cycle 24 h after transfection. Cell synchronization was performed in order to assess whether any interaction between the proteins of interest was cell cycle stage specific. Immunoprecipitates were analysed by SDS-PAGE and Western blotting with anti-Nek6, anti-Nek7, anti-Cortactin A, anti-Hsp70 and anti-Nek9 antibodies (Figure 7.4A). This analysis revealed that Cortactin A and Hsp70 clearly coprecipitated with Nek6, but not Nek7, and that this interaction was equally strong both in G1/S and M-phase extracts with both active and inactive Nek6. In contrast, Nek9 coprecipitated with both Nek6 and Nek7 again in a manner that was independent of cell cycle status.

In order to further validate these interactions, the ability of endogenous Nek6 and Nek7 to coprecipitate with these proteins was determined. This was particularly important in the case of Hsp70 to confirm that the coprecipitation reflected a true physiological

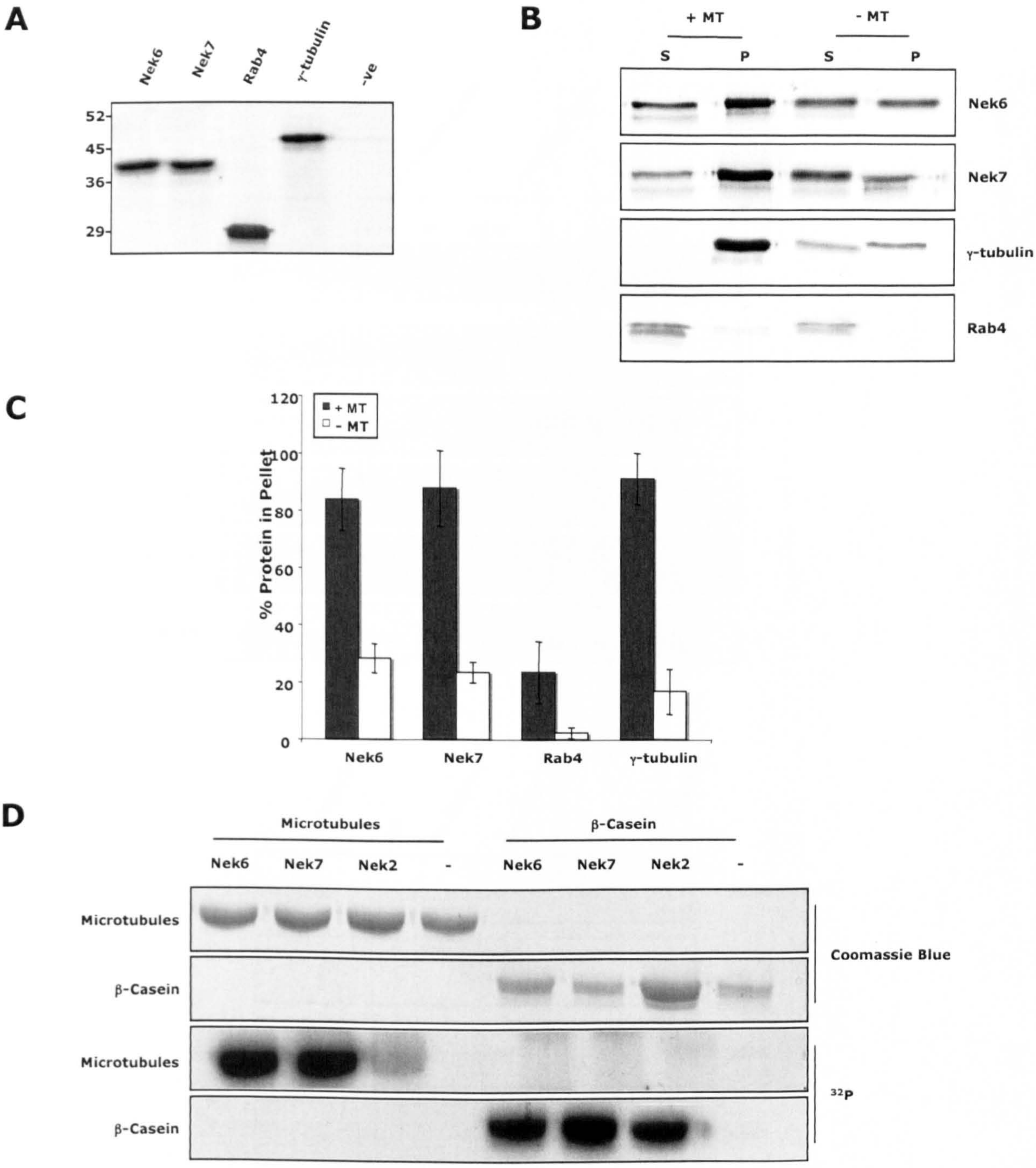
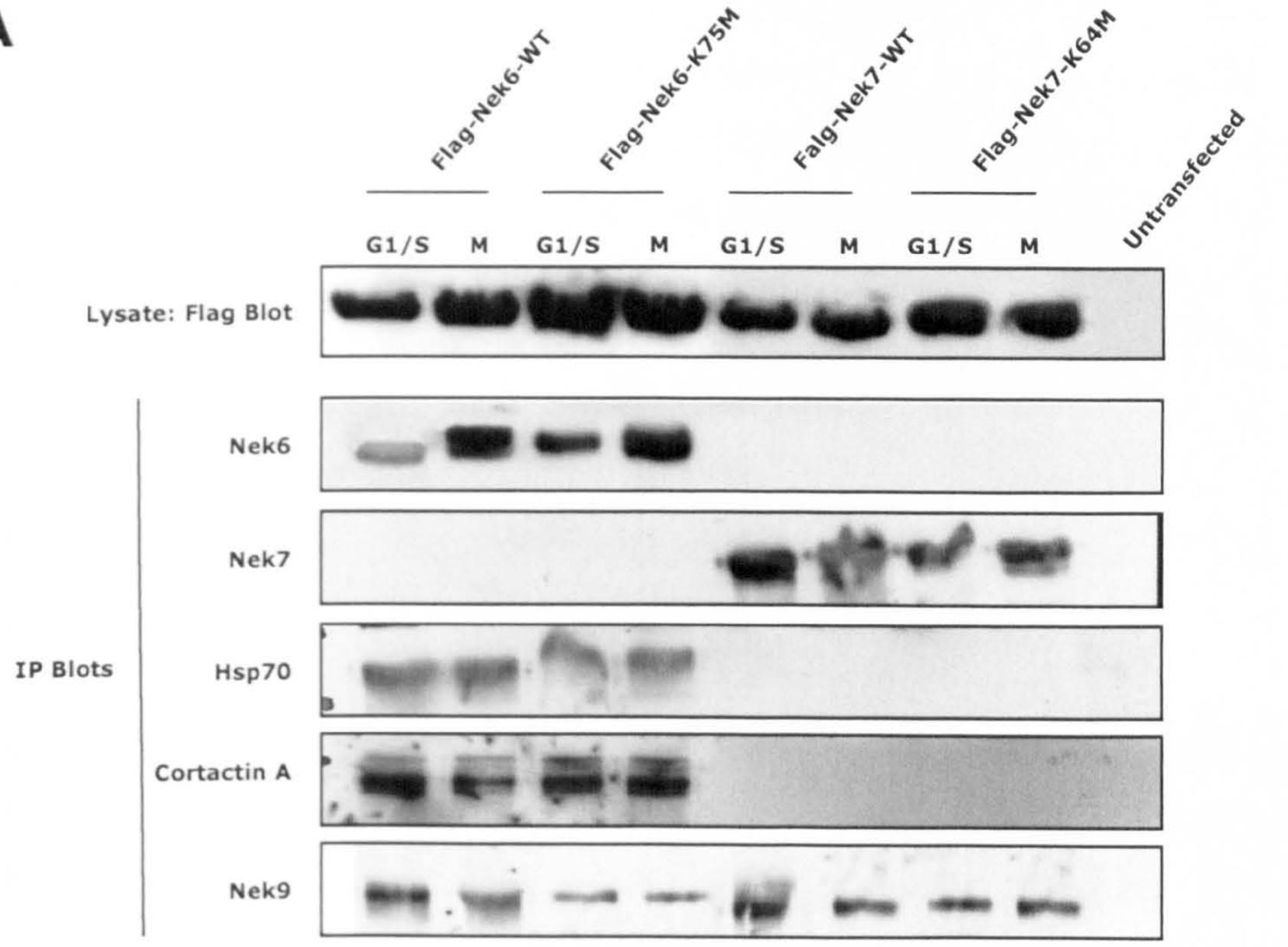


Figure 7.3 Interaction with and phosphorylation of microtubules by Nek6 and Nek7

(**A**) Nek6 and Nek7 and the control proteins, Rab4 and γ-tubulin, were generated by *in vitro* translation in the presence of [^{35}S]-methionine and analyzed by SDS-PAGE and autoradiography. -ve, no DNA added to IVT reaction. Molecular weights (kDa) are indicated on the left. (**B**) Input proteins were incubated with (+MT) or without (-MT) taxol-stabilized microtubules. Proteins were spun at 35000 rpm on a sucrose cushion for 30 min and supernatant (S) and pellet (P) fractions collected and analysed by SDS-PAGE and autoradiography. (**C**) The histogram represents the percentage of input protein found in the pellet fractions with and without taxol stabilized microtubules. Data represent mean (± S.D.) of three separate experiments. (**D**) Kinase assays were carried out with purified recombinant His_6Nek6, His_6Nek7 and His_6Nek2 kinases using purified microtubules or β-casein (positive control) as a substrate. Proteins were incubated in complete kinase buffer containing γ-^{32}P-ATP for 30 min at 30°C before being analyzed by SDS-PAGE, Coomassie Blue staining and autoradiography (^{32}P). -, no kinase.



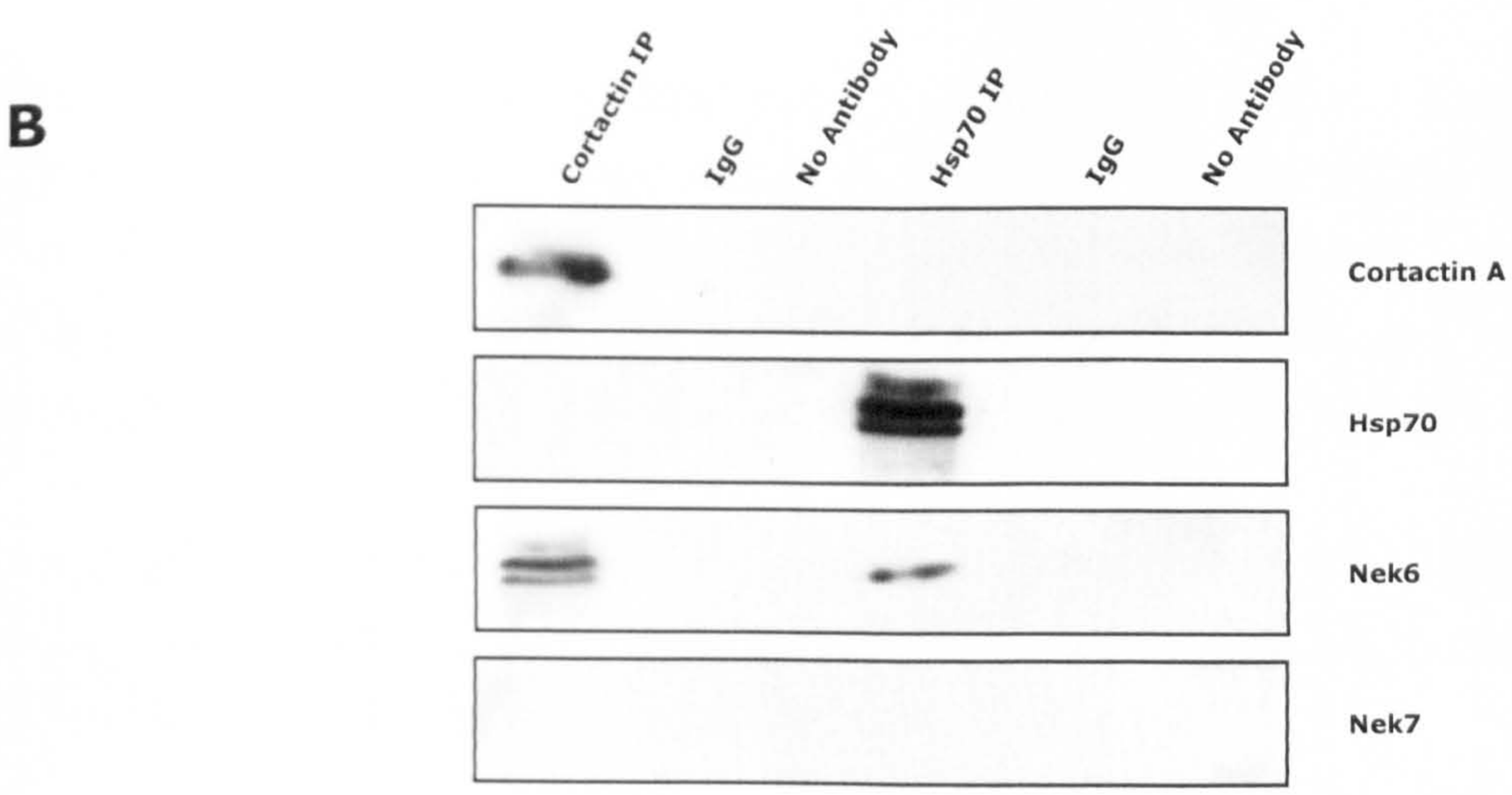


Figure 7.4 Nek6, but Not Nek7, interacts with Cortactin A and Hsp70
(**A**) HEK 293 cells were either untransfected or transiently transfected with Flag-Nek6 and Flag-Nek7 constructs, as indicated. After 24 h, cells were synchronized in G1/S or M phase of the cell cycle before cells were harvested, lysed and recombinant proteins immunoprecipitated with anti-Flag antibodies. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with anti-Nek6, anti-Nek7, anti-Hsp70, anti-Cortactin A and anti-Nek9 antibodies, as indicated. (**B**) Endogenous Cortactin A and Hsp70 were immunoprecipitated from HEK 293 cell lysates using anti-Cortactin A and anti-Hsp70 antibodies, respectively. Control immunoprecipitates were generated with rabbit IgGs or no antibodies. Immunoprecipitates were analysed by SDS-PAGE and Western blotting with anti-Cortactin A, anti-Hsp70, anti-Nek6 and anti-Nek7 antibodies, as indicated.

interaction and was not solely the result of a chaperone protein interacting with an overexpressed protein. Thus, asynchronous HEK 293 cell lysates were subjected to immunoprecipitation with anti-Cortactin A or anti-Hsp70 antibodies and immunoprecipitates analyzed by SDS-PAGE and Western blotting with anti-Nek6, anti-Nek7, anti-Cortactin A and anti-Hsp70 antibodies (Figure 7.4B). This analysis indicated that endogenous Nek6, but not Nek7, coprecipitates with both Cortactin A and Hsp70. Furthermore, Cortactin A did not appear to be present in Hsp70 immunoprecipitates or vice versa. Intriguingly, whilst a single migrating form of Nek6 was identified in Hsp70 immunoprecipitates, a Nek6 doublet was observed in the Cortactin A immunoprecipitates, suggesting that Cortactin A may preferentially bind the phosphorylated form of Nek6 predominant in mitosis.

In order to confirm the *in vitro* phosphorylation of Cortactin A and Hsp70 by Nek6, but not Nek7, recombinant, bacterially-expressed His_6Hsp70 and His_6CortactinA were generated by cloning the ESTs for the two proteins into pETM11 and expressing proteins in BL21 *E. coli*. This led to the recovery of approximately 9 mg of Hsp70 and 4 mg of Cortactin A per litre of *E. coli* culture (Figure 7.5A).

The ability of recombinant His_6Nek6, His_6Nek7 or His_6Nek2 to phosphorylate these purified proteins was then assayed by incubation in complete kinase buffer containing γ-^{32}P-ATP and analysis by SDS-PAGE, Coomassie Blue staining and autoradiography (Figure 7.5B & C). This analysis demonstrated that Nek6, but not Nek7 or Nek2, strongly phosphorylated both purified Hsp70 and Cortactin A *in vitro*.

Thus, both Cortactin A and Hsp70 specifically interact with, and are phosphorylated by, Nek6 but not Nek7. This then provides the first evidence for an important functional difference between these highly similar protein kinases.

7.2.5 Cell cycle-dependent localization of Hsp70 and Cortactin A

Hsp70 can bind microtubules and potentially regulate microtubule dynamics (Liang and MacRae, 1997). Cortactin A, is known to be a regulator of actin dynamics, but has not previously been studied in respect of mitosis (Daly, 2004; Buday and Downward, 2007). In view of the interaction with Nek6 and the potential role of Nek6 and Nek7 in mitotic progression, the cell cycle localization of both Hsp70 and Cortactin A was examined via immunofluorescence microscopy. HeLa cells were stained with anti-Cortactin A or anti-Hsp70 antibodies and costained with either anti-α-tubulin or anti-γ-tubulin antibodies. This revealed that endogenous Hsp70 localized to spindle poles and microtubules in metaphase (Figure 7.6A) with a redistribution to the spindle midzone in anaphase, and

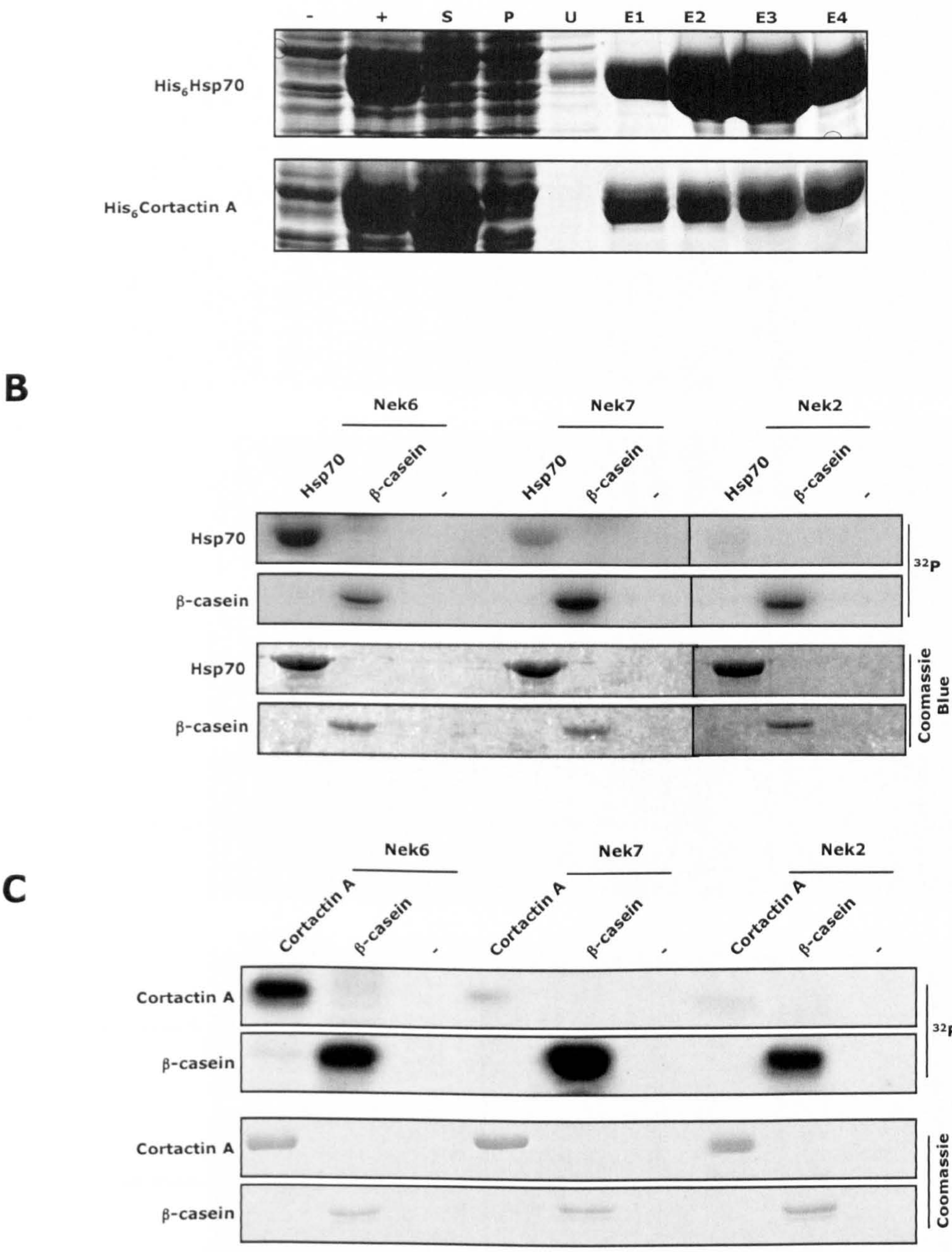


Figure 7.5 Cortactin A and Hsp70 as substrates of Nek6 but not Nek7

(**A**) His_6Hsp70 (upper panel) and His_6cortactin A (lower panel) were expressed in BL21 *E. coli* for 4 h at 18°C following induction with 100 μM IPTG. Following lysis and purification over a nickel column, protein recovery at the various steps was analyzed by SDS-PAGE and Coomassie Blue staining. -, uninduced sample; +, induced sample; S, soluble protein in supernatant following lysis; P, insoluble protein in pellet following lysis; E1-E4, elution fractions 1-4. Purified Hsp70 (**B**) or Cortactin A (**C**) protein was used a substrate in kinase assays with purified recombinant His_6Nek6, His_6Nek7 and His_6Nek2. Kinase assays with β-casein or no substrate (-) were carried out as controls. Proteins were incubated in kinase buffer containing γ-^{32}P-ATP for 30 min at 30°C before being analyzed by SDS-PAGE, Coomassie Blue staining and autoradiography (^{32}P).

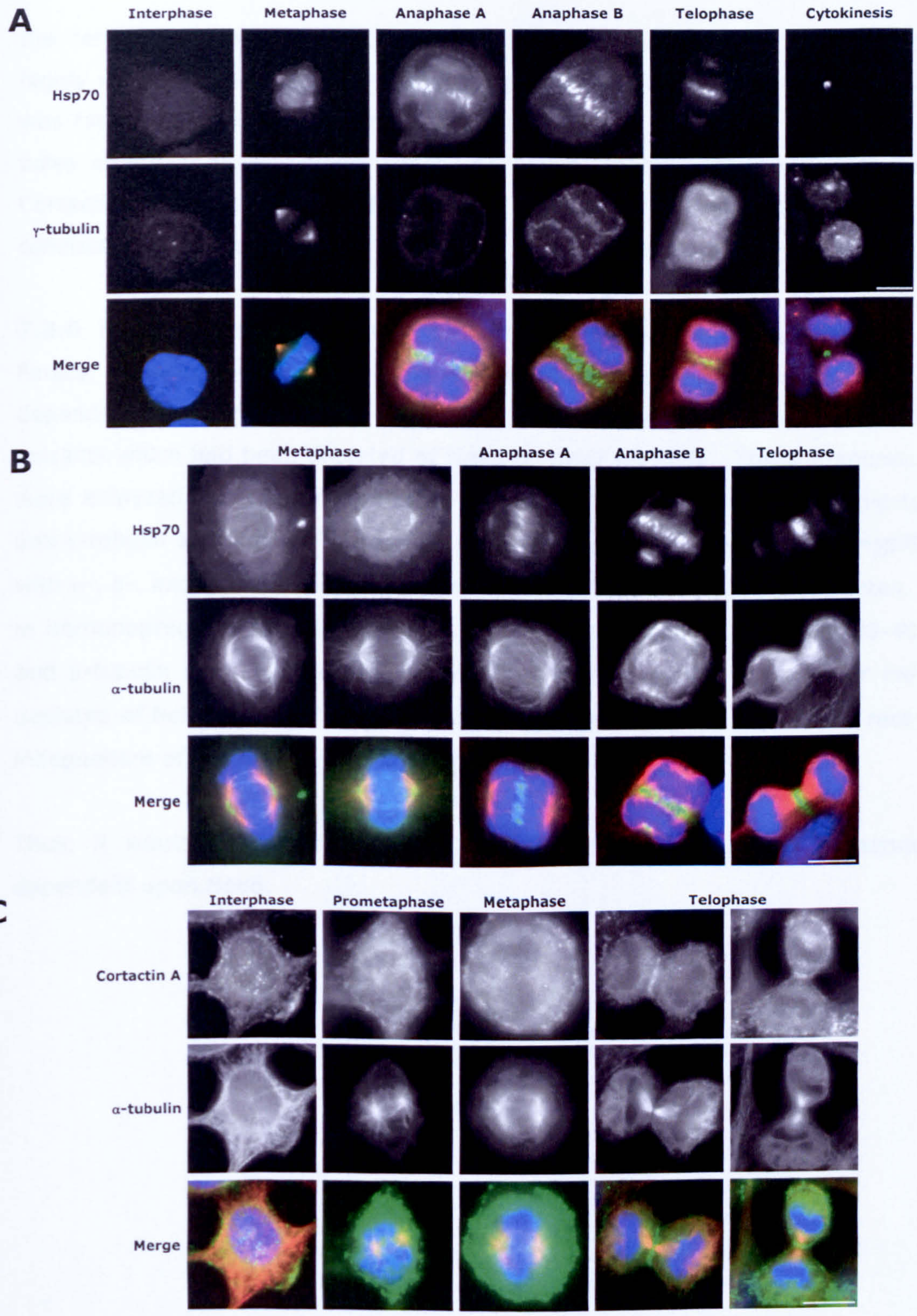


Figure 7.6 Cell cycle localization of Hsp70 and Cortactin A

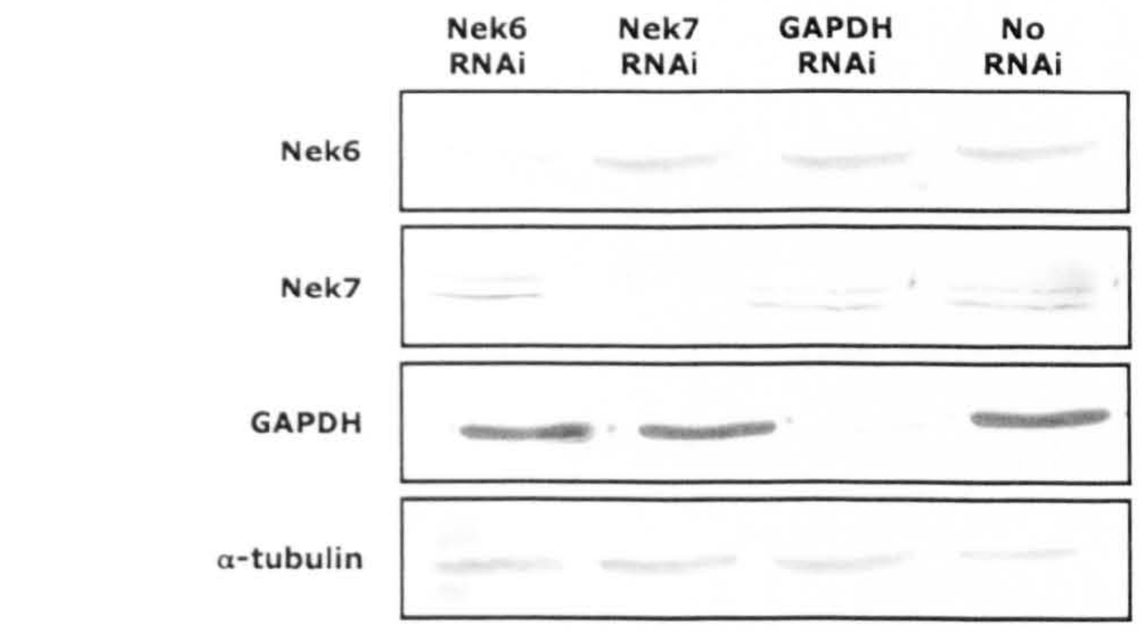
(**A**) HeLa cells were fixed and permeabilised with ice-cold methanol and processed for immunofluorescence microscopy with anti-γ-tubulin (red) and anti-Hsp70 (green) antibodies. DNA was stained with Hoechst 33258 (blue). (**B**) HeLa cells were fixed and permeabilised with ice-cold methanol and processed for immunofluorescence microscopy with anti-α-tubulin (red) and anti-Hsp70 (green) antibodies. DNA was stained with Hoechst 33258 (blue). (**C**) HeLa cells were fixed and permeabilised with ice-cold methanol and processed for immunofluorescence microscopy with anti-α-tubulin (red) and anti-Cortactin A (green) antibodies. DNA was stained with Hoechst 33258 (blue). Scale bars, 10μm.

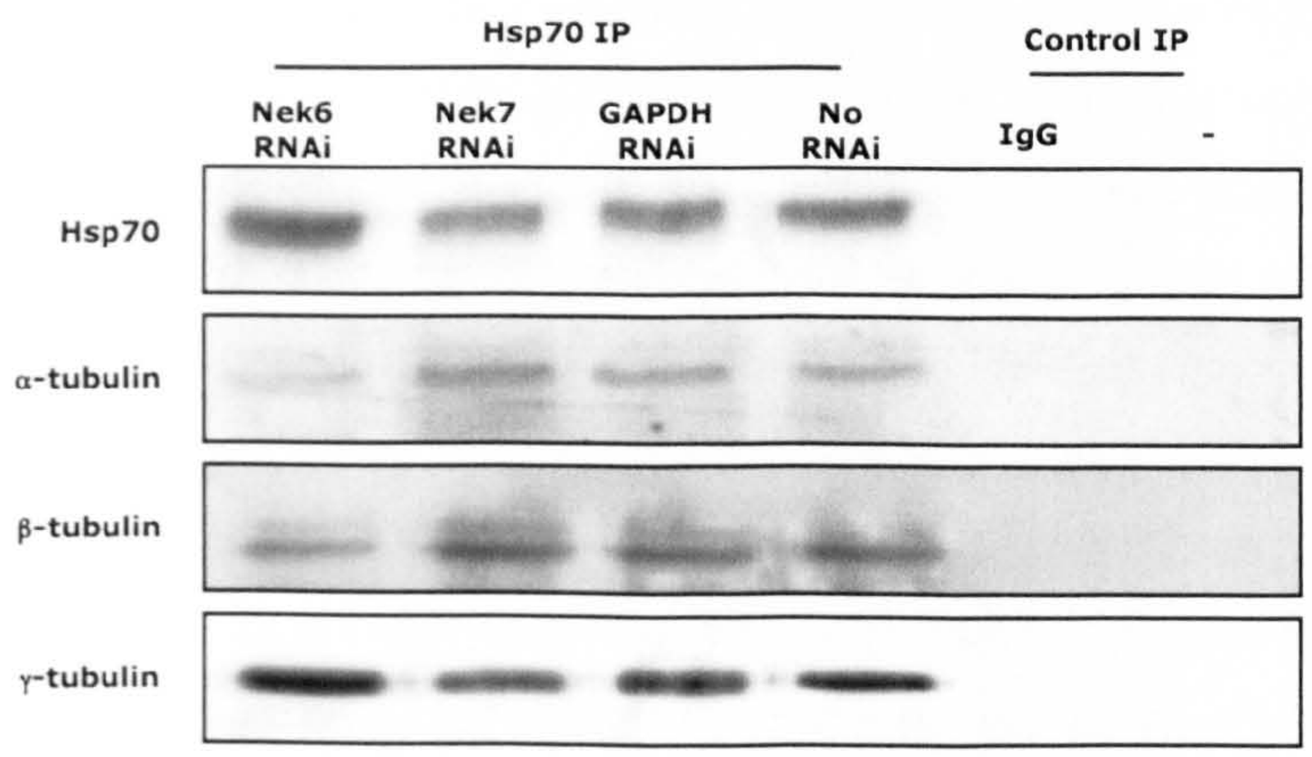
the central spindle and midbody in late mitosis (Figure 7.6B). This distribution was highly reminiscent of that seen with endogenous Nek6. Cortactin A, on the other hand, was rather diffusely distributed in early mitosis, with no specific localization to spindle poles or microtubules (Figure 7.6C). However, as cells progressed into late mitosis, Cortactin A became concentrated at the contractile ring and cleavage furrow, which is consistent with a potential role for Nek6 in regulating cytokinesis.

7.2.6 Hsp70 interacts with α/β-tubulin in a Nek6-dependent manner

Finally, we wished to examine whether the interaction of Hsp70 with microtubules was dependent on Nek6. To this end, Hsp70 was immunoprecipitated from HEK 293 cell extracts which had been depleted of Nek6 or Nek7 by RNAi. These immunoprecipitates were analyzed by Western blotting with anti-Hsp70, anti-α-tubulin, anti-β-tubulin and anti-γ-tubulin antibodies (Figure 7.7A & B). This analysis revealed that Hsp70 interacts with α-, β-, and γ-tubulin as all three were present in the immunoprecipitates. However, in immunoprecipitates prepared from cells depleted of Nek6, typically 30-40% less α- and β-tubulin was recovered compared to control depleted extracts, or indeed those depleted of Nek7 (Figure 7.7B & C). In contrast, the amount of γ-tubulin recovered way independent of the presence or absence of Nek6 or Nek7.

Thus, it would appear that Hsp70 interacts with α/β-tubulin in a manner that is dependent upon Nek6.





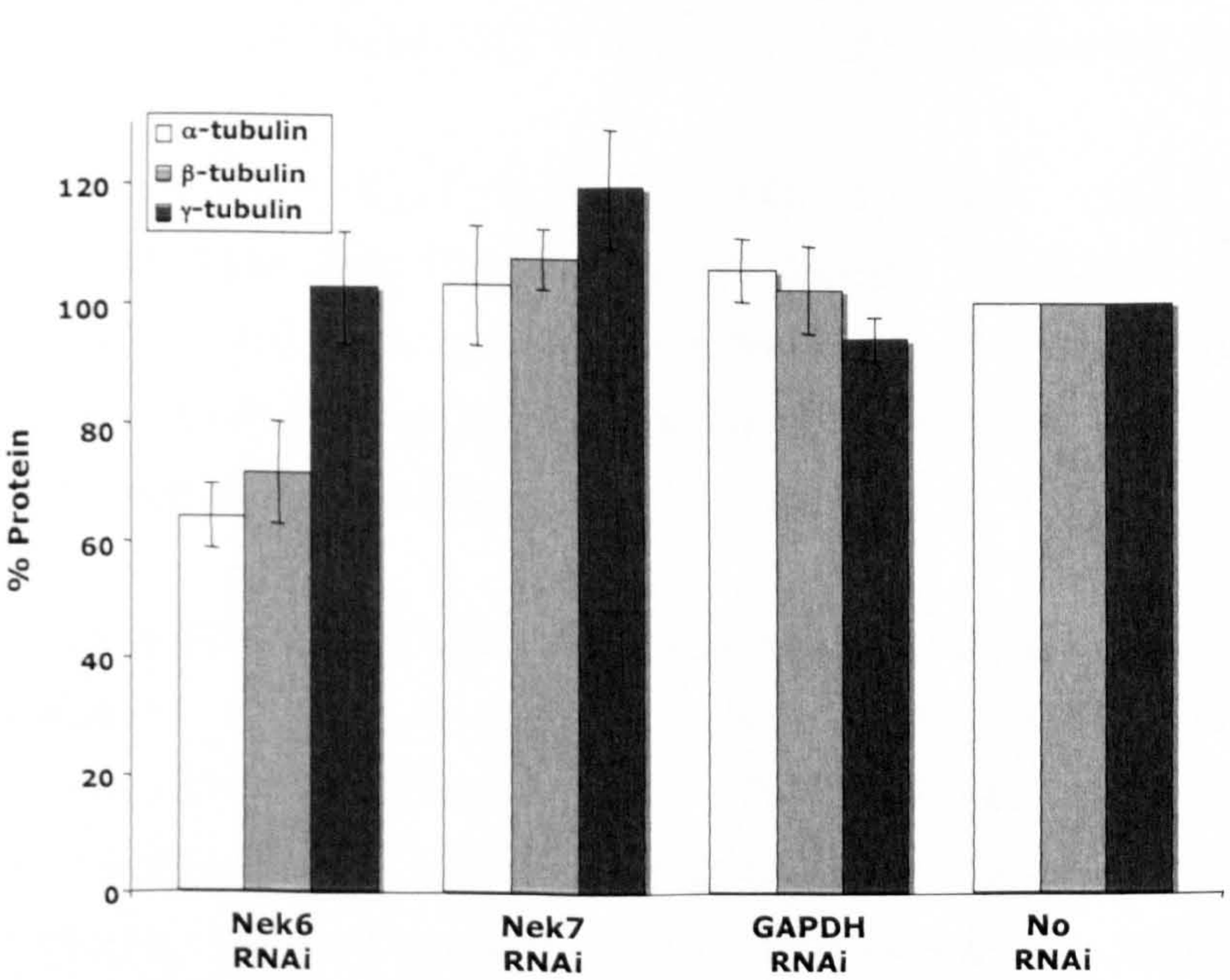


Figure 7.7 Depletion of Nek6 reduces interaction of Hsp70 with α- and β-tubulin

(**A**) HeLa cells were untransfected (no RNAi) or transfected with Nek6, Nek7 or GAPDH siRNA oligos for 72 h. Cells were lysed and samples of the lysate were analyzed by Western blot as indicated to verify the knockdown. (**B**) Remaining lysates were subjected to immunoprecipitation with anti-Hsp70 antibodies. Control IPs were carried out with mouse whole IgG and no antibody on mock-depleted lysates. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with anti-Hsp70, anti-α-tubulin, anti-β-tubulin and anti-γ-tubulin antibodies, as indicated. (**C**) The levels of protein in the immunoprecipitates were quantified by densitometry measurement using ImageJ software. The amount of protein is expressed as a percentage of protein immunoprecipitated from mock-depleted extracts, normalised to the amount of Hsp70 protein precipitated. Data represent mean (±S.D.) of three separate experiments.

7.3 Discussion

A key feature of functional characterization of a given protein kinase is substrate identification. Protein kinases represent approximately 2% of the 30000 proteins encoded by the human genome and approximately one third of mammalian proteins are phosphorylated. Thus, broadly speaking, each protein kinase has an average of 20 substrates (Knebel *et al.*, 2001).

However, substrate identification remains a major challenge to biochemists despite the emergence of various new methodologies to screen for potential substrates. One such technique involves screening sequential fractions of cell lysates to identify proteins specifically phosphorylated by a given kinase *in vitro*. This approach is known as a kinase substrate tracking and elucidation (KESTREL) assay. It has previously been employed for the successful identification of novel substrates of a variety of protein kinases, including the identification of vimentin as a substrate of Aurora-A (Troiani *et al.*, 2005). Vimentin is an intermediate filament protein shown to be phosphorylated by many key mitotic regulators including Plk1 (Yamaguchi *et al.*, 2005), Cdk1 (Tsujimura *et al.*, 2001), Aurora B (Goto *et al.*, 2003) and Rho-associated kinase (Goto *et al.*, 1998).

No substrates of Nek6 or Nek7 have yet been described. Hence, having identified potential roles for Nek6 and Nek7 in robust spindle formation and cytokinesis, we undertook a KESTREL screen to identify substrates that might underpin the involvement of Nek6 and Nek7 in these regulatory pathways. This screen was carried out by our collaborator, Dr Axel Knebel at Kinasource Ltd, Dundee.

We first showed that Nek6 and Nek7 were able to use Mn^{2+} as a cofactor. This, together with the availability of other kinases with structurally similar kinase domains, made them good candidates for this screen. The KESTREL analysis resulted in the unambiguous identification of four Nek6 substrates: β-tubulin, Hsp70, Cortactin A and actin. No substrates for Nek7 were identified using this screen, although this may have been as a result of the recombinant Nek7 used being of lower specific activity than Nek6.

Microtubules are polymers of heterodimers of α- and β-tubulin arranged laterally to form hollow cylinders. Thus, the functional data on Nek6 together with the identification of a core component of the mitotic spindle, β-tubulin, as a potential Nek6 substrate, suggests that Nek6 may have a direct role in phosphorylation of spindle microtubules themselves. Furthermore, we have shown that Nek6, and indeed Nek7, cosediment with microtubules, and are capable of phosphorylating taxol-stabilized microtubules.

Phosphorylation of microtubules may not directly alter microtubule dynamics but may regulate the recruitment of and interaction with effector proteins. Hence, microtubule phosphorylation may play a role in directing mitotic events such as spindle positioning, spindle dynamics, microtubule stabilization and cytokinesis, in part by targeting effector proteins to a subset of microtubules (Verhey and Gaertig, 2007).

In view of the interaction between Nek6, and indeed Nek7, with γ-tubulin, another possible role for Nek6 may be to promote the interaction of γ-tubulin complexes with pre-existing spindle microtubules, thus facilitating the proposed amplification of spindle microtubules through their nucleation from within the spindle itself (Goshima *et al.*, 2007). Indeed, in *Arabidopsis*, the Nek6 orthologue has been colocalised with γ-tubulin complex proteins on cortical microtubules and an interaction reported with the microtubule-dependent armadillo repeat-containing kinesin motor protein ARK1 (Motose *et al.*, 2008; Sakai *et al.*, 2008).

The second potential substrate of Nek6 identified in the KESTREL screen was Hsp70. Importantly, Hsp70 was also identified as an interacting partner and substrate of Nek6 in our co-immunoprecipitation/mass spectrometry analysis. Heat shock proteins were first observed as proteins preferentially synthesized in response to heat or other physiological stresses (Tissieres *et al.*, 1974). However, they have since been shown to be synthesized constitutively and involved in a wide variety of cellular processes as a result of their properties as molecular chaperones (Liang and MacRae, 1997). The Hsp70 family of molecular chaperones is highly conserved and the most prominent amongst all the stress proteins (Lindquist and Craig, 1988; Agueli *et al.*, 2001). They are important in assisting the folding of newly synthesized proteins and the assembly, and indeed disassembly, of protein oligomers, as well as in directing misfolded or denatured proteins for degradation and in promoting protein transport across organelle membranes (Oka *et al.*, 1998). There is also significant evidence for an intimate relationship between Hsp70 and the cytoskeleton and a role for Hsp70 in mitosis (Rattner, 1991; Brown *et al.*, 1996; Perret *et al.*, 1995; Liang and MacRae, 1997; Sconzo *et al.*, 1999; Agueli *et al.*, 2001).

Several Hsp70s were initially identified as MAPS (Liang and MacRae, 1997) and Hsp70 has been shown to localize to microtubules and MTOCs (Weller, 1988; Rattner, 1991; Perret *et al.*, 1995;Sconzo *et al.*, 1991; Agueli *et al.*, 2001). We found that Hsp70 localized to microtubule-based structures, including spindle microtubules and spindle poles, in both metaphase and anaphase cells, as well as to the central spindle and midbody in late mitotic cells. Microinjection of anti-Hsp70 antibodies into sea urchin

embryos impairs mitosis and results in the formation of abnormal spindle asters (Sconzo *et al.*, 1999), whilst temperature sensitive Hsp70 loss-of-function mutants of *Saccharomyces cerevisiae* exhibit aberrant mitotic spindle formation resulting in abnormal nuclear distribution at mitosis (Oka *et al.*, 1998).

Thus, it would appear that Hsp70, like Nek6, functions in mitosis to regulate the formation and action of the mitotic spindle. Furthermore, RNAi depletion of Nek6, but not Nek7, appeared to result in a reduced affinity of Hsp70 for α- and β-tubulin, raising the possibility that the interaction of Hsp70 with spindle microtubules is dependent on Nek6 function. Therefore, although the basis for, or exact nature of, the interaction between Nek6 and Hsp70 is unknown, it is intriguing to speculate that Nek6 may regulate the localization or interactions of Hsp70 with crucial targets at different points in mitosis, thereby further contributing to mitotic spindle organization and cytokinesis.

We have recently used mass spectrometry to identify the site in Hsp70 phosphorylated by Nek6 as Thr-66. Thus, it will be intriguing to examine the localization Hsp70 T66 mutants as well as testing the effect of overexpressing this mutant on mitotic progression.

The final Nek6 substrates identified as a result of the KESTREL screen were Cortactin A and actin, with Cortactin A being identified as the best Nek6 substrate based on initial phosphorylation rates. Actin is a major component of the cytoskeleton with a well-documented role in cytokinesis, as a structural component of the contractile ring (Schroeder, 1973). It has also been reported to play an important role in initiation of mitotic spindle formation by driving centrosome separation at the onset of mitosis (Uzbekov *et al.*, 2002; Whitehead *et al.*, 1996).

Cortactin A is considered to be a scaffold protein which recruits different proteins to the site of actin polymerization thus regulating actin cytoskeletal networks necessary for endocytosis, cell migration and invasion, adhesion, synaptic organization and cell morphogenesis (Buday and Downward, 2007; Lua and Low, 2005). However, there is currently very little data concerning a role for Cortactin A in mitosis. Although it has recently been proposed that Cortactin A may function to recruit actin to centrosomes and thus facilitate centrosome separation upon entry into mitosis (Wang *et al.*, 2008).

Intriguingly, analysis of the localization of Cortactin A showed that it is concentrated to the vicinity of the contractile ring and midbody of late mitotic cells. In view of the interaction of Cortactin A and actin, the localization of both Nek6 and Cortactin A, and

the cytokinesis delay seen upon interference with Nek6, we propose that Nek6 may have a previously unsuspected role in controlling cytokinesis through Cortactin A phosphorylation. Whether this function may be achieved through the alteration of the localization of Cortactin A or perhaps its interaction with other regulators of cytokinesis remains to be determined. Crucial to elucidation of this pathway will be the analysis of the effect of interference of Nek6 function on Cortactin A localization and also analysis of sites of phosphorylation to allow characterization of the effect of expression of phosphorylation site mutants on cell abscission.

Another protein identified as a Nek6 substrate using the co-immunoprecipitation approach, rather than the KESTREL screen was the Neurofilament middle (NF-M) protein. NF-M is an intermediate filament protein preferentially expressed in the neurons of the central nervous system. This was an unexpected finding in view of the fact the HEK 293 cells are considered to be kidney-like cells which, as such might not be expected to express proteins normally associated with neurons. However, it has been shown that HEK 293 cells do indeed express NF-M, as well as the other neurofilament subunits, NF-L, NF-H, α-internexin (Shaw *et al.*, 2002). Interestingly, these proteins are subject to post-translational modifications, including phosphorylation which are believed to be important for regulation of their function and organization (Sihag *et al.*, 2007). Furthermore, vimentin is another intermediate filament protein whose phosphorylation at mitosis by key mitotic regulators including Plk1, Cdk1, and Aurora A and B is essential for the dramatic cytoskeletal reorganization that occurs in mitosis (Yamaguchi *et al.*, 2005; Tsujimura *et al.*, 2001; Goto *et al.*, 2003; Troiani *et al.*, 2005). Thus, in view of the demonstrated association of Nek6 with actin and Cortactin A, this may reflect a general role for Nek6 in regulation of cytoskeletal dynamics.

An interesting finding from the KESTREL screen was that in all three columns, the Nek6 substrates co-eluted, which may suggest that they exist in a complex. Moreover, these substrates were present in two distinct peaks from the gel filtration column indicating that at least 2 different sized complexes contain these proteins. All four proteins were present in the second gel filtration peak, however, Cortactin A was not obviously present in the first peak, suggesting that Nek6 may exist in two complexes, one with Cortactin A and one without. Again this may reflect different roles for Nek6 in mitotic spindle organization and cytokinesis.

However, it is important to note that, whilst an interaction was detected between Nek6 and Cortactin A and Nek6 and Hsp70, anti-Cortactin A immunoprecipitates did not obviously contain Hsp70 and neither did anti-Hsp70 immunoprecipitates contain

Cortactin A. Again this suggests the presence of different Nek6 complexes, one containing Hsp70 and one containing Cortactin A.

The KESTREL screen was only useful in identification of substrates of Nek6; no substrates of Nek7 were identified, possibly as a result of insufficient levels of activity. However, whilst Nek7 appeared not to either interact with or phosphorylate Hsp70 or Cortactin A, there did appear to be an interaction with Nek7 and α/β-tubulin. Nek7 can therefore immunoprecipitate with γ–tubulin, and coprecipitate with α- and β-tubulin and phosphorylate taxol-stabilized microtubules *in vitro*. Furthermore, interference with both Nek6 and Nek7 function appears to result in similar phenotypes, which may be as a result of both proteins acting in a dominant-negative manner to inhibit their common upstream activator, Nek9. Indeed immunoprecipitation experiments demonstrated that both Nek6 and Nek7 do indeed bind to Nek9. Assuming that Nek9 is required for their activation (Belham *et al.*, 2003), expression of Nek6 and Nek7 mutants could therefore interfere with this event.

Their differing localization may suggest that whilst Nek6 is more intimately involved in microtubule nucleation within the spindle itself, the role of Nek7 is more focused on microtubule nucleation and organization at the spindle pole. Identification of substrates of Nek7 will be key to elucidating how this kinase acts to regulate mitotic spindle formation and function.

The identification of Hsp70 and Cortactin A as specific substrates of Nek6 but not Nek7 is the first significant evidence of a functional distinction between these two highly similar proteins. It has not been clear whether two such closely related proteins are likely to have entirely redundant roles or whether their distinct N-termini allow differences in their regulation and function (O'Regan *et al.*, 2007). Given the sequence similarity of the kinase domain of these two proteins, it seems likely that any differences between them would arise as a result of their distinct N-termini and thus that the interaction between Nek6 and Cortactin A and Hsp70 may be regulated by the N-terminus of the protein. The generation of chimeric Nek6 and Nek7 proteins in which the N-termini are exchanged would facilitate the exploration of this possibility.

In summary, this chapter has described the identification and preliminary examination of four putative Nek6 substrates: β-tubulin, Cortactin A, Hsp70 and actin. One of these, β-tubulin is also likely to be a Nek7 substrate. Nek6 may form a multisubunit complex with β-tubulin and Hsp70 to facilitate mitotic spindle organization. Nek6 may also exist in a distinct complex with Cortactin A and actin to regulate cell abscission, perhaps

through regulation of membrane trafficking events during cytokinesis or alternatively through regulation of central spindle microtubules. Finally, the identification of distinct substrates for Nek6, which do not appear to either bind or be phosphorylated by Nek7, provides exciting evidence that these two highly similar kinases, may in fact be functionally distinct.

Chapter 8

Discussion

Of the eleven NIMA related kinases encoded by the human genome, four have so far been implicated in regulation of mitotic progression. These include Nek2, Nek9, Nek6 and Nek7 (O'Regan *et al.*, 2007). Nek6 and Nek7 are highly similar to one another and are unusual in that their sequence encodes little more than a catalytic domain and lacks the regulatory sequences typical of other family members. This similarity between Nek6 and Nek7 suggests that they may have arisen from a recent gene duplication event and that they may have redundant functions. Previous data had implicated Nek6 and Nek7 in regulation of mitosis downstream of another NIMA-related kinase, Nek9, which is thought to be responsible for the activatory phosphorylation of Nek6 and Nek7 in mitosis (Belham *et al.*, 2003). However, published data on Nek6 and Nek7 function was limited, often contradictory and little was known about the events downstream of these kinases. The work presented in this thesis therefore represents the first comprehensive analysis of the regulation and function of Nek6 and Nek7 kinases.

8.1 Cell cycle-dependent regulation of Nek6 and Nek7 activity

Previous data on the cell cycle-dependent regulation of Nek6 and Nek7 has often been conflicting. We have shown that consistent with a role in mitotic regulation, both proteins are upregulated in mitosis in terms of both protein abundance and kinase activity. It has been reported that Nek6 and Nek7 are activated by phosphorylation of two residues within their activation loop (T202 and S206 in Nek6 and T191 and S195 in Nek7) and that phosphorylation of the S206 residue is indispensable for Nek6 activity with T202 phosphorylation being of secondary importance. These residues are emerging as potentially conserved Nek phosphorylation sites important for activity. Of the equivalent sites in Nek2, S171 and T175, phosphorylation of T175 is likewise essential for Nek2 activity with S171 also phosphorylated but of lesser importance (Rellos *et al.*, 2007); the same is true of the equivalent two sites, S206 and T210, in Nek9 (Roig *et al.*, 2005). Analysis of the kinase activity of these sites in Nek6 and Nek7 in this study showed that the T206A and T195A mutants in Nek6 and Nek7, respectively, did indeed exhibit lower residual activity compared to the S202A and S191A mutants. We also confirmed that both Nek6 and Nek7 interact with Nek9. The activation loop of a protein kinase is a region of 20-25 amino acids defined by the presence of two flanking tripeptide motifs DFG and APE motifs (DLG and SPE in Nek6 and Nek7), respectively (Johnson *et al.*, 1996) and activation loop phosphorylation is the most common mechanism of kinase activity regulation (Nolan *et al.*, 2004). Phosphorylation of activation loop residues typically acts to alter the conformation of the kinase such that either the active form is stabilized or the inactive form destabilized. The ends of the activation loop are referred to as anchor points, and these are highly conserved among serine/threonine kinases and indeed between serine/threonine

kinases and tyrosine kinases (Nolan *et al.*, 2004). The N-terminal anchor contains a magnesium binding loop essential for binding the magnesium ion required to position phosphate groups for addition during phosphorylation. This is followed by a short region of β-sheet which typically is disrupted when the protein is in an inactive conformation, thus causing the collapse of the activation loop and distorting the magnesium binding loop (Hanks and Hunter, 1995; Johnson *et al.*, 1996; Nolan *et al.*, 2004). The C-terminal anchor contains regions essential for the interaction between the kinase and its substrate. The region between these segments is highly divergent, allowing for precise regulation and differing substrate specificities (Nolan *et al.*, 2004). Phosphorylation of activation loop residues causes the loop to change conformation, positioning itself for substrate binding, whilst being tethered at either end by the anchor points.

Identification of other potential upstream activators of Nek6 and Nek7 will also provide important insights into their function. Recently, further Nek7 activation loop phosphorylation sites were identified during a phosphoproteome analysis carried out to analyze to cell cycle regulation of protein kinases and identify phosphorylation sites (Daub *et al.*, 2008). This may suggest that Nek7, and indeed Nek6 are activated by other upstream regulators which may include members of the other classes of mitotic kinases. Several of the key mitotic regulators are known to have mechanisms of regulation that are interrelated; Plk1 is believed to regulate Cdk1 activity at the G2/M transition, for example, and the activation of Nek2 at the G2/M transition may be mediated, at least in part by the activity of Cdk1. Furthermore, it has been suggested that Cdk1 may also be an upstream activator of Nek9 (Roig *et al.*, 2002). Identification of different upstream kinases for Nek6 and Nek7 may provide an alternative basis for observed differences in localization and interaction.

8.2 Nek6 and Nek7 regulate mitotic spindle formation

Studies on the cell cycle-dependent localization of Nek6 and Nek7 have highlighted the first important difference between them: Nek7 appears to be a centrosomal protein (Yissachar *et al.*, 2006; Kim *et al.*, 2007), whilst Nek6 associates primarily with the microtubules of the mitotic spindle. The data presented here regarding localization of Nek7 to centrosomes is not conclusive as many cells did not show such localization. However, this may reflect the small levels of the total cellular pool of Nek7 which actually associate with the centrosome. Nek9, for example, was initially identified as a predominantly cytoplasmic protein with no specific localization to any intracellular structures either in interphase or mitosis (Roig *et al.*, 2002). However, the development of an antibody specific for the phosphorylated, activated form of Nek9 allowed the

identification of a small portion of total Nek9 at the centrosome during mitosis (Roig *et al.*, 2005). This may also apply to Nek7.

From a functional perspective, we have demonstrated that interfering with Nek6 or Nek7 either by RNAi depletion or expression of catalytically-inactive mutants, results in metaphase arrest and apoptosis. Furthermore, the apoptosis is a direct result of the delay in mitosis as inhibiting apoptosis resulted in a large increase in mitotic cells, whilst preventing cells from entering mitosis prevented apoptosis. This mitotic arrest phenotype is similar to that described upon interference with Nek9 function (Roig *et al.*, 2002; Roig *et al.*, 2005) or indeed, on interference with other mitotic kinases, such as Plk1 (Sumara *et al.*, van Vugt *et al.*, 2004) or Aurora A (Giet *et al.*, 2002; Lu *et al.*, 2006).

To ensure accurate distribution of sister chromatids, the SAC is activated until all kinetochores are attached to spindle microtubules. Once this is accomplished, activation of the APC/C then mediates the separation of sister chromatids, allowing anaphase to proceed (Mussacchio and Hardwick, 2002; Hoyt, 2001). Thus, a delay at metaphase is indicative of prolonged activation of the SAC. Hence, Nek6 and Nek7 kinase activity may be required for recruitment or activation of SAC components. Indeed several mitotic checkpoint proteins have been shown to regulate mitosis by phosphorylation (Abrieu *et al.*, 2001; Yin *et al.*, 2003). Alternatively, prolonged checkpoint activation may be due to insufficient kinetochore attachment or lack of tension across the mitotic spindle. The latter alternative is consistent with reports that Nek7 is involved in centrosome maturation and spindle nucleation (Kim *et al.*, 2007; Yissachar *et al.*, 2006) and also with the idea of a mitotic signaling cascade involving Nek9, which also appears to function in spindle organization through both centrosome- and Ran-GTP-dependent pathways (Roig *et al.*, 2005). Examination of the localization and activity of SAC components such as Mad2, will provide more detailed insights into the mechanism of the metaphase arrest.

Many key mitotic regulators are also centrosomal proteins which control microtubule nucleation and anchoring activity. Interference with their function typically results in defects in spindle assembly often accompanied by a reduction in centrosomal γ-tubulin. Here, the metaphase arrest seen prior to apoptosis in cells depleted of Nek6 or Nek7 or expressing catalytically-inactive mutants appeared to result from the formation of a less robust mitotic spindle. This was demonstrated both by quantitative imaging of spindle microtubule density and through testing spindle integrity in response to increasing doses of microtubule poisons. Importantly, there was also a reduction in centrosomal γ-

tubulin, although the effect on the microtubule nucleating activity of centrosomes has yet to be determined. Nonetheless, in view of the fact that Nek7 has been reported to recruit γ-tubulin to spindle poles and that Nek9 can interact with γ-tubulin (Kim *et al.*, 2007; Roig *et al.*, 2005), it seems likely that the mechanism underlying the metaphase defect may reflect a function for these kinases in centrosome maturation and/or microtubule nucleation and as such may arise at least in part from aberrant microtubule nucleation.

Among the fungal NIMA-related kinases, Fin1 is believed to have a role in microtubule nucleation and mitotic spindle formation. Indeed a *fin1* temperature-sensitive mutant was identified which formed monopolar spindles due to the fact that mutants were only able to nucleate spindle microtubules from one of the two SPBs (Grallert and Hagan, 2002). There is also evidence to suggest that NIMA itself may be directly involved in mitotic spindle formation as overexpression of NIMA results in formation of transient spindle-like structures (Osmani *et al.*, 1988).

However, the localization of Nek6 to microtubules of the mitotic spindle, along with its ability to sediment with microtubules and phosphorylate β-tubulin *in vitro*, suggests that Nek6 may act within the spindle itself to promote and amplify microtubule nucleation or stability (Goshima *et al.*, 2008; Mahoney *et al.*, 2006). This could result from phosphorylation and/or recruitment of tubulin isoforms, spindle-associated γ-TuRC binding factors such as Augmin, or other MAPs, whose microtubule association is required for spindle integrity. Indeed, the *Arabidopsis* Nek6 orthologue has been shown to localize to, and interact with, γ-tubulin complexes on cortical microtubules (Motose *et al.*, 2008). Moreover, this kinase can interact with the microtubule-dependent armadillo repeat-containing kinesin motor protein, ARK1 (Sakai *et al.*, 2008). Kinesins are important regulators of the mitotic spindle apparatus as well as of centrosome separation. Hence, Nek6 may have widely conserved roles in microtubule and spindle organization in animal and plant cells.

8.3 Nek6 and Nek7 regulate cytokinesis

The generation of mutants with intermediate levels of kinase activity, so called hypomorphic mutants, resulted in the serendipitous discovery that Nek6 and Nek7 may also be required for mitotic exit. Hence, they may, like many other mitotic kinases, act to control more than one aspect of mitosis. Indeed, roles in cytokinesis have been proposed for NIMA-related kinases in lower eukaryotes: fission yeast Fin1, for example, regulates components of the SIN pathway, whilst *Drosophila* DmNek2 is required for

proper organization of anillin and actin at the cleavage furrow (Grallert *et al.*, 2004; Prigent *et al.*, 2005). It should be noted that we have not yet ruled out the possibility that the failure in mitotic exit results from aberrant metaphase progression. Nonetheless, it is exciting to speculate that Nek6, and possibly Nek7, may function in cytokinesis, perhaps to stabilize the microtubules of the central spindle and midbody.

In this respect, our results are similar to those seen upon depletion of Borealin, a member of the CPC. RNAi depletion of Borealin resulted in cells which progressed through the early stages of mitosis normally, ultimately forming an apparently normal metaphase plate; however, the spindle then began to lose its integrity resulting in aberrant mitotic exit (Gassmann *et al.*, 2004). This phenotype is believed to relate to the function of the CPC in delivering Aurora B to the sites of MAPs, where it may regulate their activity by phosphorylation (Lan *et al.*, 2004). Furthermore, the requirement of the CPC for cytokinesis appears to arise, at least in part, from its role in microtubule stabilization. During mitotic exit, the CPC associates with material that coats interpolar microtubules and the spindle midzone and may act to stabilize these microtubules during cytokinesis (Ruchard *et al.*, 2007).

Proper microtubule organization is critical not only during early mitosis but also to the proper regulation of cytokinesis. In particular, the astral and interpolar microtubule populations contribute to cytokinesis progression. Astral microtubules typically elongate during anaphase in order to touch the cortex and extend towards the equator of the cell which is believed to be important for microtubule signaling and thus regulation of microtubule morphogenesis during cytokinesis (Schuster and Burgess, 1999; Strickland *et al.*, 2005). The interpolar microtubules bundle into the microtubules of the spindle midzone and eventually into the midbody where they play essential roles in signaling as well as in keeping separated genomes apart (Straight *et al.*, 2003). Although initially formed from bundled interpolar microtubules, the midzone soon becomes self-organizing and it is likely that new microtubules are then nucleated from within the midzone itself: γ-tubulin has been shown to transiently associate with the spindle midzone during cytokinesis and this association is essential for its proper completion (Desai and Mitchison, 1997; Rusan and Wadsworth, 2005; Shu *et al.*, 1995; Straight *et al.*, 2003; Eggert *et al.*, 2006). This is reminiscent of an organizational pathway observed in the metaphase spindle in which the localization of γ-tubulin to the mitotic spindle microtubules themselves is thought to reflect another non-centrosomal pathway for spindle microtubule generation in which augmin and γ-tubulin function cooperatively to amplify microtubule numbers (Lajoie-Mazenc *et al.*, 1994; Luders *et al.*, 2006; Goshima *et al.*, 2008; Mahoney *et al.*, 2006). In view of the cytokinesis defect seen and

the fact both Nek6 and Nek7 specifically interact with γ-tubulin at all stages of the cell cycle, we speculate that the fragile metaphase spindle phenotype seen upon interference with Nek6 function may reflect a failure in this pathway and thus a role for Nek6 in promoting the interaction of γ-tubulin with spindle microtubules. If a similar pathway of microtubule nucleation acts during cytokinesis, it is interesting to speculate that the cytokinesis defect seen upon interference with Nek6 and Nek7 function may reflect a conserved role in this pathway. Thus, the functions of Nek6 at during both early and late mitosis may similarly reflect a conserved role in microtubule nucleation either at the spindle poles or from within the metaphase spindle or the central spindle.

Alternatively, the interaction of Nek6 with Cortactin A and actin may underpin a completely unexpected role of Nek6 in membrane trafficking or actin organization during cell abscission. An emerging theme among the mitotic kinases is that those which play roles in organizing the centrosome and the early mitotic spindle also appear to have roles in cytokinesis (Glotzer, 2005). Polo-like kinases for example, localize to the MTOCs in early mitosis and are important for centrosome maturation (Donaldson *et al.*, 2001; do Carmo Avides *et al.*, 2001; Lane and Nigg, 1996; Casenghi *et al.*, 2003) and as cells progress through mitosis typically the Plks become redistributed to the central spindle and midbody and are important for cytokinesis. Thus, the fission yeast Plo1 is thought to act upstream of the SIN (Tanaka *et al.*, 2001), *Drosophila* Polo is important for localization of kinesins to the midbody and contractile ring formation (Carmena *et al.*, 1995), and Plk1 appears to regulate the localization of RhoA to the contractile ring (Brennan *et al.*, 2007; Petroczki *et al.*, 2007; Santamaria *et al.*, 2007). Further, Aurora kinases also play important roles in stabilization of the mitotic spindle microtubules and are essential for cytokinesis (Giet and Prigent, 2000;Gasmann *et al.*, 2004; Adams *et al.*, 2001; Sampath *et al.*, 2004; Giet and Glover, 2001; Carvalho *et al.*, 2003). Among the NIMA-related kinases, Fin1 is also important in modulating the SIN pathway (Grallert *et al.*, 2004), and indeed there is some evidence that Nek2, which has well-defined roles in centrosome function, may play a role in cytokinesis as specific depletion of the Nek2B isoform delays mitotic exit (Fletcher *et al.*, 2005).

In summary, it appears that Nek6 and Nek7 may function at early and late stages of mitosis both to regulate the formation and stability of the mitotic spindle and to regulate cytokinesis.

8.4 Identification of Nek6 substrates

In an exciting development, we used KESTREL screening and coimmunoprecipitation approaches to identify the first physiological substrates of human Nek6. This is a critical step in the functional characterization of a kinase as only identification of downstream targets properly reveals how they may carry out particular functions. Thus, the identification not only of β-tubulin, but also Hsp70, Cortactin A and actin as putative Nek6 substrates has provided important new insights into how this kinase may regulate different aspects of mitotic progression.

The first substrate of Nek6 identified as a result of the KESTREL screen was β-tubulin. The functional data on Nek6 together with the identification of a core component of the mitotic spindle, β-tubulin, as a potential Nek6 substrate, suggests that Nek6 may have a direct role in regulation of spindle microtubules themselves. Both Nek6 and Nek7 cosedimented with microtubules and were capable of phosphorylating taxol-stabilized microtubules. Microtubule regulation is not only carried out through the post-translational modification of MAPs but also through post-translational modification of microtubules themselves (MacRae, 1997; Westermann and Weber, 2003). Such modifications may act either to directly affect microtubule dynamics or to regulate the recruitment of and interaction with effector proteins (Verhey and Gaertig, 2007). In this manner, tubulin phosphorylation may play a direct role in controlling mitotic events such as spindle positioning, spindle dynamics, microtubule stabilization and cytokinesis. Microtubules themselves can be phosphorylated by several kinases (Westermann and Weber, 2003). Notably, Cdk1 phosphorylates β-tubulin on Ser172 and this is believed to be important for regulation of microtubule dynamics during mitosis (Fourest-Lieuvin *et al.*, 2006; Verhey and Gaertig, 2007). Thus, the phosphorylation of β-tubulin may reflect another route by which Nek6, and indeed perhaps Nek7, may regulate mitotic spindle stability.

Both the KESTREL screen and the coimmunoprecipitation approach identified Hsp70 as another substrate of Nek6. Indeed, preliminary evidence suggests that Hsp70 and Nek6 may act together to regulate formation and action of the mitotic spindle. Hsp70 was found to localize to microtubule based structures, including spindle poles and microtubules, in both metaphase and anaphase cells, as well as the central spindle and midbody of late mitotic cells. Likewise, interference with Hsp70 function has been shown to result in aberrant mitosis, often as a result of the formation of abnormal, fragile spindles (Sconzo *et al.*, 1999; Oka *et al.*, 1998). Furthermore, several Hsp70s are well-characterized MAPs (Liang and MacRae, 1997) and RNAi depletion of Nek6, but not

Nek7, resulted in a decreased affinity of Hsp70 for α- and β-tubulin *in vitro*, suggesting that the interaction with, or localization to, microtubules of Hsp70 is Nek6-dependent. Therefore, although the exact basis for this interaction is unknown, we speculate that Nek6 may regulate the localization or interaction of Hsp70 with microtubules at different points in mitosis, thereby contributing to spindle organization and cytokinesis. Moreover, the fact that the putative Nek6 substrates cofractionated in the KESTREL screen, raises the possibility that these proteins exist in a complex with Nek6 perhaps acting to dock β-tubulin and Hsp70 together.

Intriguingly, the best substrate identified for Nek6 in the KESTREL screen was Cortactin A. This was quite unexpected as this actin binding protein is implicated in regulation of membrane dynamics during endocytosis rather than in regulation of mitosis (Daly, 2004; Buday and Downward, 2007). However, it is known that cell abscission requires careful coordination of actomyosin ring contraction with membrane delivery (Albertson *et al.*, 2005). As hypomorphic Nek6 mutants resulted in a delay in cytokinesis and both Nek6 and Cortactin A localized to the cleavage furrow and midbody in late mitotic cells, we postulate that Nek6 may have a role in regulating cell abscission through Cortactin A phosphorylation. Furthermore, this implies that Cortactin A is important for membrane delivery during abscission. Thus, the function of Nek6 in cytokinesis may be either in coordination of membrane trafficking events, or in stabilization of central spindle microtubules or, perhaps, a combination of the two.

Intriguingly, it was recently shown that tyrosine-phosphorylated Cortactin A interacts with γ-tubulin, localizes to centrosomes and spindle poles and is important for actin-mediated centrosome separation in early mitosis (Wang *et al.*, 2008). However, no defect in centrosome separation was observed upon interference with Nek6 function in our experiments. Nevertheless, it remains a possibility that the interaction of Nek6 with Cortactin A may have a function related to γ-tubulin localization. The results of the KESTREL screen indicate that Nek6 may exist in the cell in two complexes, one with Cortactin A and one without. Again, this may reflect the different roles for Nek6 in mitotic spindle organization and cytokinesis. The substrates of Nek6 and their potential contribution to the mitotic functions of Nek6 are summarized in figure 8.1.

Whilst Nek7 did not appear to either interact with or phosphorylate Hsp70 or Cortactin A, Nek7 did cosediment with microtubules and interact with α/β/γ-tubulin. Furthermore, interference with both Nek6 and Nek7 function resulted in similar phenotypes. However, this may be the result of both proteins acting in a dominant-negative manner to inhibit their common upstream activator, Nek9. Indeed, immunoprecipitation

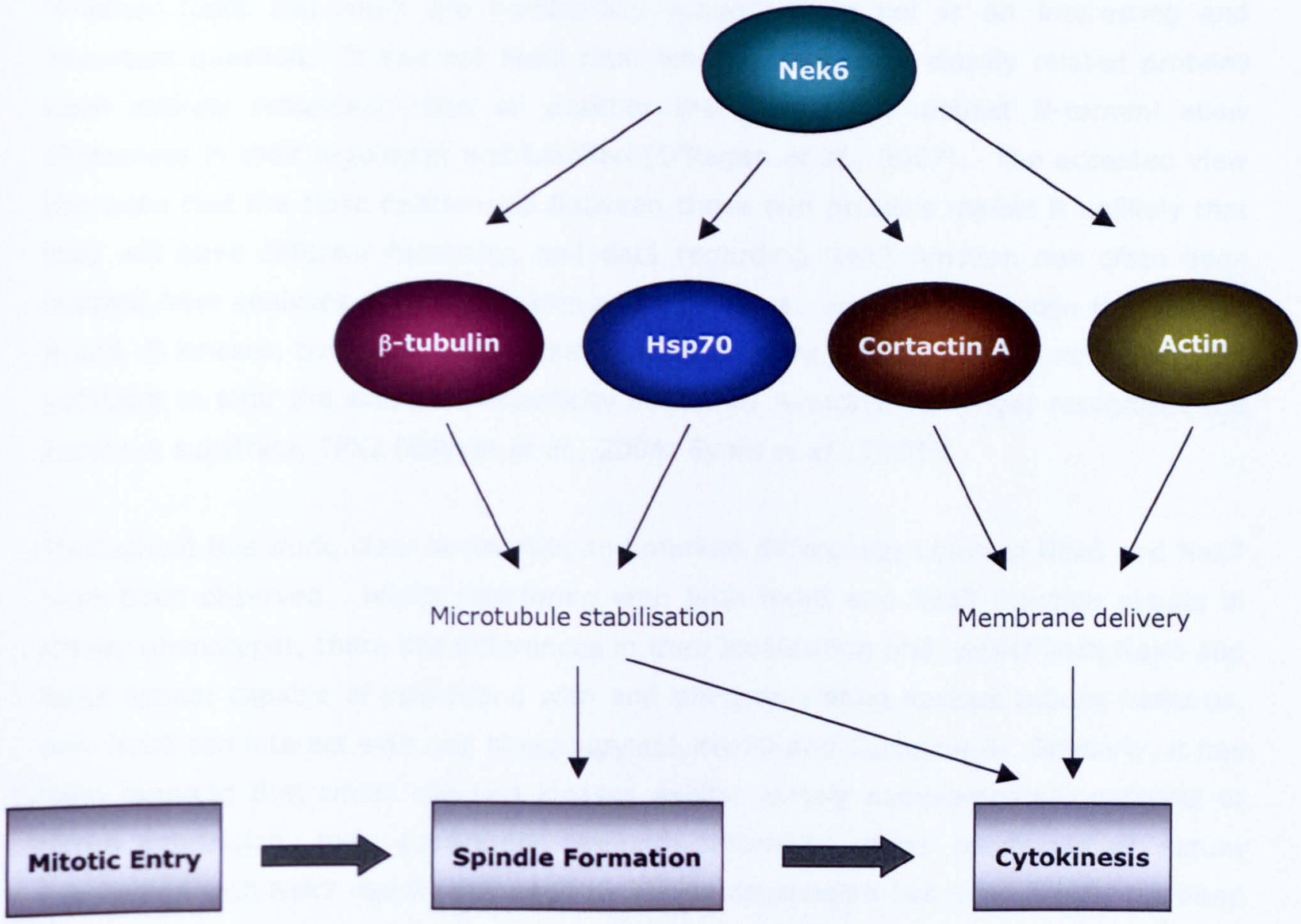


Figure 8.1 Nek6 substrates and their possible contributions to mitosis
Nek6 phosphorylation of β-tubulin and Hsp70 may act in early mitosis to recruit them to the mitotic spindle, to each other, or to facilitate the recruitment of other microtubule stabilizing elements to the mitotic spindle to contribute to spindle formation and integrity. In late mitosis these may act in a similar manner to stabilize the microtubules of the central spindle and midbody and so contribute to cytokinesis. However, Nek6 phosphorylation of Cortactin A and actin raises the possibility of an unsuspected role in recruitment of elements necessary for membrane delivery to the cleavage furrow.

experiments demonstrated that both Nek6 and Nek7 do indeed bind to Nek9. Further, consistent with Nek9 being required for their activation (Belham *et al.*, 2003), we found that expression of Nek6 or Nek7 mutants interfered with activation of the complementary kinase.

8.5 Nek6 and Nek7: are they functionally distinct?

Whether Nek6 and Nek7 are functionally redundant or not is an interesting and important question. It has not been clear whether two such closely related proteins have entirely redundant roles or whether their short but distinct N-termini allow differences in their regulation and function (O'Regan *et al.*, 2007). The accepted view has been that the close relationship between these two proteins makes it unlikely that they will have different functions, and data regarding Nek7 function has often been inferred from analyses of Nek6 function and vice versa. However, between the Aurora-A and -B kinases, two other very closely related kinases, a single amino acid change is sufficient to alter the substrate specificity such that Aurora-B no longer recognizes the Aurora-A substrate, TPX2 (Bayliss *et al.*, 2004; Eyers *et al.*, 2005).

Throughout this work, clear similarities and marked differences between Nek6 and Nek7 have been observed. Whilst interfering with both Nek6 and Nek7 function results in similar phenotypes, there are differences in their localization and, whilst both Nek6 and Nek7 appear capable of interacting with and phosphorylating various tubulin isoforms, only Nek6 can interact with and phosphorylate Hsp70 and Cortactin A. Similarly, it has been reported that whilst the two kinases exhibit largely complementary patterns of tissue expression, they apparently respond differently under conditions of serum deprivation with Nek7 rapidly activated by serum deprivation but Nek6 rapidly inhibited (Feige and Motro, 2002; Minoguchi *et al.*, 2003).

The fact the RNAi depletion of either Nek6 or Nek7 resulted in a strong phenotype, namely a delay in metaphase progression followed by apoptosis, suggests that loss of either kinase is not complemented by the presence of the other. It is though possible that the combined amount of Nek6 and Nek7 activity is critical and depletion of one or the other is sufficient to give a haploinsufficient phenotype. However, the identification of differences in mitotic localization between Nek6 and Nek7 as well as Nek6-specific substrates suggests that there is a functional difference between these two kinases. This raises the question of why almost identical phenotypes were observed upon expression of either Nek6 or Nek7 kinase-inactive mutants. The most obvious explanation for this would be that Nek6 and Nek7 act in a dominant-negative manner to inhibit a common upstream activator, which may well be Nek9. It has been shown, both

in this report and in other published data, that both Nek6 and Nek7 bind to Nek9. Thus, assuming that Nek9 is indeed required for activation of endogenous Nek6 and Nek7, expression of either Nek6 or Nek7 mutants could effectively sequester the entire cellular pool of Nek9 and thus interfere with this event.

In conclusion, identification of differences in Nek6 and Nek7 localization and substrates provides evidence for significant differences between these two proteins (summarized in Figure 8.2). Identification of Nek7 specific substrates as well as determining whether it is the distinct N-termini of Nek6 and Nek7 which result in these differences will provide new insights into the basis for these differences.

8.6 Nek6 and Nek7 as chemotherapeutic targets

Interference with Nek6 and Nek7 induces a marked growth inhibition, mitotic arrest and apoptosis. This has been shown in stable cell lines as well as following transient expression (this study; Yin *et al.*, 2003). Furthermore, apoptosis followed mitotic arrest and was dependent upon entry into mitosis. Thus, Nek6 and Nek7 represent exciting new targets for the development of chemotherapeutic small molecule inhibitors that could be used to treat hyperproliferative disorders such as cancer.

Small molecules can act to inhibit kinases in several ways, for example via competition with ATP for the ATP-binding site in the catalytic pocket of the kinase or stabilization of the kinase in an inactive conformation. As a result there are a rapidly increasing number of kinases being developed as drug targets. Of the more than 500 kinases present in the human genome, 25 are the subject of clinical trials with putative inhibitors, whilst several such compounds are currently licensed for clinical use (Dancey and Sausville, 2003). Aside from the ability to induce apoptosis in dividing cells, a correlation between aberrant expression or mutation of a gene and the initiation or progression of cancer is an important criterion to be considered when identifying new therapeutic targets in human cancer. The Nek2 kinase has been shown to be upregulated in cell lines derived from a variety of different cancer cells and its expression is significantly increased in the majority of breast tumours (Hayward *et al.*, 2004). Overexpression of Nek2 causes aneuploidy, whilst interference with Nek2 function has been shown to inhibit cell growth in HeLa cells (Fletcher *et al.*, 2004), making it an attractive target for chemotherapeutic intervention in breast cancer (Hayward *et al.*, 2004).

It is reported that Nek6 interacts with the peptidyl-prolyl isomerase, Pin1, in the hepatocellular carcinoma (HCC) cell line, Hep3B, and that both of these proteins are upregulated in HCC (Chen *et al.*, 2006). Not only is Pin1 essential for cell cycle

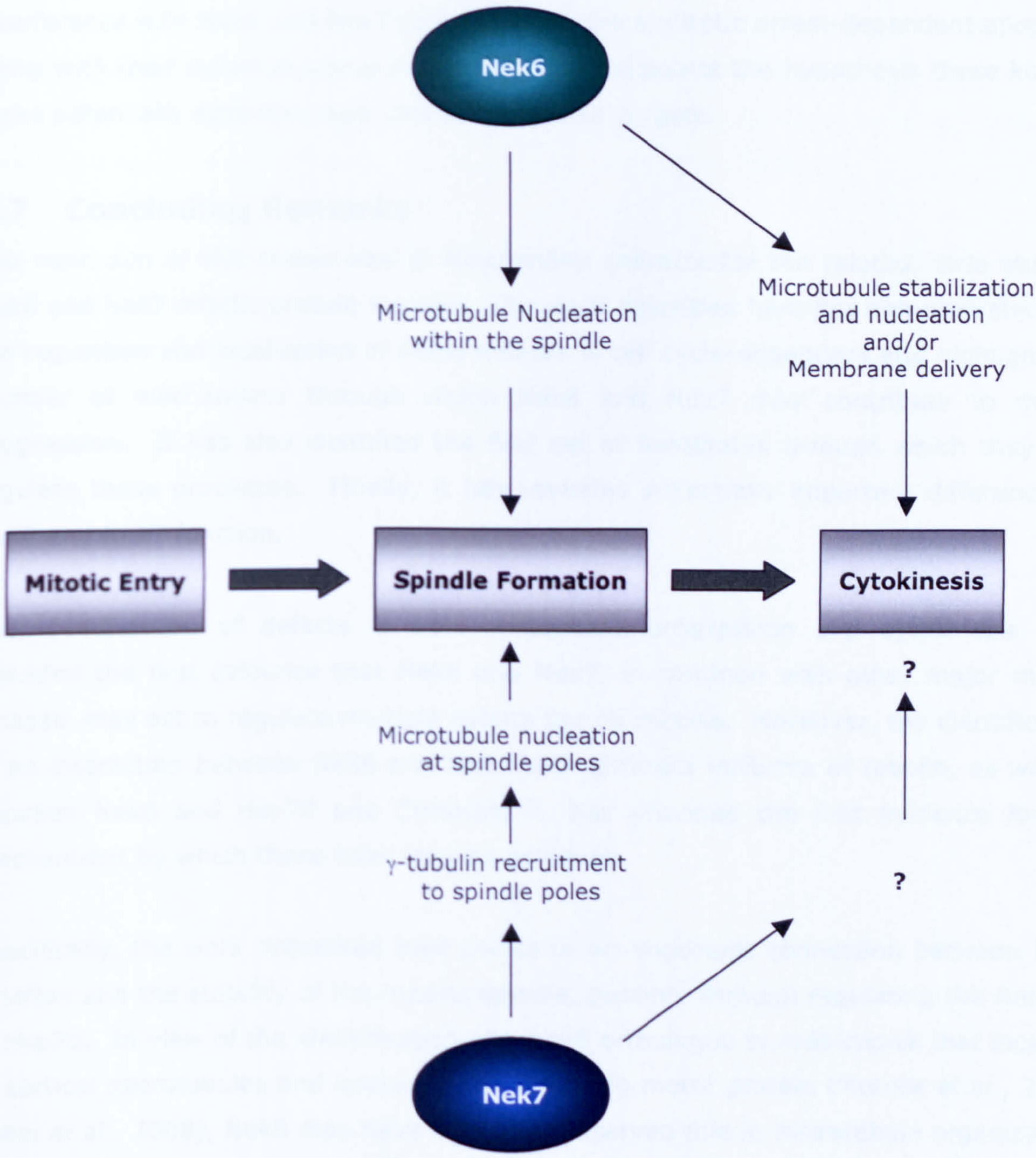


Figure 8.2 Differences in the postulated roles of Nek6 and Nek7
Whilst the localization and substrates of Nek6 suggest that its role in mitotic spindle organization may involve nucleation and stabilization of microtubules within the spindle itself, the centrosomal localization of Nek7 points to a role in microtubule nucleation from spindle poles. Nek6 may also act at cytokinesis either to stabilize the microtubules of the central spindle and midbody or in coordination of membrane delivery to the cleavage furrow. Whether Nek7 also has a role in cytokinesis is, as yet, unclear.

progression in yeast and mammalian cells, but its depletion induces a similar mitotic arrest/apoptosis phenotype as seen upon interference with Nek6 and Nek7 function (Shen *et al.*, 1998). Pin1 is prevalently overexpressed in many human cancers and may be involved in hepatocarcinogenesis (Lu, 2004; Pang *et al.*, 2004). Hence, the fact that interference with Nek6 and Nek7 function produces a mitotic arrest-dependent apoptosis along with their potential upregulation in cancer, supports the hypothesis these kinases make potentially attractive new chemotherapeutic targets.

8.7 Concluding Remarks

The main aim of this thesis was to functionally characterize the related, little studied, Nek6 and Nek7 mitotic protein kinases. The work described here has demonstrated that the regulation and localization of these kinases is cell cycle-dependent and highlighted a number of mechanisms through which Nek6 and Nek7 may contribute to mitotic progression. It has also identified the first set of substrates through which they may regulate these processes. Finally, it has revealed potentially important differences in Nek6 and Nek7 function.

The identification of defects in both metaphase progression and cytokinesis have provided the first evidence that Nek6 and Nek7, in common with other major mitotic kinases, may act to regulate multiple events during mitosis. Moreover, the identification of an interaction between Nek6 and Nek7 with different isoforms of tubulin, as well as between Nek6 and Hsp70 and Cortactin A, has provided the first evidence for the mechanisms by which these roles may be achieved.

Specifically, the work presented here points to an important connection between Nek6 function and the stability of the mitotic spindle, possibly through regulating the function of Hsp70. In view of the identification of a Nek6 orthologue in *Arabidopsis* that localizes to cortical microtubules and interacts with a kinesin motor protein (Motose *et al.*, 2008; Sakai *et al.*, 2008), Nek6 may have a widely conserved role in microtubule organization. Meanwhile, the interaction of Nek6 with Cortactin A, along with the cytokinesis defects observed upon interference with Nek6 function, may reflect a previously unsuspected role for Nek6 in membrane delivery during cytokinesis.

Importantly, this work provides the first evidence for real differences between the closely related Nek6 and Nek7 and suggests that these two kinases may act in different, but related, pathways to effect nucleation and organization of the mitotic spindle. Hence, the functions of Nek6 and Nek7 may reflect a broader conservation of functionality among Neks in the organization of microtubules (O'Regan *et al.*, 2007).

Clearly, much work remains to be done in order to fully elucidate the pathways by which Nek6 and Nek7 contribute to mitotic regulation. In particular, the identification of substrates of Nek7 would provide an important new perspective on its role in mitosis. Moreover, validation of the Nek6 substrates through phosphosite mapping and mutagenesis studies is clearly essential. However, the work presented here has nonetheless provided important new insights into the mechanisms by which Nek6 and Nek7 may regulate multiple events at the different stages of mitosis.

Finally, I have demonstrated that, as inhibition of either Nek6 or Nek7 leads to apoptosis specifically in dividing cells, these kinases make exciting new candidates for the development of anti-cancer drugs.

Chapter 9

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Appendix

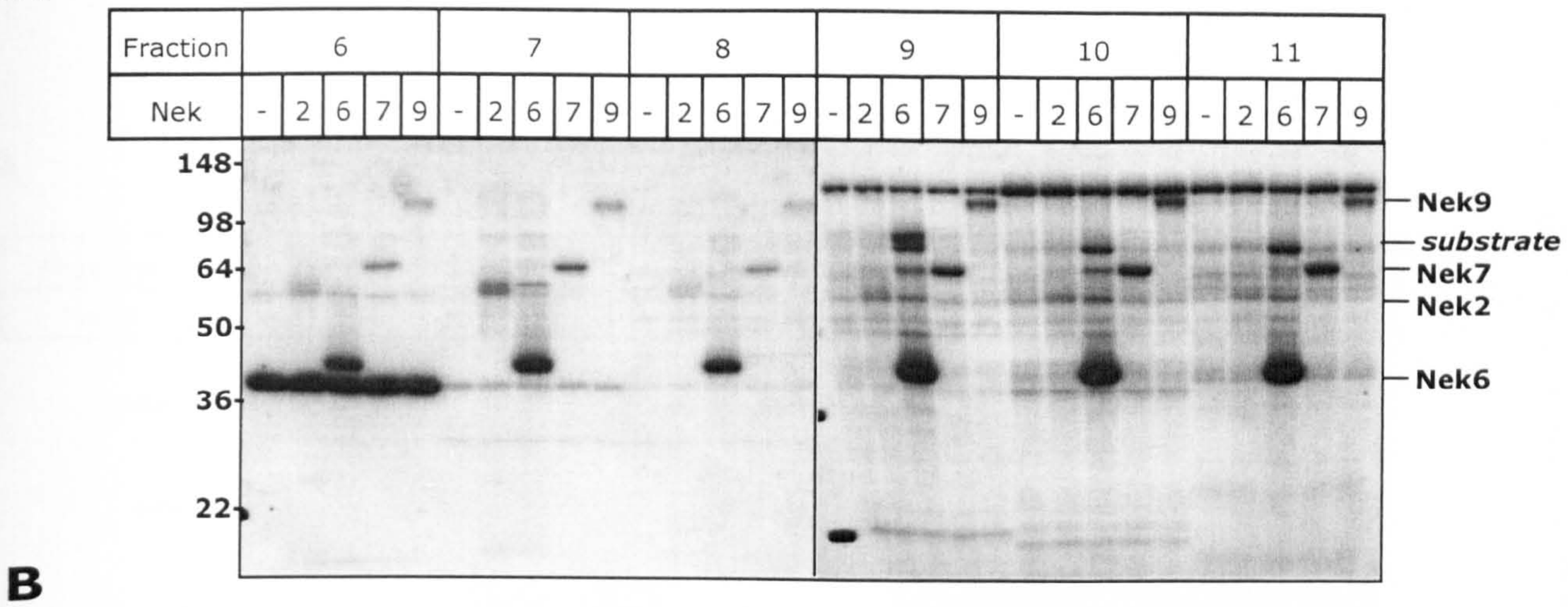
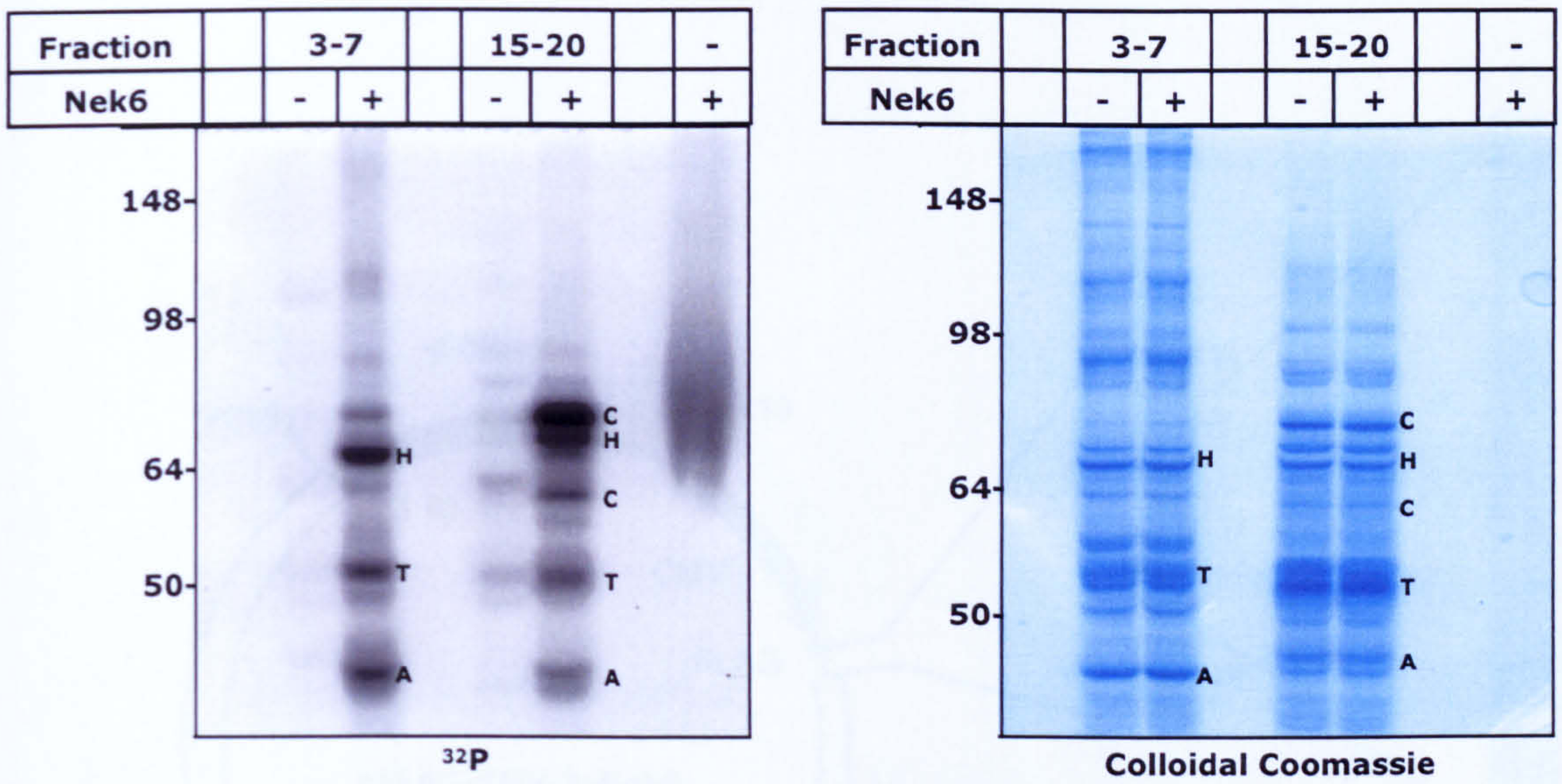


Figure A.1 KESTREL analysis for putative Nek6 and Nek7 substrates

(**A**) Desalted HEK 293 cytosolic extract was chromatographed on heparin sepharose. Aliquots of the fractions were incubated with Mn^{2+}-[γ^{32}P]-ATP in the absence (-) or presence of 1 μg/ml Nek2, Nek6, Nek7 or Nek9, before analysis by SDS-PAGE and autoradiography. (**B**) 1000 mg HEK 293 cytosol was prepared, desalted, chromatographed on heparin sepharose and analysed for the presence of Nek6 substrates. The desalted extract (Id) and fractions 1 – 23 are shown. A cluster of substrates (substr.) eluted in fractions 13 – 20 and later. (**C**) Heparin fractions 16 to 18 were pooled, desalted, chromatographed on Source 15 Q and analysed as in (B). (**D**) Fractions 16 to 18 of the Q-column were subjected to chromatography on a Superdex 200 column and analysed as in (B). Experiments were performed by Dr Axel Knebel, Kinasource Ltd, Dundee.

A



B

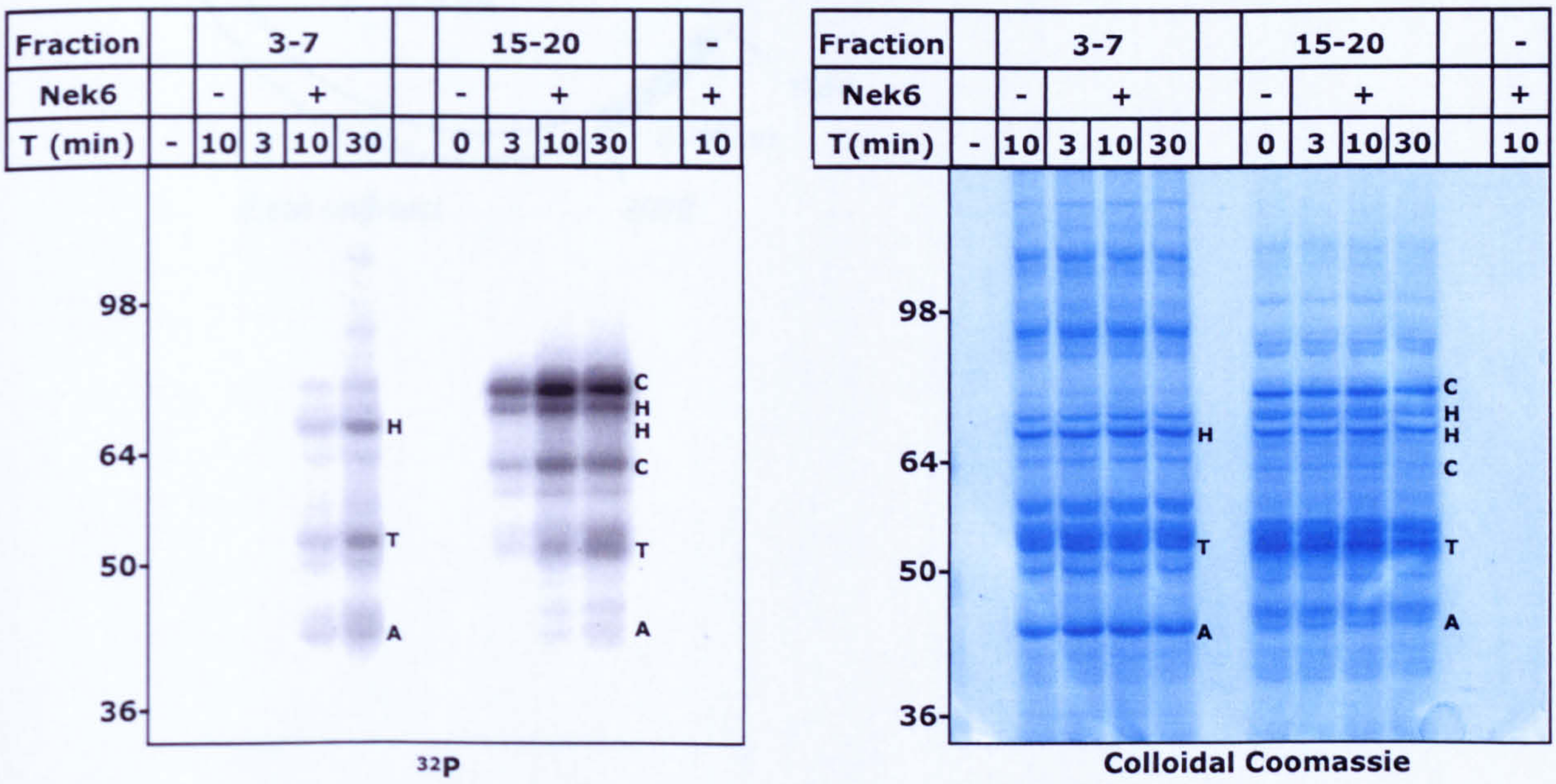
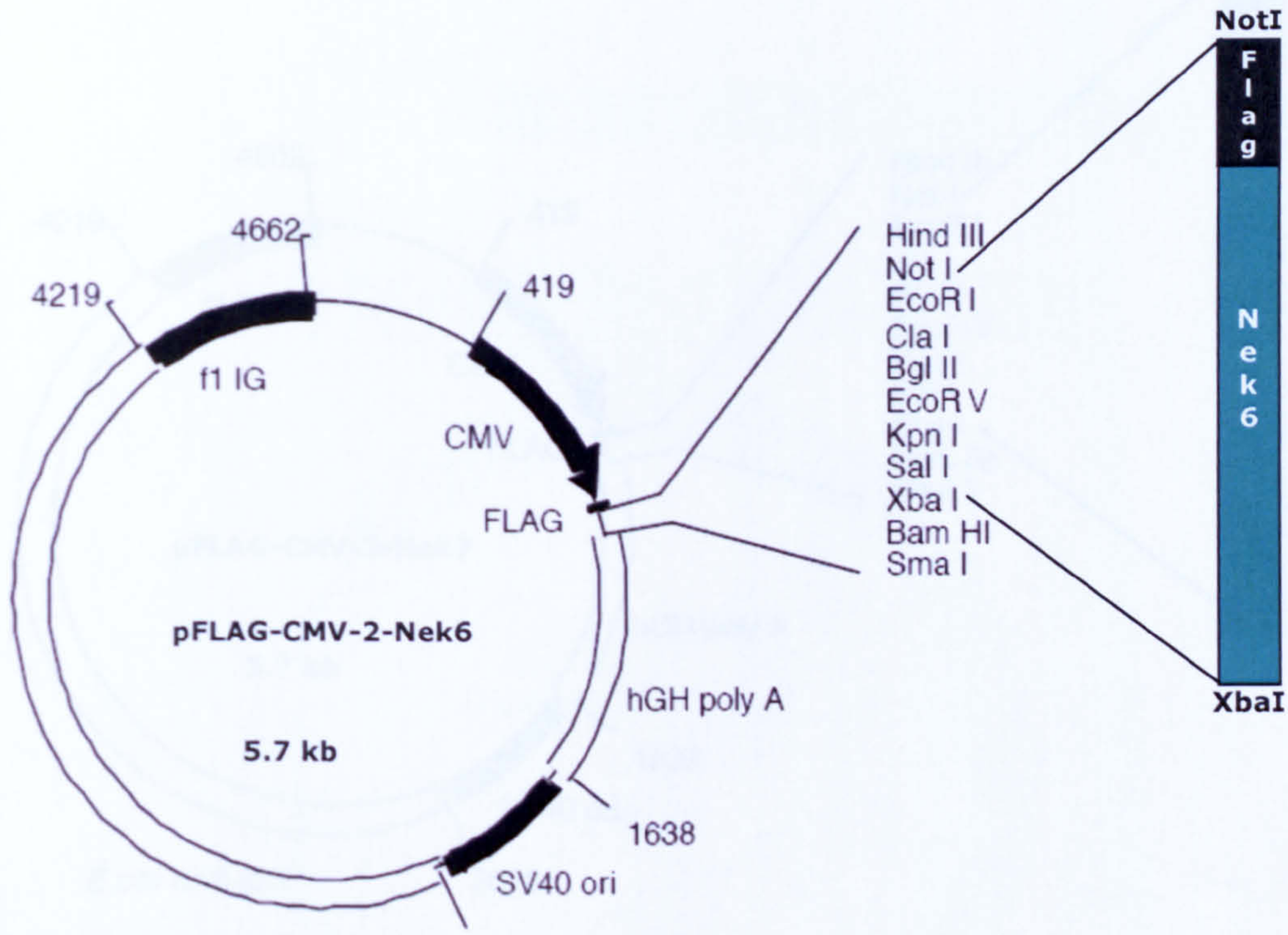


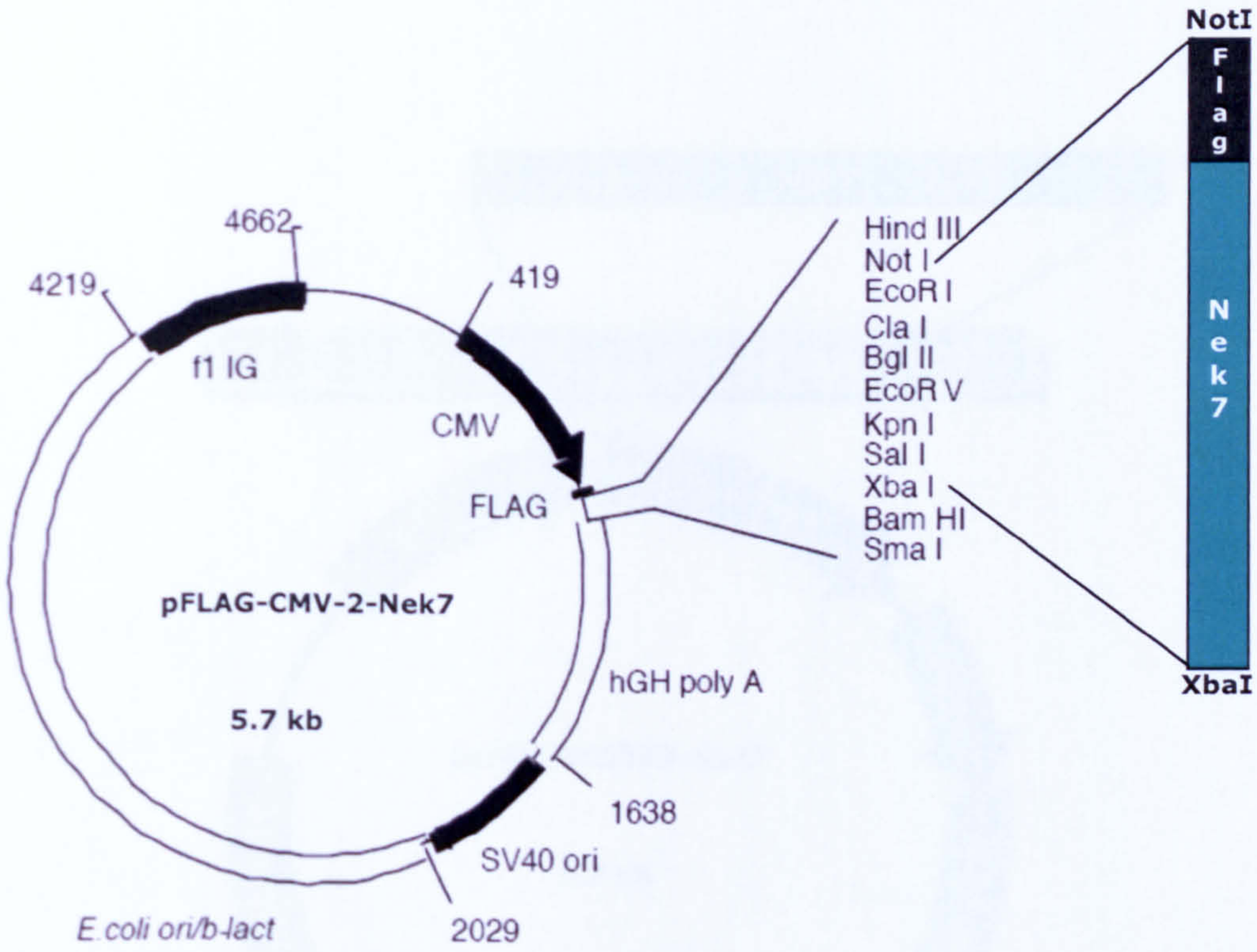
Figure A.2 Identification of Nek6 substrates using a KESTREL screen

(**A**) Substrate-containing Superdex fractions 3 to 7 or 15 to 20, respectively, were pooled, desalted, incubated with Mn^{2+}-[γ^{32}P]-ATP in the absence (-) or presence (+) of 1 μg/ml Nek6 and separated by SDS-PAGE. The gel was stained with colloidal Coomassie Blue and analysed by autoradiography. Bands which were radiolabelled in the presence of Nek6 were excised, digested with trypsin and subjected to identification by Mass Spectrometry Fingerprinting. Several bands at 70 kDa were identified as Hsp70 (H), bands at 80 kDa and at 62 kDa were cortactin A (C), a band at 55 kDa was β-tubulin (T) and bands at 45-47 kDa were isoforms of actin (A). (**B**) The pooled Superdex fractions from B were incubated with 0.1 mM Mn^{2+}-[γ^{32}P]-ATP for 3, 10 or 30 min and analysed as in B. Experiments were performed by Dr Axel Knebel, Kinasource Ltd, Dundee.

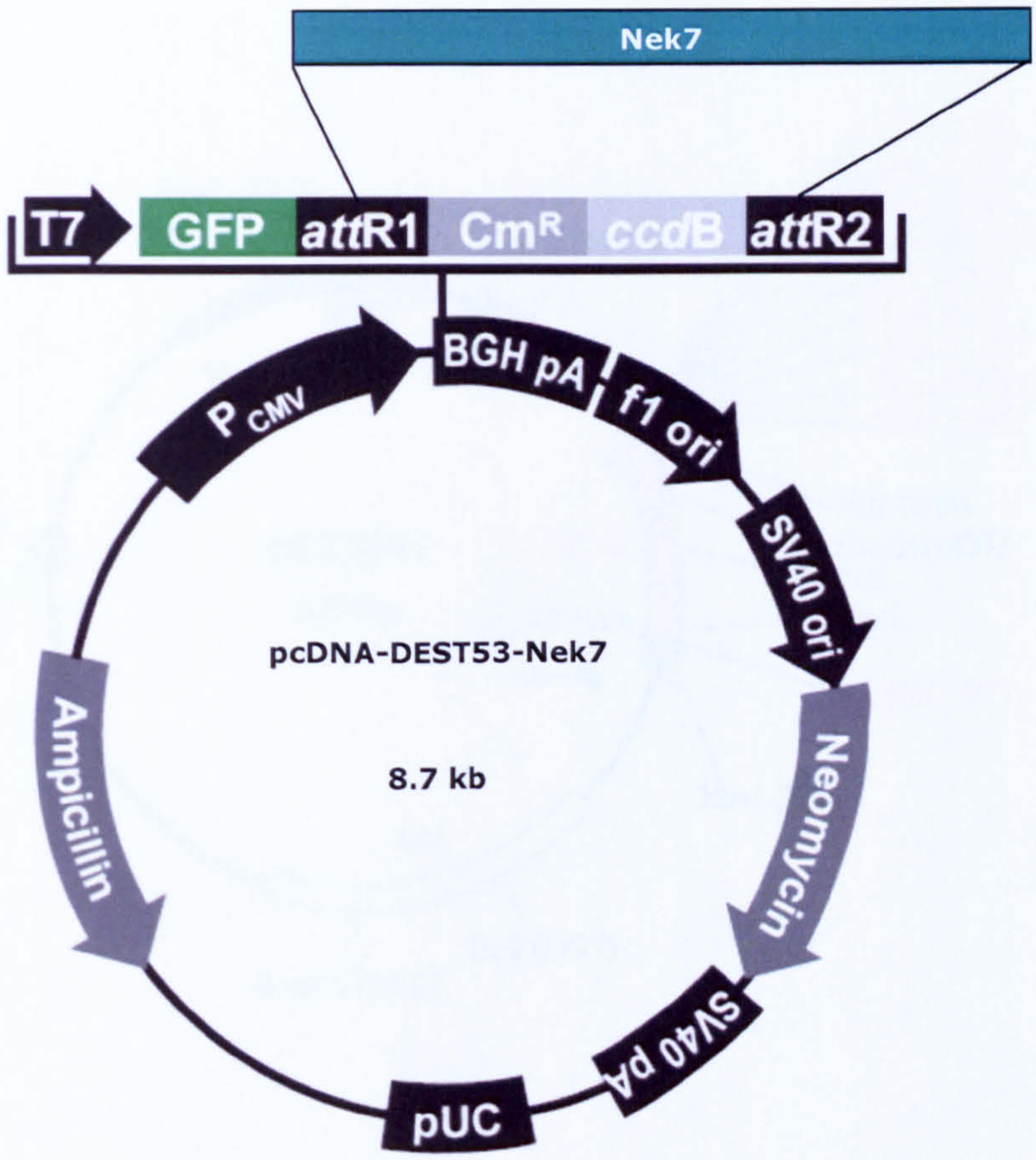


E.coli ori/b-lact	2029

pFLAG-CMV-2-Nek6	generated by cloning Nek6 into pFLAG-CMV-2 using NotI and XbaI restriction sites
pFLAG-CMV-2-Nek6-K74M	generated via site directed mutagenesis of Lysine-74 (AAG) to methionine (ATG)
pFLAG-CMV-2-Nek6-K75M	generated via site directed mutagenesis of Lysine-75 (AAG) to methionine (ATG)
pFLAG-CMV-2-Nek6-K74MK75M	generated via site directed mutagenesis of Lysine-74 and Lysine-75 (AAG) to methionine (ATG)
pFLAG-CMV-2-Nek6-S202A	generated via site directed mutagenesis of Serine-202 (AGC) to Alanine(GCC)
pFLAG-CMV-2-Nek6-T206A	generated via site directed mutagenesis of Serine-206 (AGC) to Alanine(GCC)



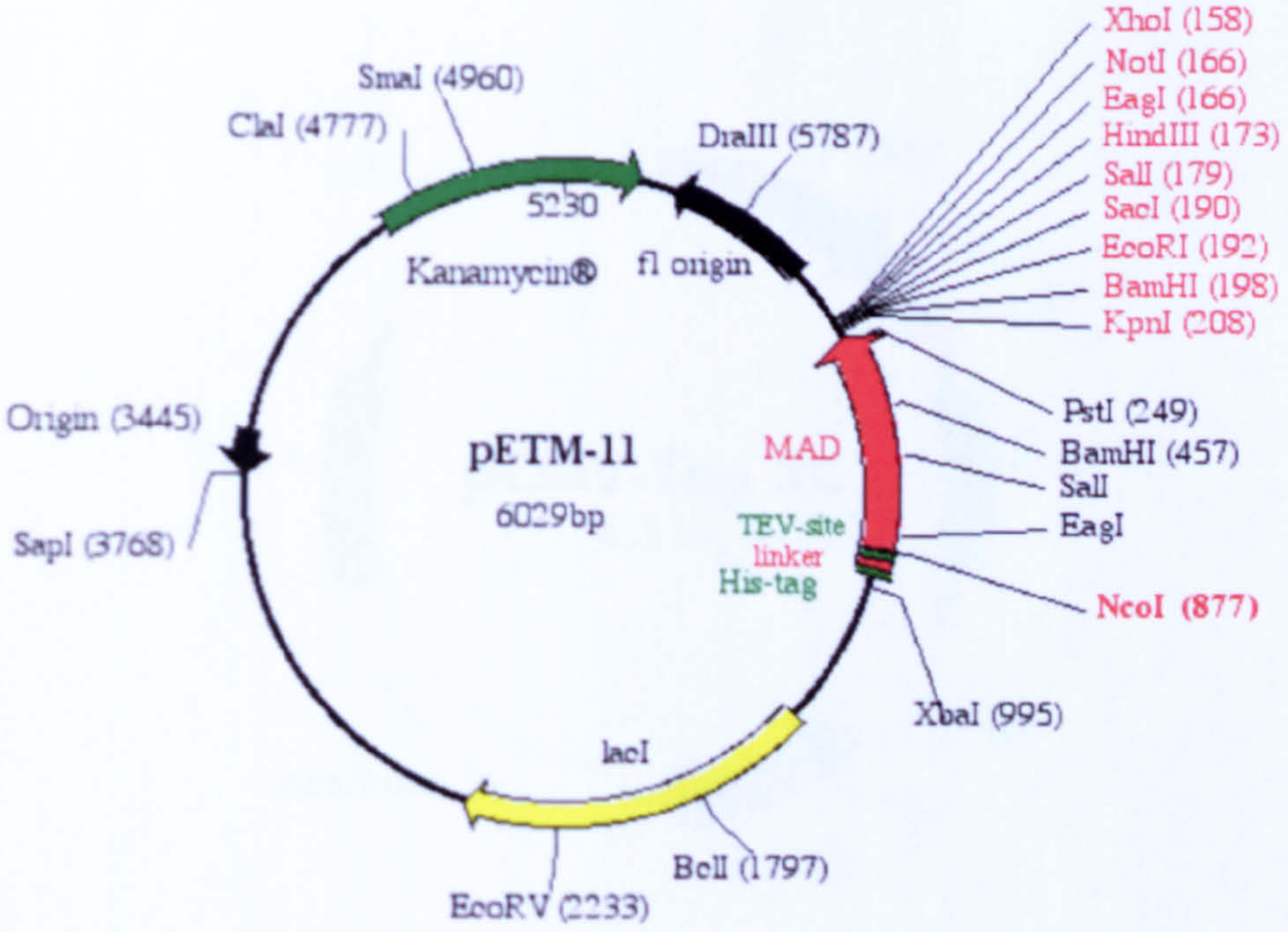
pFLAG-CMV-2-Nek7	generated by cloning Nek7 into pFLAG-CMV-2 using NotI and XbaI restriction sites
pFLAG-CMV-2-Nek7-K63M	generated via site directed mutagenesis of Lysine-63 (AAG) to methionine (ATG)
pFLAG-CMV-2-Nek7-K64M	generated via site directed mutagenesis of Lysine-64 (AAG) to methionine (ATG)
pFLAG-CMV-2-Nek7-K63MK64M	generated via site directed mutagenesis of Lysine-63 and Lysine-64(AAG) to methionine (ATG)
pFLAG-CMV-2-Nek7-S191A	generated via site directed mutagenesis of Serine-191 (AGC) to Alanine(GCC)
pFLAG-CMV-2-Nek7-T195A	generated via site directed mutagenesis of Serine-195 (AGC) to Alanine(GCC)



pcDNA-DEST53-Nek7 generated by inserting Nek7 into pcDNA-DEST-53 using recombination at attR1 and attR2 sites

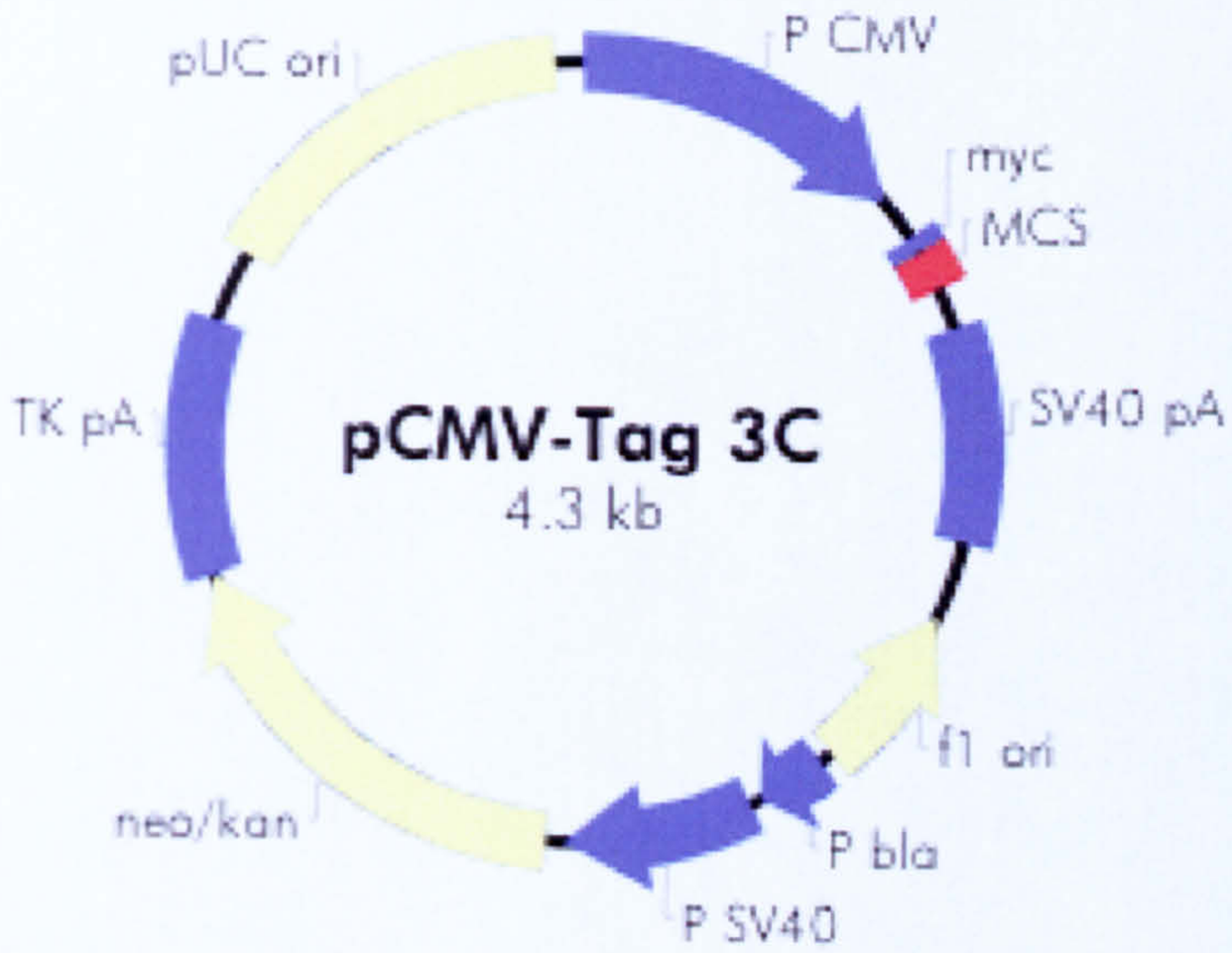
pcDNA-DEST53-Nek7-K63M generated via site directed mutagenesis of Lysine-63 (AAG) to methionine (ATG)

pcDNA-DEST53-Nek7-T195A generated via site directed mutagenesis of Threonine-195 (ACG) to Alanine (GCC)



pETM-11-Cortactin A generated by cloning Cortactin A into pETM-ii using Not1 and Nco1 restriction sites.

pETM-11-Hsp70 generated by cloning Hsp70 into pETM-11 using Nco1 and HindIII restriction sites.



pCMV-Tag 3C-Nek6 generated by cloning Nek6 into pCMV-Tag 3C using Not1 and Nco1 restriction sites.

pCMV-Tag 3C-Nek7 generated by cloning Nek7 into pCMV-Tag 3C using Not1 and Nco1 restriction sites.